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Cholinesterase Inhibiting Terpenoid from *Albizia kalkora*

Muhammad Afzal, Ejaz Ahmed*, Ahsan Sharif
Institute of Chemistry, University of the Punjab, Quaid-e-Azam Campus, Lahore, 54590, Pakistan.
dr.ejaz.ahmed@gmail.com*

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Summary: *Albizia kalkora* is potent source of phytocompounds. In recent phytochemical investigation one new triterpenoid Albizinoic acid A was isolated from the chloroform soluble fraction of medicinal plant *Albizia kalkora*. The structure was elucidated by physical, chemical, and spectroscopic analysis (FT-IR, 1D& 2D-NMR, Mass spectrometry). This bioactive compound exhibited inhibitory potential against Alzheimer's disease (AD) causing enzymes acetylcholinesterase (AChE) and butyrylcholinesterase (BChE). IC_{50} of Albizinoic acid A against cholinesterase were found 25.3± 0.02, AChE and 19.0±0.03, BChE while galanthamine as a positive control (0.5 ± 0.05, AChE and 8.5±0.01, BChE). This enzymes assay indicated that Albizinoic acid A has great potential of pharmacological characteristics.

Keywords: Albizia kalkora, Triterpene, Albizinoic acid, 1D and 2D NMR, Cholinesterase enzyme inhibition.

Introduction

Alzheimer's disease (AD) caused by low level of acetylcholine (ACh) is characterized by memory impairment and emotional disturbance[1]. The level of acetylcholine (ACh) decreased due to production of acetylcholinesterase (AChE) enzyme [2]. Recent research showed that butyrylcholinesterase (BChE) also responsible for advanced level of AD. Therefore, the increase the level of ACh in brain by inhibition of AChE and BChE. Some drugs used for treatment of AD by inhibition of acetylcholinesterase (AChE) such as tacrine, donepzil, rivastigmine, physostigmine and galanthamine [3]. Phytochemical constituents obtained from medicinal plants also play key role for curing of diseases.

Albizia kalkora (jullibrissin) is medium size flowering plant belongs to *Fabaceae* family which is third largest plant family of the world. *A. kalkora* is a generally 6 to 9-meter high plant with 1-2 cm yellowish green leaves and pink colored flowers widely distributed from northern to southern hemisphere, in Pakistan it is especially present in the Cholistan desert [4] The Pharmacological aspects of *A. kalkora (jullibrissin)* showed that it is an important medicinal plant, commonly used as Chinese medicines to cure various diseases [5]. The phytochemical and pharmacological literature studies revealed that *A. kalkora* has number of biological active constituents, belongs to numerous classes of natural products such as terpenoids, steroids, saponins, phenolics, alkaloids, carbohydrates and carboxylic acids [6]–[8]

These studies prompted us to carry out phytochemical investigation on the *Albizia kalkora* plant. Presently we reported bioactive triterpenoid Albizinoic acid **A** isolated from chloroform soluble fraction of *Albizia kalkora* and structure elucidated by physical, chemical, and spectroscopic mean. Furthermore, the compound showed potent inhibition against Acetylcholinesterase (EC 3.1.1.7) and butyrylcholinesterase (EC 3.1.1.8) enzymes using Galanthamine (positive control)

Experimental

Materials, methods, and instruments

Melting point apparatus model Buchi 535 was used to examine the M.P. of extracted compounds with the help of glass capillaries. The optical rotations of purified compounds were measured by digital polarimeter (JASCO DIP-360). All chemicals and enzymes for enzyme inhibition activities were purchased from Sigma Aldrich (MO, St. Louis, USA). The Ultraviolet visible spectrophotometer (Hitachi U-3200) was used to obtain the UV-visible Spectra. Infrared spectra were recorded on Infrared spectrophotometer (JASCO A-302). Nuclear Magnetic Resonance spectrometers (Bruker AM-400 and AM-500) were used to record ^{1}H-NMR in CD_3OD using TMS as standard and ^{13}C –NMR spectra were recorded in $CDCl_3$, CD_3OD at 100 and 125 MHz whereas 2D-NMR (COSY, NOESY, HMQC and HMBC) were recorded in $CDCl_3$, CD_3OD at 400-500 MHz in the same instrument.

