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Original Research Article

Isolation, characterization, pharmacological evaluation and *in silico* modeling of bioactive secondary metabolites from *Ziziphus oxyphylla* a member of Rhamnaceae family

Irfan Khan[1], Muhammad Zahoor[1,2], Alam Zeb[2], Muhammad Umar Khayam Sahibzada[3]*, Wasim Ul Bari[1], Sumaira Naz[2]

[1]Department of Chemistry, [2]Department of Biochemistry, University of Malakand, Chakdara, Dir Lower, KPK 18800, [3]Department of Pharmacy, Sarhad University of Science and Information Technology, Peshawar, KPK 25000, Pakistan

For correspondence:* ***Email:* *umar.sahibzada@gmail.com*

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Abstract

Purpose: *To investigate the pharmacological properties of the medicinally active metabolites of Ziziphus oxyphylla.*
Methods: *Compound I-IV were isolated form the root of Ziziphus oxyphylla (compound I = Stigmasterol, II = Betulinic acid, III = 1,2,3 benzene triol and IV = 5-Pentadecanoic acid). Various spectroscopic techniques were used to identify and characterize the isolated compounds. DPPH (2,2-diphenyl-1-picrylhydrazyl) and ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) assays were employed to determine the antioxidant potentials of these compounds. The acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) inhibition potential of the isolated compounds were also evaluated.*
Results: *Amongst the isolated compounds, compound IV was the most potent antioxidant against DPPH and ABTS free radicals, exhibiting half-maximal concentration (IC_{50}) values of 64 and 65 µg/mL, respectively. All the compounds exhibited good inhibition of acetylcholinesterase and butyrylcholinesterase. However, stigmasterol was more potent than the other isolated compounds, showing IC_{50} of 85.10 ± 1.45 and 84.81 ± 1.17, respectively, against AChE and BChE.*
Conclusion: *Although, all isolated compounds inhibited the selected free radicals (DPPH and ABTS) and cholinesterases, stigmasterol and 5-penatadecanoic acid were more potent than other two compounds. Thus the former can potentially be used to treat oxidative stress and neurodegenerative diseases.*

Keywords: *Ziziphus oxyphylla, Stigmasterol, 5-Pentadecanoic acid, Antioxidant, Acetyl Cholinesterase, Butyryl cholinesterase*

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INTRODUCTION

Medicinal plants are valuable sources of bioactive compounds and are used in crude form as traditional medicines. Being factories of natural compounds they provide active ingredients of modern drugs as well [1]. Due to their versatile applications and low incidences of side effects, plant derived substances are preferred by local communities around the world

and are used as drug for the treatment of many diseases [2]. Plants being the renewable sources, provides low cost drugs and other biologically important substances [3]. Approximately, 80 % inhabitants of the developing countries are using medicinal plants as remedies of different ailments.

A number of bioactive phytochemicals were isolated from plants during the first half of the nineteenth century [4-6]. Many of them are now used as active ingredients in modern drug formulations. A number of herbal drugs have been effectively used for the treatment coronary heart diseases and cancer [7]. Oxidative stress that is caused by the increased production of free radicals in human bodies due to environmental changes and modern food habits, can be controlled by the intake of certain phytochemicals, collectively known as antioxidants. Although, a number of synthetic antioxidants are used as preservatives in the food industry but genotoxic and carcinogenic effects have been reported about them in experimental animals. On the other hand, natural antioxidants that can be obtained from plant, are thus considered safe for systemic uses and are preferred over synthetic antioxidants [3,7].

Alzheimer disease (AD) which can be characterized by common behavioral symptoms like cognitive dysfunction, turbulence, and limitation in the usual activities, is a neurodegenerative disease of the old age [8]. The common indicator of AD is the reduction in the amount of acetylcholine (ACh) as a result of its excessive hydrolysis by AChE and BChE. ACh, being a neurotransmitter, affect the conduction of neuro impulses across the synapses. In such situations, the inhibition of AChE and BChE are desired to minimize the complications associated with AD. Currently, there are five such inhibitors available in the market for the treatment of AD. Amongst them galantamine and rivastigmine are natural products of plant origin [9,10]. None of these five drugs are 100% potent, therefore, improved inhibitors from plants are being sought to develop a new efficient drug for the treatment of AD.

