CPQ Nutrition (2019) 3:4
Experimental Paper

CIENT PERIODIQUE

In Vitro Evaluation of the Antioxidant and Urease Inhibition Activities of Three Triterpenes from the Flowers of *Alstonia Scholaris*

Nighat Sultana[1*] & Muhammad Saleem[1,2]

[1]*Pharmaceutical Research Center, PCSIR Laboratories Complex, Karachi-75280, Pakistan*

[2]*Departments of Biotechnology, University of Karachi, Karachi, Pakistan*

[*]**Correspondence to:** Dr. Nighat Sultana, Pharmaceutical Research Center, PCSIR Laboratories Complex, Karachi-75280, Pakistan.

Copyright

© 2019 Dr. Nighat Sultana. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Received: 16 March 2019

Published: 10 April 2019

Keywords: *In Vitro Evaluation; Urease Inhibition; Flowers*

Reinvestigation of the flowers of *Alstonia scholaris* of Pakistan origin have resulted in the isolation of three triterpenoids, two of the ursane type, 3β-acetate- 24-nor- urs-4, 12-diene ester triterpene (1), 3β -hydroxy-24-nor-urs-4,12,28-triene triterpene (2) and one of the oleanane type 3,28- β -diacetoxy-5-olea-triterpene (3) together with two known triterpenes α-amyrin acetate (4) and ursolic acid (5). These compounds were analyzed for antioxidant and antiurease inhibition tests. Compounds 2 and 4 were subjected to enzymatic bioassays. Compounds 2 and 4 were found to be antioxidant with IC_{50} 107±0.39 and >500μM respectively, while compounds 2 and 4 were also found to be urease inhibitors with IC_{50} 175 ± 0.26 and 218 ± 0.14μM, respectively.