*To whom all correspondence should be addressed.

Collection of plant material and Isolation

Albizia kalkora L. was collected from Cholistan desert near district Bahawalpur, Pakistan. This whole plant was air dried, crushed. 500 g of fine powder of plant was soaked in 2 liters of methanol for three weeks with continuous shaking at 100 rpm. The soaked plant material was filtered through cheese cloth with subsequent filtration through Whattman's filter paper. The filtrates were concentrated through rotary evaporator at 45ºC under reduced pressure. 79 grams of *A. kalkora* thick gummy methanolic extracts were obtained.

Preparation of sub-fractions from methanolic fraction

For sub-fractions take 50 grams of partially dried gummy methanolic extract dipped with 200 mL of distilled water. Firstly, n-hexane solvent was added in methanolic extracted water solution. Separate the n-hexane soluble compound from aqueous phase by 1 L volume of separating funnel. This process repeated till all n-hexane soluble compounds were separated. Afterword the residual extract was sequentially separated with chloroform, ethyl acetate, ethanol, and n-butanol. The sub-fractions were evaporated by rotary evaporator and obtained 5 grams n-hexane, 8 grams chloroform, 7 grams ethyl acetate, 6 grams ethanol and 5 grams n-butanolic sub-fractions. The aqueous was also separated from the residual aqueous material and yielded 3 g [9].

Chloroform soluble fraction (5 grams) was chromatographed over 60-200 mesh size silica gel using n-hexane, n-hexane chloroform, and chloroform-methanol gradients for elution in increasing order of polarity to obtained fifteen semi pure fractions. The semi pure fraction 5 at 1.2:7.8(n-hexane-chloroform) was again re-chromatographed over preparative TLC yielded compound1 in 25 mg.

Compound **(1)** Colourless white solid, showed positive Bromine water, Ferric chloride, Litmus and Liebermann-Burchard's tests. $[\alpha]_D^{25}$ +40.0 (c = 1.50, MeOH); IR (KBr) ν_{max} cm^{-1} 3340, 2950, 1720, 1630, 1512, 1380, 1485, 1250, 801, 777. HREIMS, $[M^+]$ at *m/z* 486.3580 (calcd. for $C_{30}H_{48}O_4$ 486.3538); EIMS *m/z* 486 (12), 474 (52), 468 (35), 456 (78), 450 (66), 375 (54), 264 (89), 224 (91), 91 (100), 45 (25). ^1H and ^{13}C NMR ($CDCl_3$, 400 MHz) see Table-1.

Acetylcholinesterase and Butyrylcholinesterase Bioassays

The AChE and BChE inhibition activities were performed by Ellman method with slight modifications. The total volume of reaction mixture was 100 μL. it contained 60 μL Na_2HPO_4 buffer with concentration of 50 mM and pH 7.7. 10 μL sample tested compound was added with additional 10 μL enzyme. All contents were mixed and pre inhibition reading took at 405 nm. The mixture was pre-incubated for 10 min at 37 ºC. the inhibition reaction was stated by the addition of 10 μL of 0.5mM/well substrate and 10 μL DTNB. After 30 min of incubation at 37 ºC absorbance was measured by spectrophotometer (405 nm) using 96 well plate reader synergy HT, Biotek, USA. The experiments were repeated three time with respective controls. Galanthamine (0.5 mM/well) was used as positive control. Percentage inhibition was calculated by following equation. IC_{50} values were calculated by EZ-Fit Enzyme kinetic software (PSIA, USA). [10].

$$\text{Inhibition (\%)} = \frac{\text{Absorbance of negative control - Absorbance of sample compound}}{\text{Absorbance of negative control}}$$

Statistical Analysis

IC_{50} and ±SEM values were calculated by using Microsoft excel program (Two way AVOVA).

Results and Discussion

The colorless crystalline solid compound (1) was isolated from chloroform soluble fraction. Positive bromine water test for unsaturation, litmus and ferric chloride test indicated the presence of carboxylic acid moiety whereas Liebermann-Burchard's test confirmed the triterpenoidal ring.

FT-IR spectrum showed strong adsorption band for hydroxyl, carbonyl and olefinic moiety at 3340 cm^{-1}, 1720 cm^{-1}, 1630 cm^{-1} respectively.