Ziziphus oxyphylla which belongs to the family Rhamnaceae is a very important medicinal plant and is under scientific investigations for the last two decades due their medicinal uses in the folklore medicines [11,12]. Various species of *Ziziphus* genus are used for the treatment of weakness, gastrointestinal tract, and liver complications. It has also been used to reduce obesity and to treat urinary tract and skin infections, diabetes, fever, diarrhea, insomnia etc. Antibacterial, antifungal, and phytotoxic activities of some species of this genus have already been reported [13].

A number of compounds have also been isolated from this genus. Amongst them nummularine, zizynummin, lotusanine, adouctine, mauritine, dachuine, franganine and frangufoline are the important compound that have exhibited medicinal properties in animal models [13,14]. Devi and her team isolated nummularine-K, nummularine-R, and amphibinesorbitol H from *Z. nummularia* while frangufoline from *Ziziphus jujuba* [15]. Singh and his team isolated nummularine-T from *Ziziphus nummularia* [16]. Ghedira *et al* isolated lutisine from *Ziziphus lotus* [17]. Jubanine-C, an alkaloid was isolated by M. Tripathi and his team from *Ziziphus jujuba* [18]. The alkaloid lotusine G was isolated from by Crouéour from *Ziziphus lotus* [19].

To the best of our knowledge, the root of *Ziziphus oxyphylla* has not been subjected to isolation of biologically active compounds. The medicinal importance of this plant promoted us to isolate biologically active compounds from this plant. Four compounds were isolated in pure form, which were screened for their antioxidant and anticholinesterase potentials.

EXPERIMENTAL

In column isolation, the adsorbent silica (particle = 70 – 230 mesh size, acquired from Merck, Germany) was used. To visualize the isolated compounds, TLC plates (60 PF254, Merck), cesium sulphate, iodine, and UV lamp (local made) were used. Different spectroscopic techniques ($^{1}H/^{13}C$ NMR, COSY, NOESY, etc.) were employed for the structural elucidation of isolated compounds.

Plant material

Ziziphus oxyphylla, root samples were collected from Barimkai village of Dir District, KPK, Pakistan. The plant was authenticated by a botanist, Dr. Mohammad Nisar (voucher specimen no. 1022HU) and stored in the Herbarium of the University of Malakand, were followed (UOM/HU/Eth/Coll.0321).

The root samples were cleaned with water, shade dried and then ground to a fine powder using grinder. The fine powder was then soaked in 95 % methanol/water solvent system for 75 h. The mixture was then filtered and the leftover solid residues were dipped again in then mentioned solvent system for additional 75 h. The filtrate obtained of both the steps were

mixed togather and converted into a semisolid mass at 40 ºC using a rotary evaporator (Switzerland, Modal R-200 Buchi, Rotavapor). The crude methanolic extract was then subjected to fractionation using different solvents. About 700 g of the methanolic extract was dissolved in 2000 mL distilled water and then subjected to solvent-solvent extraction. The different fractions obtained were concentrated under vacuum in a rotary evaporator. The semisolid masses obtained were air dried and were kept in refrigerator till further use.

HPLC analysis were used to determine the distribution of phytochemicals amongst the different extract fractions (HPLC system used: Agilent 1260). The ethyl acetate extract had a large number of phytochemicals (based on HPLC findings) and was thus loaded to silica gel column for the isolation. The column was eluted with n-hexane/ethyl acetate solvent system. The eluted fractions were analyzed on TLC plates and identical fractions were combined to get 25 fractions. After passage through pencil silica columns, fraction 13 was found to contain stigmasterol (57 mg) that was eluted with EtOAc/n-hexane (3:7) mixture. Fraction 7 contains two compounds: 1,2,3 benzene triol and 5-pentadecanoic acid (EtOAc/n-hexane, 6: 8). Betulinic acid was purified from fraction 11 and eluted with EtOAc/n-hexane mixture in ration of 5:5.