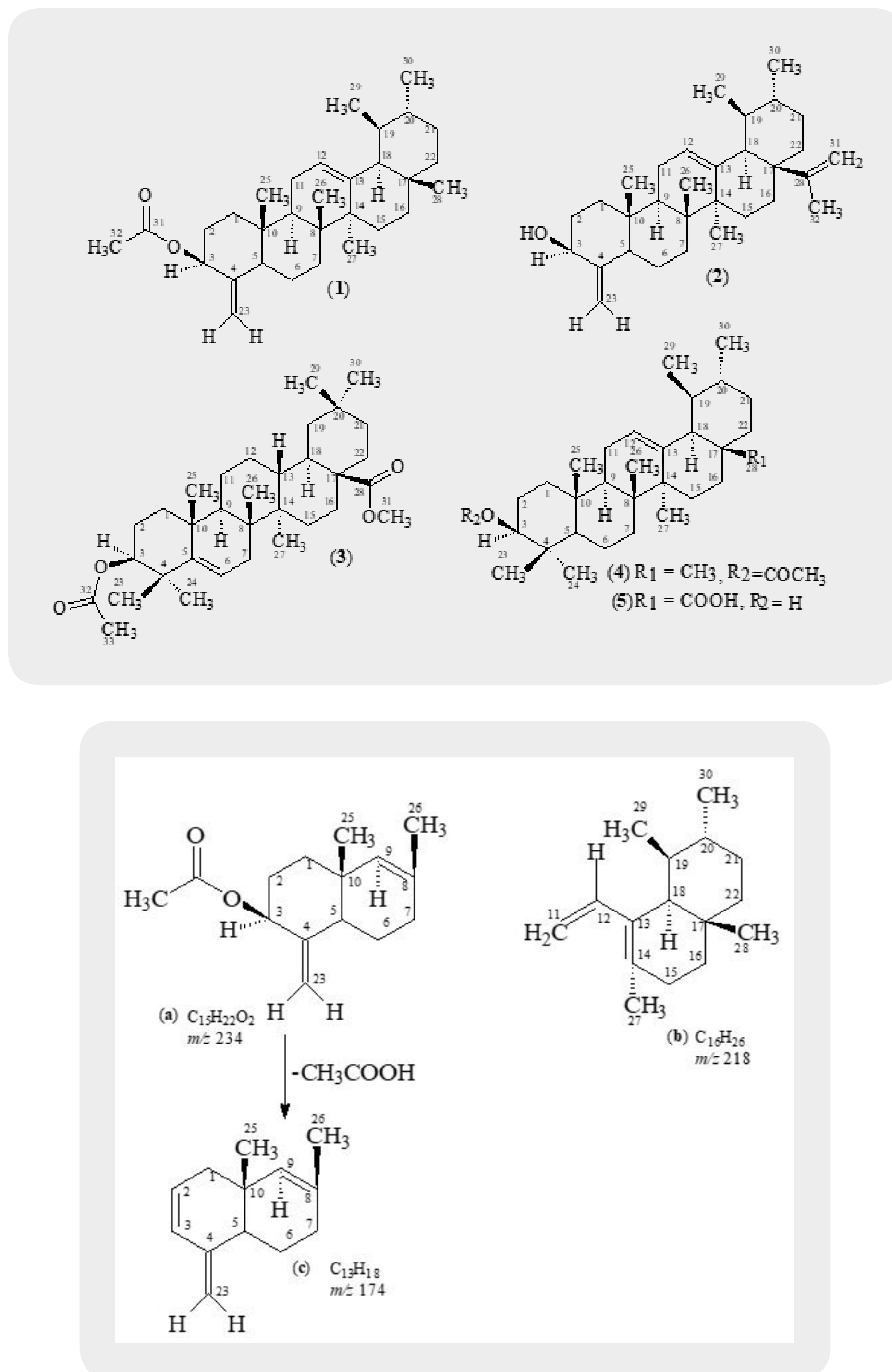

Figure 1: *Some selected mass fragments of 1*

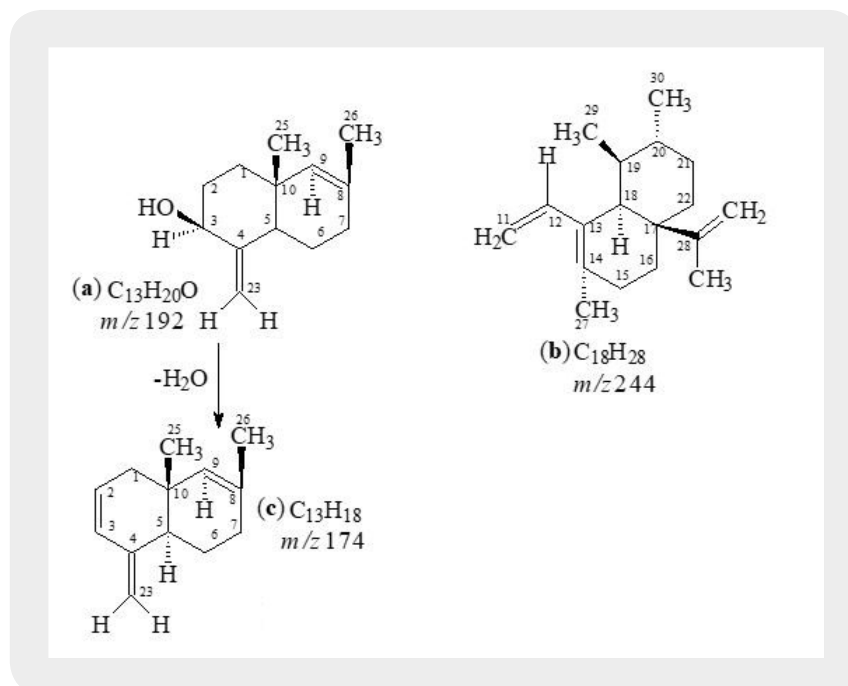

Figure 2: *Some selected mass fragments of 2*

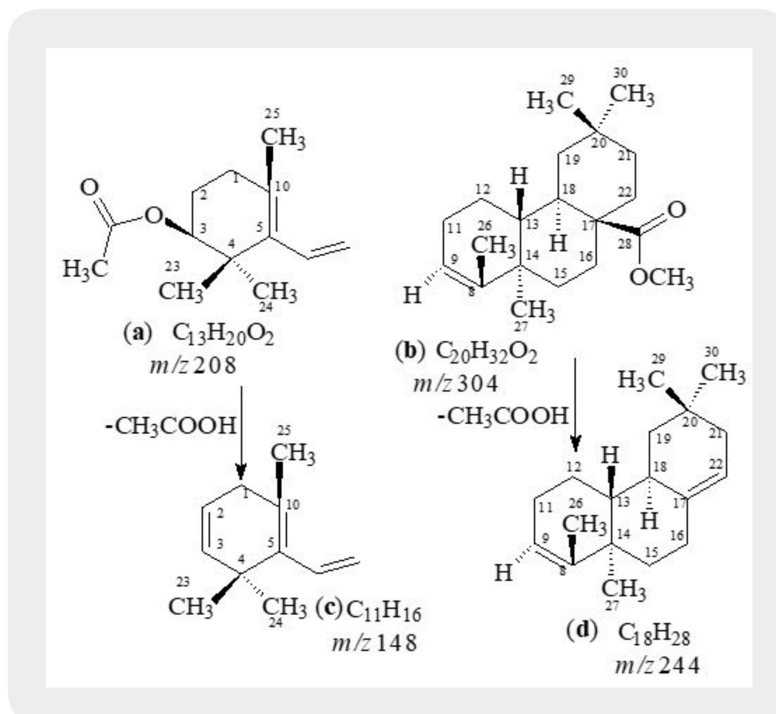

Figure 3: *Some selected mass fragments of 3*

The genus *Alstonia* comprises about twelve species. *Alstonia scholaris* Linn. R. Br. belongs to family Apocynaceae [1], grows throughout India, in deciduous and evergreen forests, also in plains [2,3]. Its timber is a non-durable hardwood, suitable for light indoor construction purposes, pulp and paper production. The wood has been used for school blackboards, hence the name 'scholaris'. They are milk bearing shrubs or trees, with large, entire, generally whorled leaves, and terminal cymes of white flowers.

The plant *Alstonia scholaris* has been used in different system of traditional medication for the treatment of diseases and ailments of human beings. It is reported to contain various alkaloids, flavonoids and phenolic acids. The flowers of *Alstonia scholaris* are known to contain lupeol and β-amyrin [4]. The bark contains alkaloids: ditaine, echitenine, echitamine (ditamine) and echitamidine together with triterpenes β-amyrin and lupeol [5-9]. It has been reported as antimicrobial, antiamoebic, antidiarrhoeal, antiplasmodial, hepatoprotective, immunomodulatory, anti-cancer, antiasthmatic, free radical scavenging, antioxidant, analgesic, anti-inflammatory, anti-ulcer, anti-fertility, anaemia, chronic diarrhea, dysentery, menstrual disorders, malarial fever, colic and acute arthritis and wound healing activities [10-14]. There are also reports available for the traditional use of this plant for its cardiotonic, anti-diabetic and anti-arthritic properties. The bark yields a tonic and antiseptic medicine. A concentrated decoction of trunk bark is used as a wash in furunculosis and impetigo, and as a gargle in dental caries. The bark, leaves and milky exudates of *Alstonia scholaris* are used in India [1-3].