The EIMS spectrum exhibited the molecular ion peak at *m/z* 486. The HREIMS spectrum gave molecular ion peak at 486.3580 corresponding to the molecular formula $C_{30}H_{48}O_4$ (calcd. 486.3538) indicated eight degrees of unsaturation. The EIMS gave characteristic fragment ion peaks at *m/z* 224 and 262 produced due to the retro Diels Alder cleavage of C ring [11]. The loss of carboxylic moiety and water from fragment B confirmed their attachment to the ring D or E, while the loss of two water molecules and three methyls from fragment A confirmed their attachment to the ring A or B. The EIMS spectrum also displayed prominent peaks at *m/z* 474, 468, 456, 450 and 375 due to the successive losses of water and methyl groups from the parent molecule.

The ^{1}HNMR spectrum of compound (**1**) revealed the signals for four tertiary methyl groups at δ 1.24 1.07, 1.04, and 0.82, while one secondary methyl appeared at δ 1.06 as a doublet having J = 6.5 Hz. One trisubstituted olefinic proton was observed as

a triplet at δ 5.52 having J value 6.0 Hz while an oxymethine proton was appeared at 3.72 as a double doublet having J = 11.0 and 7.0 Hz. An oxymethine proton at δ 4.30 as a double doublet having J= 6.0 and 4.2 Hz indicated the hydroxyl (OH) moiety in the molecule in α-configuration. The oxymethylene protons showed signals at δ 4.00 (1H, d, J = 11.3 Hz) and 3.35 (1H, d, J = 11.3 Hz). One terminal olefinic methylene protons were inferred at δ 4.98 as a doublet (J = 10.2 Hz).

The ^{13}CNMR spectrum indicated the presence of thirty carbon signals comprising five methyl, ten methylene, seven methine and eight quaternary carbons. Four tertiary methyls appeared at δ 24.5, 22.4, 21.5, and 20.3, while one secondary methyl was observed at δ 17.0. The carbonyl of carboxylic group was observed at δ 180.6. The olefinic carbons were inferred at δ 150.2, 140.1, 124.0, and 109.1 while the hydroxymethine carbons observed at δ 69.1 and 72.9. This data clearly indicated the ursolic acid with one additional hydroxyl moiety and terminal methylene in the molecule[11]. The identification and the attachment of hydroxyl, olefinic and other moieties in the molecule were determined by HMBC, HMQC and proton-proton COSY spectrum.

Table-1: 1D (^{1}H, ^{13}C-NMR) and 2D-NMR (HMQC, HMBC) data of compound **(1)** in $CHCl_3$ + MeOH (1:1).