Antioxidant screening

DPPH is a synthetic free-radical that is used to assess the antioxidant potential of various samples including plant extracts [9]. About 20 mg/100 mL DPPH solution was prepared in distilled methanol. About 3 mL from this solution was taken and diluted with distilled methanol to adjust its absorbance to 0.700 at 515 nm. The stock solution was then accordingly diluted and was kept in dark for overnight (the time needed to generate DPPH free radical). To prepare the isolated compound stock solutions, 5 mg of each was dissolved in 5 ml methanol. Different dilutions of each compound (1000, 500, 250, 125, and 62.5 µg/mL) were prepared and used in the subsequent experiments as working standards. Then 2 mL DPPH solution was mixed with 2 mL working standards in a number of test tubes and incubated in dark for 15 min. Then the absorbance of each sample was recorded at 515 nm using UV/visible spectrophotometer. The samples antioxidant potential was calculated using equation 1.

$$R (\%) = (A-B)/A \times 100 \quad \ldots\ldots\ldots\ldots\ldots (1)$$

Where “A” denotes the absorbance of oxidized form of DPPH, while “B” is the absorbance of DPPH after mixing with test samples.

ABTS is also a synthetic free radical employed for the *in vitro* estimation of antioxidant potential of different substances. The free radical stock solution was prepared by a method described by Ovais and his team [9]. About 2 mL ABTS were mixed with 2 mL of the working standards and incubated for a specified interval of time (25 min). The absorbance of resulting mixture after incubation was determined using UV/visible spectrophotometer. Equation 1 was used to calculate the antioxidant potential of tested samples.

Evaluation of anticholinesterase inhibition potential

Ellman’s assay was utilized to estimate anticholinesterase potentials of a given substance. The hydrolysis of acetylthiocholine iodide results in the formation of thiocholine which make a color complex with an anion formed from DTNB (5-thio-2-nitrobenzoate) [20]. From different dilutions of each compound, 1 mL was incubated for 15 min at 25°C with 100 µL of DNTB and AChE/BChE. After incubation, the substrate; acetylcholine/butyrylcholine iodide (100 µL) were added to reaction mixtures and incubated for additional 15 min. The absorbance of reaction mixtures were recorded at 412 nm using spectrophotometer. Equations 2-4 were used to calculate enzyme inhibition.

$$\text{Rate of reaction} = \Delta A/\Delta T \quad \ldots\ldots\ldots\ldots\ldots\ldots (2)$$

$$Ea (\%) = (\text{Rate of reaction})/(\text{Maximum rate of reaction}) \times 100 \quad \ldots\ldots\ldots\ldots\ldots\ldots (3)$$

$$Ei = 100 - \%Ea \quad \ldots\ldots\ldots\ldots\ldots\ldots (4)$$

where Ea = Enzyme activity, Ei = Enzyme inhibition, A = Absorbance, V = rate of reaction in the presence of inhibitor, Vmax = rate of reaction in the absence of inhibitor.

Phosphate buffer (8.0 pH and 0.1 M) was used to prepare different solution. The pH of solutions were adjusted with potassium hydroxide. The concentrations of enzyme used were; 0.01 and 0.03 Unit/mL of AChE and BChE respectively. Different solutions used in the experiments were prepared in distilled water (0.0005 M acetyl/ butrylthiocholine iodide, 0.0002273 M DTNB). All the solutions were stored at 4°C in a refrigerator till further use. As a positive control, galantamine have been used.

Molecular docking simulations

To establish a correlation between the experimentally observed percent inhibition of AChE/BChE and the possible binding orientations of the compounds with the enzymes crystal structures (PDB: 1ACL, 4BOP: from the RCSB Protein Data Bank), simulation software (Schrödinger) was used. All the ligands studied were in neutral form and optimized in the force field of OPLS-3. To prepare the selected enzyme crystal structures suitable for protonation at pH 7, protein preparation (Schrödinger) was used. The receptor grid box (a 20 Å box containing active site water molecule in its center) was defined. The docking was performed with Glide (Schrödinger) using XP extra precision with evasion settings and glide scoring function, reporting the 15 top ranked poses for each ligand. Visual review of the binding pose and generation of figure was done with (Schrödinger) Maestro [21].

RESULTS

The spectral data of the isolated compounds are presented as follows:

Compound I (Stigmasterol)

Proton NMR: In ^{1}HNMR **(**DMSO, δ ppm), the multiplet observed at δ_H 5.35 is due to Exocyclic Methylene Protons (H-6). The detail of remaining peaks is as follow; at δ_H 5.60 (m, 2H, H-22,23), 4.86 (s, 1-OH, H-3), 3.47 (m), 0.88 (3H, d, *J* = 6.52 Hz), 0.92 (3-H, d, *J* = 6.82 Hz), 0.78 (3-H, s, CH_3-18), 0.88 (3-H, t, *J* = 6 Hz), 1.02 δ_H (3-H, s), 0.83 (3-H, d, *J* = 6 Hz). Methylene protons were observed between δ_H 1.20- 2.65.