3β -Acetate-24-nor-urs-4,12-diene ester triterpene (1), $C_{31}H_{48}O_2$, was obtained as a white solid mass by column and preparative thin-layer chromatography of the ethanolic extract of A. scholaris flowers.

The molecular composition of 1 was determined as $C_{31}H_{48}O_2$ by high-resolution electron-impact mass measurements of the M^+ m/z 452.3652 (calcd. 452.3654) which indicated eight degrees of unsaturation in the molecule.

The ^{1}H-NMR spectrum [11,12] ($CDCl_3$, 500MHz) of 1 showed five three-proton singlets (δ 2.11, 0.85, 0.95, 0.84, 1.23) and two methyls as doublets (δ 0.80, $J_{29,19}$ = 6.5Hz, 0.91, $J_{30, 20}$= 6.5Hz) indicating the presence of five tertiary methyls and two secondary methyls in the molecule as expected in a pentacyclic triterpenoidal skeleton (see experimental section).

The ^{13}C-NMR spectra (broad-band decoupled and DEPT [11,12] $CDCl_3$, 125MHz) of 1 exhibited signals for all 31 carbons and further supported the formula derived by mass spectrometric observations. DEPT spectra showed the presence of seven methyl, ten methylene, seven methine and (by difference from the broad-band decoupled spectrum) seven quaternary carbons (see experimental section).

Two-dimensional NMR techniques such as COSY 45°, HOHAHA, HMQC and HMBC [12-14] were used to obtain more structural information. The Homonuclear Hartmann Hahn spectrum (HOHAHA) [12-15] recorded with mixing delay of 100ms showed that the C-3 methine proton is coupled with four protons i.e. with the C-2 and C-1 methylenic protons (δ 1.23/1.25 and 1.61/1.81) respectively [16-18]. These observations led to define the structure of 1 as 3β -acetate-24-nor-urs-4,12diene ester triterpene.