Position No.	^{1}H[a] (HMQC)	Multiplicity and J (Hz)	^{13}C[b]	HMBC[c] (H→C)
1.	1.49	m	37.2	J^2 (C-2, C-10)
				J^3 (C-3, C-26, C-5)
2.	1.71	m	26.9	J^2 (C-1, C-3)
				J^3 (C-4, C-10)
3.	3.72	dd, (11.0, 7.0)	72.9	J^2 (C-2, C-4)
				J^3 (C-1, C-5)
4.	-	-	42.0	-
				-
5.	0.80	m	48.1	J^2 (C-6, C-4, C-10)
				J^3 (C-3, C-7, C-24, C-23)
6.	1.45	m	17.9	J^2 (C-5, C-7)
				J^3 (C-4, C-8, C-10)
7.	1.39	m	32.8	J^2 (C-6, C-8)
				J^3 (C-5, C-9, C-14)
8.	-	-	40.1	-
				-
9.	1.69	dd, (10.9, 6.8)	45.7	J^2 (C-8, C-10, C-11)
				J^3 (C-1, C-7, C-5, C-12, C-14, C-26)
10.	-	-	36.5	-
11.	2.20	m	22.5	J^2 (C-9, C-12)
				J^3 (C-8, C-10, C-13)
12.	5.52	t, (6.0)	124.0	J^2 (C-11, C-13)
				J^3 (C-9, C-14, C-18)
13.	-	-	140.1	-
14.	-	-	43.1	
15.	1.88	m	28.6	J^2 (C-14, C-16)
				J^3 (C-13 C-17,)
16.	1.94	m	29.2	J^2 (C-15, C-17)
				J^3 (C-14, C-18, C-22)
17.	-	-	48.9	-
18.	2.99	d, (4.4)	51.5	J^2 (C-13, C-17, C-19)
				J^3 (C-12, C-14, C-22, C-20)
19.	1.68	d (4.4)	39.6	J^2 (C-18, C-20, C-29)
				J^3 (C-13, C-17, C-21, C-30)
20.	-	-	150.2	-
				-
21.	4.30	dd, (6.0, 4.2)	69.1	J^2 (C-20, C-22)
				J^3 (C-19, C-17, C-30)
22.	2.16	dd, (6.1, 4.2)	39.0	J^2 (C-17, C-21)
				J^3 (C-16, C-18, C-20, C-28)
23.	3.35 (11.3)	d	64.6	J^2 (C-4)
	4.00 (11.3)	d		J^3 (C-3, C-5, C-24)
24.	1.07	s	21.5	J^2 (C-4)
				J^3 (C-3, C-5, C-23)
25.	0.82	s	24.5	J^2 (C-8)
				J^3 (C-9, C-7, C-14)
26.	1.24	s	22.4	J^2 (C-10)
				J^3 (C-1, C-5, C-9)
27.	1.04	s	20.3	J^2 (C-14)
				J^3 (C-8, C-13, C-15)
28.	-	-	180.6	-
29.	1.06 (6.5)	d	17.0	J^2 (C-19)
				J^3 (C-18, C-20)
30.	4.98 (10.2)	d	109.1	J^2 (C-20)
				J^3 (C-19, C-21)

[a] ^{1}H NMR carried out at 400 MHz
[b] ^{13}C NMR carried out at 100 MHz
[C] HMBC carried out at 400 MHz

The olefinic proton at δ 4.98 showed J^{2} correlation with quaternary carbon and methine carbon at C-14 and C-11 respectively. Hydroxymethine proton was observed at δ 4.30 showed J^{2} correlation with quaternary carbon at δ 150.2 and methylene at δ 39.0 while J^{3} correlation with C-17 (δ 48.9), C-19 (δ 39.6) and C-30 (δ 109.1) indicating the exact location of OH group at C-21 (δ 72.3) position. The hydroxymethine proton at δ 3.72 showed J^{2} correlation with methylene at δ 26.9 and quaternary carbon at δ 42.0 while J^{3} correlation with hydroxymethylene at δ 64.6 and with the tertiary methyl at δ 21.5 and δ 48.1 (C-5) indicated the attachment of hydroxyl moiety at C-3, also proved by biogenetic nature of the terpenoid systems.

The stereochemistry of the molecule was confirmed by NOESY spectrum. The proton at δ 4.00 showed correlation with the methyl at δ 3.72 and proton at δ 0.80 at C-5 position. The hydroxymethine proton at δ 4.30 showed correlation with the methyl at δ 2.99 (H-18) and at δ 1.06 at C-29 (17.0) position indicating that the hydroxyl group at C-21 (69.1) position was present in the α-configuration.

Based on these evidence albizinoic acid A was identified as 3β,21α,23α-trihydroxyl ursane-12(30)diene, 28-oic acid.

The enzyme inhibitory activity of albizinoic acid A against cholinesterase was determined and the IC_{50} value was found 25.3 ± 0.02, AChE and 19.0 ± 0.03, BChE respectively, using galanthamine as a positive control (0.5 ± 0.05, AChE and 8.5 ± 0.01, BChE).

The results clearly exhibited that IC_{50} values of Albizinoic acid A are high against both enzymes as compared to standard galanthamine drug.

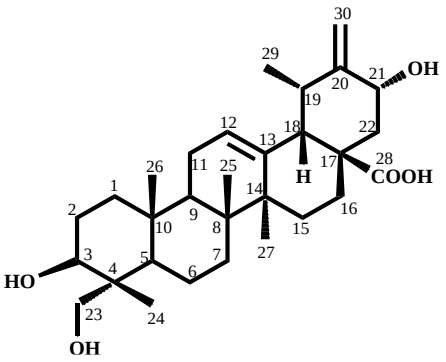

Fig. 1: Structure of Albizinoic acid A (**1**).

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