Carbon-13 NMR: The detail of peaks observed in ^{13}CNMR (DMSO, δ ppm) spectrum are as follow: 33.23 (C-1), 33.04 (C-2), 72.46(C-3), 51.75(C-4), 139.80 (C-5), 122.45 (C-6), 30.40 (C-16,7), 35.12 (C- 8), 57.39 (C-9), 49.29 (C-10), 19.33 (C-11), 49.43 (C-12), 51.78 (C-13), (C14,17), 12.67 (C-18), 12.34 (C-19), 49.15 (C-20), 19.48 (C-21), 33.23 (C-26,22), 41.92 (C-23), 52.81 (C-24), 47.30 (C-25), 33.28 (C-27), 37.44 (C-28), 12.53 (C-29).

Figure 1 represents the chemical structure of compound **I**, which have also been confirmed through HMBC, HSQC and COSY.

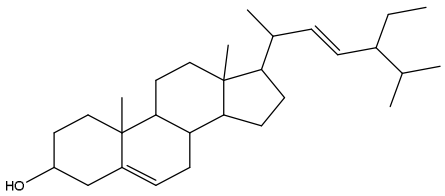

Figure 1: Compound **I** (Stigmasterol)

Compound II (Betulinic acid)

The melting point of compound **II** was approximately 316 to 318°C. The IR spectra showed characteristic peaks at 3408, 1628, 1379 and 1065 cm^{-1} presenting unsaturation and OH groups. Its molecular formulae is $C_{30}H_{48}O_3$. The observed spectral data was in accordance with the reported IR data of the same compound [20].

^{1}H-NMR (DMSO, ppm): The details of proton spectrum (DMSO, δ ppm) is given as follow: the δH at 0.77 (s, 3-H, H-28), 0.88 (s, 3-H, H-29), 0.97 (s, 6-H, H-25 & 26), 1.02 (s, 3-H, H-30), 0.93 (s, 1-H, H-4), 1.06 (s,1-H, H-12), 1.42 (m, 8-H, H-6, 8,15 & 20), 1.58 (m, 4-H, H-7 & 14), 1.35 (m, 2-H, H-19), 1.71 (s, 3-H, H-24), 1.94 (m, 2H, H-16), 1.56 (m, 4-H, H1 & 13), 2.33 (m, 1H, H-21), 2.25 (m, 1H, H-18), 3.03 (m, 1H, H-2), 4.61 (s, 1-OH, H-27), 3.15 (dd, 1-H, H-23), and 3.03 (t, 1-H, H-23), representing different location and environment of proton present.

^{13}C-NMR (DMSO, ppm): The detail ^{13}CNMR (DMSO, δ ppm) is as follow; 15.12 (C-30), 16.10 (C-28), 16.67 (C-7), 16.73 (C-29), 19.46 (C-14), 19.57 (C-24), 22.11 (C-25,26) 26.93 (C-13), 28.07 (C-1), 28.62 (C-15), 30.86 (C-20), 31.75 (C-16), 33.39 (C-8), 35.63 (C-19),38.16 (C-6). 39.70 (C-9), 39.96 (C-11), 48.58 (C-21), 49.29 (C-18), 50.51 (C-10), 56.91 (C-4), 57.53 (C-17), 79.70 (C-2), 110.3 (C-23), 152.03 (C-22), and 180.20 (C-31). The structure of compound **II** (Betulinic acid) is presented in Figure 2. For final confirmations; HMBC, HSQC, and COSY techniques have been used.

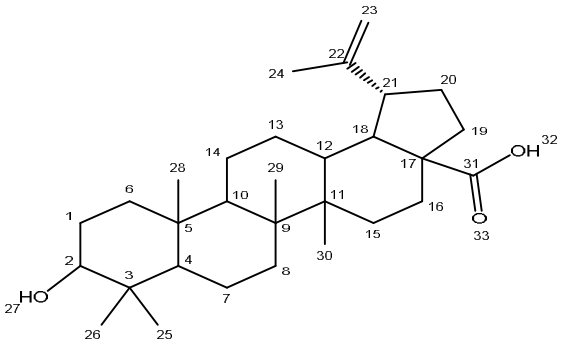

Figure 2: Compound **II** (Betulinic acid)

Compound III (1, 2, 3 benzene triol)

It is a white colored amorphous solid, soluble in water having melting point of 131-134°C. The FTIR spectrum showed peaks at 3608, 1628, and 1165 cm^{-1} indicating the presence of OH and aromatic groups.