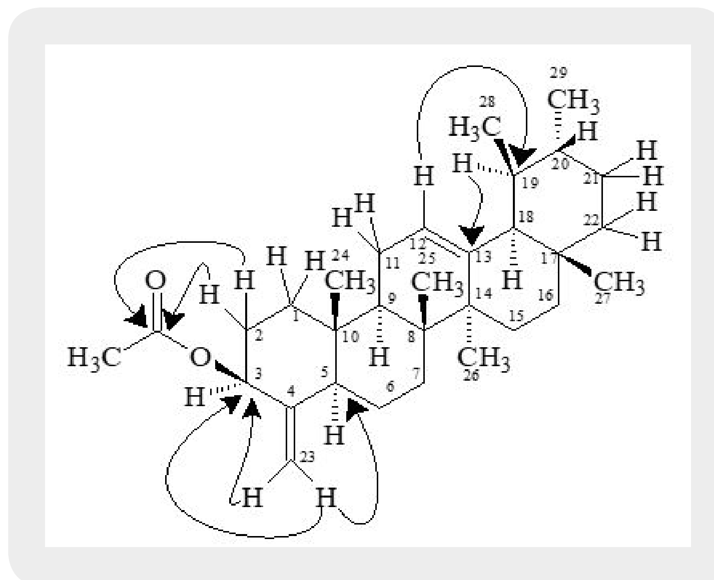

***Figure 4:** Selected multiple bond interactions of 1 observed in the HMBC experiment*

3β-hydroxy-24-nor-urs-4,12,28-triene triterpene (2), $C_{31}H_{48}O$, was obtained as a white amorphous substance by column and preparative thin-layer chromatography of the ethanolic extracts of *A. scholaris*. The molecular composition was determined as $C_{31}H_{48}O$ by high-resolution electron-impact mass measurements of the M^+ (m/z 436). The UV spectrum recorded in methanol showed terminal absorption only, while the infrared spectrum displayed absorptions for hydroxyl (OH, 3415 cm^{-1}) and C = C (1630 cm^{-1}) groups.

The ^{1}H-NMR spectrum [11,12] ($CDCl_3$, 500MHz) of 2 showed four three-proton singlets at δ 0.84, 0.95, 0.85, 1.23 and two methyls as doublets (δ 0.80, d, J = 6.8 Hz, 0.91, J = 6.6 Hz) indicating the presence of four tertiary methyls and two secondary methyls in the molecule as expected in a pentacyclic triterpenoidal skeleton. The downfield region of the spectrum contained only three signals i.e. a broad double doublet at δ 3.54 ($J_{3\alpha, 2\beta}$ = 12.4Hz, $J_{3\alpha, 2\alpha}$ = 4.5Hz), four close doublets two at δ 4.57 (d, $J_{23\alpha, 23\beta}$ = 2.4Hz), 4.68 (d, $J_{23\beta,23\alpha}$ = 2.4Hz) and another two at 4.54 (d, $J_{31\alpha, 31\beta}$ = 2.5Hz) δ 4.65 (d, $J_{31\beta, 31\alpha}$ = 2.5Hz) and a tripplet at δ 5.11 (t, J = 3.26Hz), which could be assigned to the hydroxy-bearing C-3 methine, C-23, 31 methylene and the vinylic C-12 protons, respectively. The chemical shift and coupling constants of the C-3 methine signal indicated an equatorial (β) orientation of the OH group [19].

The ^{13}C-NMR spectra (broad-band decoupled and DEPT [11,12] CD3OD, 100MHz) exhibited signals for all 31 carbons and further supported the formula derived by mass spectrometric observations. The ^{13}C-NMR broad-band spectrum ($CDCl_3$, 100MHz) of 2 showed the resonances of 31 carbon atoms in the molecule. DEPT spectra showed the presence of six methyl, eleven methylene, seven methine and (by difference from the broad-band decoupled spectrum) seven quaternary carbons. The downfield signals at δ 79.01 and 124.34 were due to the hydroxy-bearing C-3 and vinylic C-12, respectively. The six methyl carbons resonated at δ 15.72, 17.48, 28.06, 23.53, 28.11 and 19.74 in the ^{13}C-NMR spectra. The assignments to the various carbons in the molecule are presented in experimental section.

Two-dimensional NMR techniques such as COSY 45°, HOHAHA, HMQC and HMBC [11-13] were used to obtain more structural information. The one-bond $^{13}C/^{1}H$ couplings were determined by the HMQC spectrum see experimental section. The Homonuclear Hartmann Hahn spectrum (HOHAHA) [15,16,20-23] recorded with mixing delay of 100ms showed that the C-3 methine proton is coupled with four protons i.e. with the C-2 and C-1 methylenic protons (δ1.23/1.25 and 1.61/1.81) respectively. The another two downfield exocyclic methylene protons appearing in the COSY-45° spectrum (4.54 and 4.65) not only displayed geminal coupling interactions but also gave strong cross peaks with the C-32 proton (δ 1.64/4.54, 4.65) in the same 2D experiment indicating the presence of the isopropene protons. These observations led to define the structure of 2 as 3β-hydroxy-24-nor-urs-4,12,28-triene triterpene.