^{1}H-NMR (DMSO, ppm): Different peaks observed at δ_H 6.4 (t, J=12 Hz, H-5), 6.2 (d, *J* = 6 Hz, 2H, H-4 & 6), 8.6 (s, 3-OH, OH-1, 2 & 3) confirmed the compound as 1,2,3-benzene triol.

^{13}C-NMR (DMSO, ppm): Different positions and environment of carbons were confirmed from peaks at δ_C 146. (C-1 & 3), 133 (C-2), 118.5 (C-5), 107.12 (C-4 & 6). The structure of compound **III** (Figure 3) was further confirmed through HMBC, HSQC and COSY techniques.

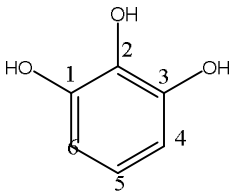

Figure 3: Compound **III** (1, 2, 3 benzene triol)

Compound IV (5-pentadecanoic acid)

Compound **IV** is a saturated fatty acid having chemical formulae; $C_{15}H_{30}O_2$. The melting point recorded for this compound was 51 to 53°C. The data obtained for Compound **IV** were in agreement with that of pentadecanoic acid already reported in literature. Pentadecanoic acid have been isolated for the first time from the *Z. oxyphylla*.

^{1}H-NMR (DMSO, δppm): 0.99 (7, 3-H H-15 J = 6 Hz), 1.30 (m, 22-H, H-2,3,4,7,8,9,10,11,12,13 & 14) 2.22 (m, 2-H, H-5 & 6).

^{13}C-NMR (DMSO, δppm): 28.11 (CH_3, C-5), 30.32 (CH_2, C-14), 30.53 (C-13), 30.58 (C-12), 30.65 (C-11), 30.73 (C-10), 30.75 (C-9) 30.82 (C-8), 33.05 (C-7), 130.6 (C-5,6), 48.57 (C-4), 49.14 (C-3), 49.43 (C-2), 180.69 (C-1). The chemical structure of compound **IV** (Figure 4) was further elucidated through HMBC, HSQC and COSY spectral techniques.

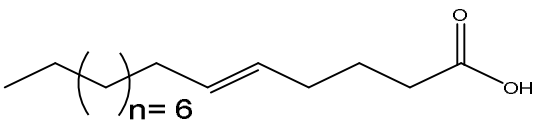

Figure 4: Compound **IV** (5-Pentadecanoic acid).

DPPH and ABTS scavenging potential

To find out whether the isolated compounds would be useful in scavenging free radical produced inside human bodies or not, their antioxidant potential was evaluated using the DPPH and ABTS assays. In DPPH assay, the DPPH free radical developed react with the test substance and after quenching the free radical the change in absorbance at 515 nm is noted. The 5-pentadecanoic acid showed maximum scavenging potentials with IC_{50} = 64 µg/mL (85.33 ± 2.60 % at 1000 µg/mL) followed by stigmasterol (IC_{50} = 65 µg/mL, and % inhibition = 84.01 ± 1.79). Compound **II** and **III** also exhibited excellent DPPH scavenging with percent inhibition of 82.19 ± 2.33 and 81.68 ± 1.90 µg/mL (IC_{50} = 90 and 93 µg/mL respectively) as shown in Table 1.

The ABTS free radical was also more potently inhibited by 5-pentadecanoic acid, which showed highest percent inhibition of 84.43 ± 1.71 at 1000 µg/mL concentration with IC_{50} value 65 µg/mL followed by stigmasterol (IC_{50} = 66 µg/mL and % inhibition = 83.23 ± 2.55). Compound **II** and **III** also exhibited notable ABTS scavenging potential with IC_{50} of 86 and 98 µg/mL respectively (Table: 1). Ascorbic acid (IC_{50} = 35 µg/mL) was used as positive control.

Cholinesterase inhibition

In a number of neurological disorders, the inhibition of AChE and BChE are desired to inhibit the hydrolysis of acetylcholine. Table 2 representing the anticholinesterases inhibitory potential of the isolated compounds isolated. Against AChE, stigmasterol was more potent and showed a percent inhibition of 85.10 ± 1.45 with IC_{50} of 63 µg/mL, followed by betulinic acid with percent inhibition of 83.26 ± 1.69 (IC_{50} =69 µg/mL). The compound **III** and **IV** also inhibited the mentioned enzyme with the IC_{50} of 72 and 73µg/mL respectively (Table 2) and were ranked as moderate inhibitors of AChE.

Galantamine was used as positive control. The isolated compounds also exhibited substantial inhibition of BChE. The stigmasterol showed remarkable inhibition with percent inhibition of 84.81 ±1.17% (IC_{50} = 67 µg/mL) followed by 5-pentadecanoic acid (IC_{50} = 69 µg/mL). Betulinic acid and 1,2,3-benzene triol were also found to have high percent inhibition with IC_{50} of 71 and 82 µg/mL, respectively (Table 2).

Table 1: Free radical scavenging activities of compounds **I** to **IV**

Compound	Conc (µg/mL)	DPPH inhibition (%, mean ± SEM)	IC_{50} (µg/mL)	ABTS inhibition (%, mean ± SEM)	IC_{50} (µg/mL)
	1000	84.01 ± 1.79^{ns}		83.23 ± 2.55 ***	
	500	73.14 ± 1.65***		73.65± 2.75 ^{n}***	
Stigmasterol	250	61.29 ± 2.17***		64.20± 1.84 ^{n}***	
	125	54.72 ± 2.54 ***	65	56.13 ± 1.34^{n}***	66
	62.5	46.11 ± 1.87*		47.64± 1.20 ^{n}***	
	1000	82.19 ± 2.33***		81.28 ± 2.15***	
	500	76.24 ± 2.65***		75.19 ± 2.55***	
Betulinic acid	250	61.47 ± 2.73***		63.09 ± 1.32***	
	125	53.66 ± 1.19***	90	53.26 ± 2.71***	86
	62.5	43.49 ± 2.72***		44.27 ± 1.51***	
	1000	81.68 ± 1.90***		80.53 ± 2.60***	
	500	71.44 ± 1.61***		69.32 ± 1.76***	
	250	64.80 ± 1.65***		59.19 ± 0.77***	
1,2,3 benzene triol	125	54.63 ± 1.87***	93	50.08± 1.21***	98
	62.5	47..05± 2.09***		43.44± 1.85***	
	1000	85.33 ± 2.60***		84.43 ± 1.71***	
	500	75.47 ± 2.98***		74.23 ± 1.60***	
5-Pentadecanoic acid	250	65.21 ± 0.96***		61.22 ± 2.89***	
	125	55.89. ± 2.98***	64	54 .17 ± 2.04***	65
	62.5	47.66 ± 2.78***		47.66± 1.70***	
	1000	94.14 ± 1.76		93.07 ± 0.53	
	500	87.87 ± 1.64		83.45 ± 2.26	
Ascorbic acid	250	78.63±1.48		75.14 ± 3.16	
	125	65.35±1.08	35	63.30 ± 2.75	35
	62.5	55.12±1.30		55.41 ± 1.39	