3,28-β-diacetoxy-5-olea- triterpene (3), $C_{33}H_{52}O_4$, was obtained as a white amorphous compound by column and preparative thin-layer chromatography of the ethanolic extract of *A. scholaris* flowers. The UV spectrum recorded in methanol showed terminal absorption only, while the infrared spectrum displayed absorptions for C=O of ester group (1735cm^{-1}) and C = C (1630cm^{-1}) groups [19,24-26]. The molecular composition was determined as C33H52O4 by high-resolution electron-impact mass measurements of the M^+ (m/z 530).

The ^{1}H-NMR spectrum (CDCl3, 500MHz) of 1 showed nine three-proton singlets at δ 2.13, 2.10, 1.15, 1.08, 0.97, 0.95, 0.94, 0.85 and 0.84 indicating the presence of nine tertiary methyls in the molecule as expected in a pentacyclic triterpenoidal skeleton. The downfield singlets at δ 2.10 and 2.13 were due to the C-31, C-33 acetate methyls. The downfield region of the spectrum also contained two signals i.e. a double doublet at 4.50 (dd, $J_{3\alpha, 2\alpha}$= 6.12, $J_{3\alpha,2\beta}$= 14.5Hz) and a broad double doublet at δ 5.62 ($J_{6,7\alpha}$ = 4.0 Hz, $J_{6,7\beta}$= 12.5 Hz), which could be assigned to the hydroxy-bearing C-3 methine and the vinylic C-6 protons, respectively [27]. A comparison of ^{1}H-NMR chemical shifts of 1 with 3α-hydroxy-D-friedoolean-5-ene was also done [10].

The ^{13}C-NMR spectra (broad-band decoupled and DEPT [10,12] $CDCl_3$, 100MHz) of 3 exhibited signals for all 33 carbons and further supported the formula derived by mass spectrometric observations. DEPT spectra showed the presence of nine methyl, ten methylene, five methine and (by difference from the broad-band decoupled spectrum) nine quaternary carbons. The downfield signals at δ 80.94, 121.70 and 170.96 were due to the acetate-bearing C-3, vinylic C-6 and acetate carbonyl C-28 respectively. The eight methyl carbons resonated at δ 31.87, 31.49, 29.20, 20.00, 19.39, 19.04, 18.04 and 12.05 in the ^{13}C-NMR spectra. Two-dimensional NMR techniques such as COSY- 45°, HOHAHA [17,18,28], HMQC and HMBC [13] were used to obtain structural information.

The Heteronuclear Multiple Quantum Coherence [13] (HMQC) spectrum of 3 displayed cross peaks between directly coupled carbon-proton pairs. HMQC interactions are presented in experimental section.

The Heteronuclear Multiple Bond Connectivity [13] (HMBC) spectrum was also very informative in determining the position of the double bond between C-5 and C-6, since the C-6 vinylic proton (δ 5.62) showed long-range interactions with C-4 (δ 40.45) and C-9 (δ 51.24). The C-3 proton (δ 4.50) showed coupling with C-5 (δ 140.78).

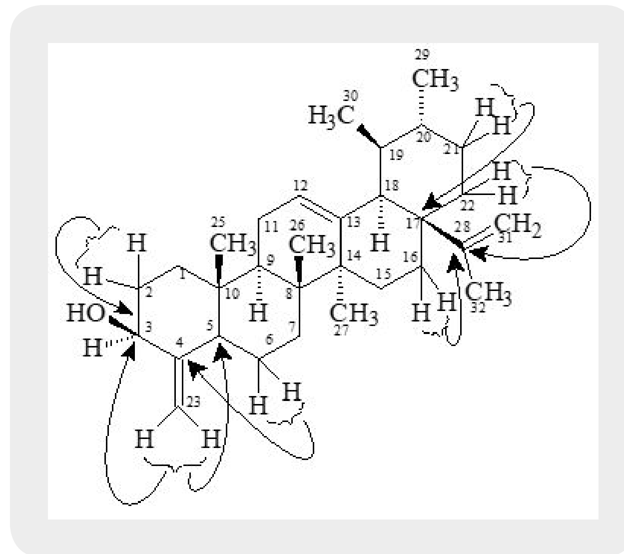

Figure 5: *Selected multiple bond interactions of 2 observed in the HMBC experiment*