Table 2: Anticholinesterase activity of compounds **I-IV**

Compound	Conc. (µg/mL)	% AChE Inhibition (mean ± SEM)	IC_{50} (µg/mL)	% BChE inhibition (mean ± SEM)	IC_{50} (µg/mL)
	1000	85.10 ± 1.45^{ns}		84.81 ± 1.17***	
	500	75.63 ± 2.60***		72.20 ± 1.90^{n}***	
Stigmasterol	250	63.01± 1.65***	63	63.59± 2.50 ^{n}***	67
	125	54.32 ± 1.89 ***		54.45 ± 0.96 ^{n}***	
	62.5	48.57 ± 2.03*		47.65± 1.74 ^{n}***	
	1000	83.26 ± 1.68***		82.67± 1.87***	
	500	75.63± 2.78***		73.43 ± 2.08***	
Betulinic acid	250	63.80 ± 1.50***	69	63.32 ± 1.60***	71
	125	54. 76 ± 0.97***		54.36 ± 2.73***	
	62.5	45.52 ± 2.67***		46.45 ± 2.62***	
	1000	81.22 ± 2.89***		79.87 ± 2.65***	
	500	73.41 ± 2.87***		70.37 ± 2.37***	
1,2,3 benzene triol	250	64.15 ± 2.10***	72	60.39 ± 1.71***	82
	125	54..86 ± 2.86***		51.10 ± 1.39***	
	62.5	46.51± 2.74***		45.52± 2.80***	
	1000	81.52 ± 1.40***		82.10 ± 1.48***	
	500	74.28 ± 2.43***		73.29 ± 1.71***	
5-penadecnoic acid	250	65.60 ± 1.82***	73	64.33 ± 2.20***	69
	125	53.25 ± 1.54***		54 .17 ± 3.11***	
	62.5	48.89 ± 2.61***		45.69 ± 2.20***	
	1000	94.11 ± 1.56		93.97 ± 0.81	
	500	86.17 ± 1.50		85.25 ± 2.06	
Galantamine	250	77.13±1.08	40	76.74 ± 2.26	40
	125	64.15±2.70		64.89 ± 1.85	
	62.5	53.02±2.31		53.71 ± 1.69	

Sim provide a scientific base for the observed *in vitro* anticholinesterase potentials of isolated compounds, molecular docking software was used to determine the most favorable binding between the enzyme active sites and the isolated compounds. The most potent inhibitor of AChE i.e compound **I** exhibited a docking score of -8.622 Kcal/mol showing the excellent fitting of

the compound in the binding pocket of enzyme protein crystal structure (Figure 1). The favorable interaction of ligand with Tyr-70, Trp-84 and Hid-440 is shown in Figure 1a and b. On the other hand, benzene triol exhibited excellent overlapping and fitness in the binding pockets of both cholinesterases with a docking score of –7.008 (AChE) and -2.746 Kcal/mol (BChE). The OH group of the said ligand form H-B (1.89 A^o) linkage with HOH-622. There is also some pi stacking interaction with PHE-330 of AChE and the ligand as presented in Figure 2a, and b. The BChE has also exhibited good interaction with 1, 2, 3 benzene triol, OH of the ligand form H-B (1.86 A^o) linkage with carbonyl of ASP-332. OH group of the same ligand also forms H-B (1.94 A^o) linkage with NH_2 of GLN-333 as presented in Figure 2c, and d.

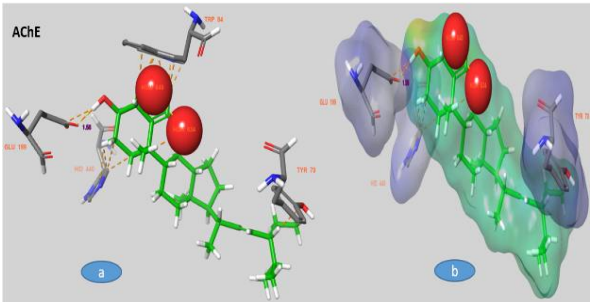

Figure 1: Molecular docking photograph of the high ranked pose of the AChE active site and compound **I** (green). (a) 2D diagram of ligand (AChE) protein and residue contact. (b) Docked ligand and active binding pocket of AChE

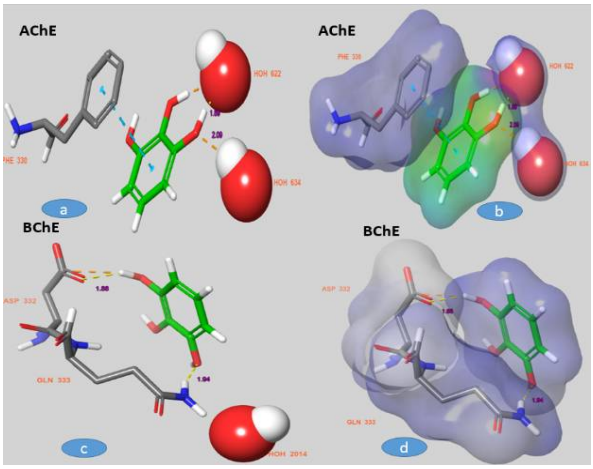

Figure 2: Molecular docking photograph of the high ranked pose of selected cholinesterases active sites and compound **III** (green) showing that it has been bound with the active site close to H_2O molecule. (a). 2D diagram of ligand (AChE) protein and residue contact; (b). Docked ligand and active binding pocket of AchE; (c). 2D diagram of ligand (BChE) protein and residue contact; (d). Docked ligand and active binding pocket of BChE

DISCUSSION

Based on the ethno pharmacological uses of *Z. oxyphylla* [5], an attempt was made to isolate phytochemicals from this plant. To the best of our knowledge, the roots of *Ziziphus oxyphylla* have not been evaluated or subjected to the isolation of phytochemicals before. The lack of information about the chemical composition of this plant and diverse medicinal uses of genus *Ziziphus, Ziziphus oxyphylla* roots in folklore medicines promoted to investigate its phytochemical constituents. The investigations led to the isolation four compounds which were confirmed through different spectroscopic techniques. First, the plant was subjected to the extraction and fractionation.

The fractions were subjected HPLC analysis and the chromatograms obtained were compared for the phytochemicals present. The ethyl acetate extract was found to contain more phytochemicals and was thus used in the subsequent isolation process. Betulinic acid, stigmasterol, 1,2,3-benzene triol and 5-penadecanoic were isolated in pure form from this fraction. Amongst the isolated compounds, the stigmasterol has been reported previously from this genus while the other three are hereby reported for the first time from the selected plant.

During metabolism a number of free radicals are formed. Normally, they are detoxified by body defense mechanisms as soon as they are formed. However, their overproduction can lead to a number of health complication including heart diseases, neurodegenerative disorders, suppression of immune system, and metabolic disorders [9]. A number of chemical substances called antioxidants, help in quenching the free radical produced thus maintaining the homeostasis of the body [22]. The isolated compounds showed substantial free radical scavenging activities against the studied synthetic free radicals; DPPH and ABTS. The lowest IC_{50} value of 64 µg/mL (85.33 ± 2.60 percent inhibition at 1000 µg/mL) was recorded for 5-pentadecanoic acid while against ABTS radical its IC_{50} value was 65 µg/mL. Substantial DPPH scavenging was also observed for compound **II** and **III** with percent inhibition of 82.19 ± 2.33 and 81.68 ± 1.90 (IC_{50} = 90 & 93 µg/mL) respectively.

The enhanced activities of acetylcholinesterase and butyrylcholinesterase are associated with many neurological complications as both of these enzymes causes the catabolism of neurotransmitter acetylcholine. Thus their inhibition is desired to alleviate the complications

associated with Alzheimer's and other neurologic diseases [22,23]. The isolated compounds showed remarkable AChE and BChE inhibitory activities as well. The AChE was more potently inhibited by stigmasterol with percent inhibition of 85.10±1.45% and IC_{50} of 63 µg/mL, followed by betulinic acid with percent inhibition of 83.26±1.69 (IC_{50} = 69 µg/mL). The compound **III** and **IV** with the IC_{50} values of 72 and 69 respectively, were ranked as moderate inhibitors of the AChE.

The isolated compounds exhibited substantial activities against the BChE. Amongst them stigmasterol showed maximum anticholinesterase activity of 84.81 ±1.17% with an IC_{50} value of 67 µg/mL followed by 5-pentadecanoic acid (IC_{50} = 69 µg/mL). Compound **II** and **III** were also found to have strong anticholinesterase potentials which is evident from their IC_{50} values (71 and 82 µg/mL respectively). Galantamine, a compound from plant origin was used as positive control.

CONCLUSION

Almost all the compounds (**I-IV)** exhibit scavenging activities against ABTS and DPPH radicals, and potentially inhibited cholinesterases. Stigmasterol and 5-pentadecanoic acid displayed the highest activities. *Ziziphus oxyphylla* is already in use as a folklore remedy in a number of health complications but further investigations are needed to isolate new biologically active secondary metabolites, which might have beneficial effects on various health disorders in human.

DECLARATIONS

Acknowledgement

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Conflicts of interest

No conflict of interest is associated with this work.

Availability of the data and materials

The findings reported in this study are part of Irfan Khan's PhD thesis and will be deposited in HEC Pakistan repository on completion of the project and thus would be available online.

Author contributions

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. I.K. & M.Z. designed the study; all the authors equally contributed in performing the experiments and write up of the paper (I.K, A.Z, W.B, M.U.K.S, S.N. & M.Z).

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