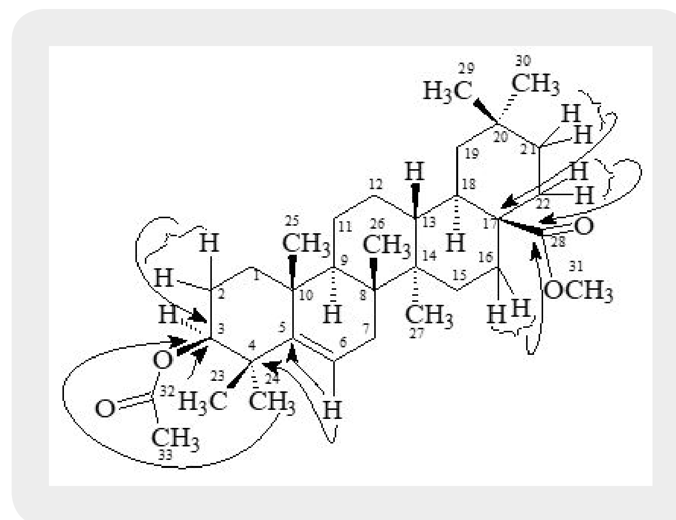

Figure 6: *Selected multiple bond interactions of 3 observed in the HMBC experiment*

α-Amyrin acetate (4) M.P. 166-170 was isolated as a white amorphous solid from the pet. ether extract of *A. scholaris* by column and thin-layer chromatography. The high resolution electron-impact mass spectrum of 4 showed the molecular ion-peak at m/z 468.3708, which corresponded to the molecular formula $C_{32}H_{52}O_2$. ^{13}C-NMR chemical shifts of C-12 and C-13 at δ 124.33 and 139.63 respectively suggested that 4 belongs to $^{\Delta}$12-α amyrin series of triterpenoids with ester group in A/B rings.

Ursolic acid (5) was isolated as a greenish white powder from the pet. ether extract of *A. scholaris* by column and thin-layer chromatography. Compounds 4 and 5 were isolated for the first time from the flowers of this plant [4] and had not been previously isolated.

These compounds were analyzed for antioxidant and antiurease inhibition test. Compounds 2 and 4 were found to be antioxidant with IC_{50} 107±0.39 and > 500μM respectively, while compounds 2 and 4 were also found to be urease inhibitors with IC_{50} 175 ±0.26 and 218±0.14 μM, respectively Table 1. The quantity of 1 and 3 was short, hence its activity could not be checked. BHA (IC_{50} = 44.3±0.09 μM) and thiourea (IC_{50} = 21.0±0.08) were used as a positive control [29,30].

Table 1: *In vitro quantitative inhibition of antioxidant and urease by compounds 1, 2 and 4*

Compound	IC_{50}(μM) Antioxidant activity	IC_{50}(μM) Urease inhibition activity
2	107 ± 0.39	176 ± 0.26
4	>500	218 ± 0.14
BHA	44.3± 0.09	-
Thiourea	-	21.9 ± 0.08

Experimental Section

General Experimental Procedures

The mass spectra were recorded on a Jeol HX-110 instrument. The ^{1}H- and ^{13}C-NMR spectra were recorded in $CDCl_3$ at 500, 400 and 125, 75MHz, respectively, on a Bruker AM-500, 400 NMR spectrometer. The UV and IR spectra were recorded on Shimadzu UV-240 and JASCO A-320 spectrophotometers, respectively. Optical rotations were measured on a polatronic D Polarimeter. The purity of the compounds was checked on TLC (si-gel, Merck PF_{254}, 0.25mm thickness). Melting points were determined in glass capillary tubes using Buchi 535 and Gallenkamp 30/MF-370 melting point apparatus.

Plant Material

The flowers of *Alstonia scholaris*, (5kg) were collected from university campus Kashmir in October 2006. A voucher specimen (AKUH # 58106) was deposited in the Herbarium of Department of Botany, University of Azad Kashmir.

Extraction and Isolation

Air-dried flowers of *Alstonia scholaris* (5kg dry weight) were extracted with MeOH (50L). The MeOH extract was concentrated to a gum (822gm), dissolved in distd. water and extracted thoroughly with pet. ether (25L). The pet. ether soluble portion was evaporated under reduced pressure to yield a gum (66.92gm) which was chromatographed on a si-gel column (Merck, 70-230 mesh, 2025.01gm). The elution of the column was initiated with pet. ether. The combined column sub-fractions 1-8 (5.91gm) obtained by elution with 5:95 ethyl acetate-pet. ether, which showed similar tlc behaviour upon spraying with ceric sulphate reagent were combined and again subjected to CC using silica gel (type 60, 70-230 mesh, 200.10gm) and column was eluted with pet.ether- ethyl acetate (99:1). The sub-fractions 6-30 (1.86gm), which showed similar tlc behaviour were combined and further purified on preparative tlc plates using a solvent system

of pet.ether- ethyl acetate (98:2) to afford pure compound 1 (19.5mg, 9.5 x 10-5% yield). The fractions obtained on elution of the column with n-hexane-ethyl acetate (10:90) were checked on TLC. Fractions 7-18 showed similar behaviour on TLC were combined and further purified by preparative TLC (Merck PF_{254} ,0.2mm) using $CHCl_3$ as eluent to afford pure compound 2 (28mg, 1.4 x 10^{-4} % yield). Elution of the major column which was loaded with 66.92g of pet.ether soluble material was eluted with 30% ethyl acetate -pet.ether yielded an impure mixture (7.83gm). This mixture was again subjected to CC (silica gel, 70-230 mesh, 60.20gm). The sub-fractions 6-30 (1.86gm), which showed similar tlc behaviour were combined and further purified on preparative tlc plates using a solvent system of pet.ether-ethyl acetate (90:10) to afford pure compound 3 (19.5mg, 9.5 x 10^{-5}% yield). The fractions obtained with 30: 70 ethyl acetate-pet.ether yielded an impure compound 4, which was further purified by preparative tlc using a solvent system of pet. ether- ethyl acetate (70:30) to obtain pure 4 (20mg, 10 x 10^{-4}% yield). Fractions 99-145 (1.91gm) obtained by elution with pet. ether- ethyl acetate (35: 65, 500ml each) were collected and again subjected to CC (70-230 mesh, 60.20gm). The sub-fractions 7-18 (0.92gm, 500ml each) obtained with 80: 20 hexane- ethyl acetate showed similar behaviour on TLC (Ceric sulphate active) were combined and further purified on preparative TLC (Merck PF_{254} ,0.2mm) using hexane- ethyl acetate 70:30 as eluent to obtain pure 5 (28mg, 1.4x 10^{-4} % yield, with R_f = 0.1).

3β-acetate-24-nor-urs-4, 12-diene ester triterpene (1)

White solid substance, $[\alpha]^{29}_D$ = 40, UV: (MeOH) λ_{max} nm: terminal absorbtion only, IR: ($CHCl_3$) ν_{max} cm^{-1}, 1732 (C=O), 1630 (C=C), HREI MS: m/z 234.1618 (calcd. 234.1619), 218.2032 (calcd. 218.2034), 174.1406 (calcd.174.1408), F.D. 452, MS: m/z 452.3652 (calcd. 452.3654); ^{1}H-NMR: ($CDCl_3$, 500MHz), ^{13}C-NMR ($CDCl_3$, 125MHz) δ; data is presented in Tables-1, 2.

Table 2: *^{1}H-NMR (# δ ppm) data of compounds 1, 2 and 3*

H. NO	1 δ^{1}H (# δ ppm) M* J(Hz)	2 δ ^{1}H (# δ ppm) M* J(Hz)	3 δ ^{1}H (# δ ppm) M* J(Hz)
1.	1.23 (m), 1.25 (m)	1.23 (m), 1.25 (m)	1.45 (m), 2.00 (m)
2.	1.61 (m), 1.81 (m)	1.61 (m), 1.81 (m)	1.65 (m), 1.85 (m)
3.	4.50 (dd, $J_{3\alpha, 2\alpha}$ = 6.12, $J_{3\alpha,2\alpha}$ 14.5 Hz)	3. 54 (dd, $J_{3\propto, 2\alpha}$ = 4.5, $J_{3\propto, 2\beta}$ = 12.4	4.50 (dd, $J_{3\alpha, 2\alpha}$ = 6.12, $J_{3\alpha,2\beta}$ =14.5)
4.	-	-	-
5.	0.92 (m)	0.92 (m)	-
6.	1.42 (m), 1.50 (m)	1.42 (m), 1.50 (m)	5.62 (dd, $J_{6,7\alpha}$ = 12.5, $J_{6,7-\beta}$ = 4.0)
7.	1.30 (m), 1.40 (m)	1.30 (m), 1.40 (m)	1.82 (m), 1.95 (m)
8.	-	-	-
9.	1.56 (m)	1.56 (m)	2.03 (m)
10.	-	-	-

11.	1.52 (m), 1.62 (m)	1.52 (m), 1.62 (m)	1.46 (m), 1.52 (m)
12.	5.11 (t, $J_{12,11}$ = 3.26 Hz)	5.11 (t, $J_{12,11}$ = 3.26)	1.38 (m)
13.	-	-	0.89 (m)
14.	-	-	-
15.	1.33 (m), 1.14 (m)	1.33 (m), 1.14 (m)	1.30 (m), 1.13 (m)
16.	1.40 (m), 1.52 (m)	1.40 (m), 1.52 (m)	1.38 (m), 1.51 (m)
17.	-	-	-
18.	2.20 (d, $J_{18,19}$ = 11.2 Hz)	2.20 (d, $J_{18,19}$ = 11.2)	1.51 (m)
19.	1.99(m)	1.99(m)	1.50-1.58 (m)
20.	1.95(m)	1.95(m)	-
21.	-	-	1.00, 1.21(m)
22.	-	-	1.20-1.31 (m)
23.	4.57 (d, $J_{23\alpha, 23\beta}$ = 2.4 Hz 4.68 (d, $J_{23\beta, 23\alpha}$ = 2.4Hz)	4.57 (d, $J_{23\alpha, 23\beta}$ = 2.4) 4.68 (d, $J_{23\beta, 23\alpha}$ = 2.4)	1.07 (s)
24.	-	-	0.85 (s)
25.	0.85 (s)	0.85 (s)	0.95 (s)
26.	0.95 (s)	0.95 (s)	0.84 (s)
27.	0.85 (s)	0.84 (s)	0.97 (s)
28.	1.23 (s)	1.23 (s)	1.15 (s)
29.	0.80 (d, $J_{29,19}$ = 6.8 Hz)	0.80 (d, $J_{29,19}$ = 6.8)	0.94 (s)
30.	0.91 (d, $J_{30,20}$ = 6.6 Hz)	0.91 (d, $J_{30,20}$ = 6.6)	1.08 (s)
31.	2.11 (s)	4.54 (d, $J_{31\alpha, 31\beta}$ = 2.5) 4.65 (d, $J_{31\beta, 31\alpha}$ = 2.5)	2.10 (s)
32.	-	1.64 (s)	-
33.	-	-	2.13 (s)

* Multiplicity

Table 3: *^{13}C-NMR (# δ ppm) data of compounds 1, 2 and 3*

C.NO	1 (# δ ppm) M♣	2 (# δ ppm) M♣	3 (# δ ppm) M♣
1.	41.55 (CH_2)	41.55 (CH_2)	39.79 (CH_2)
2.	27.12 (CH_2)	27.12 (CH_2)	28.23 (CH_2)
3.	80.98 (CH)	79.01 (CH)	80.94 (CH)
4.	150.94 (C)	156.12 (C)	40.45 (C)
5.	55.41 (CH)	55.41 (CH)	140.78 (C)
6.	18.26 (CH_2)	18.26 (CH_2)	121.70 (CH)

7.	32.88 (CH_2)	32.88 (CH_2)	24.36 (CH_2)
8.	38.51 (C)	38.51 (C)	35.88 (C)
9.	47.65 (CH)	47.65 (CH)	51.24 (CH)
10.	37.11 (C)	37.11 (C)	34.86 (C)
11.	21.09 (CH_2)	21.09 (CH_2)	21.09 (CH_2)
12.	124.34 (CH)	124.34 (CH)	37.28 (CH_2)
13.	139.64 (C)	139.64 (C)	40.46 (CH)
14.	42.86 (C)	42.86 (C)	40.05 (C)
15.	29.87 (CH_2)	29.87 (CH_2)	31.68 (CH_2)
16.	23.71 (CH_2)	23.71 (CH_2)	24.36 (CH_2)
17.	40.82 (C)	40.82 (C)	48.67 (C)
18.	59.09 (CH)	59.09 (CH)	50.11 (CH)
19.	39.62 (CH)	39.62 (CH)	42.32 (CH_2)
20.	39.66 (CH)	39.66 (CH)	29.34 (C)
21.	28.11 (CH_2)	28.11 (CH_2)	33.98 (CH_2)
22.	38.06 (CH_2)	38.06 (CH_2)	29.68 (CH_2)
23.	109.35 (CH_2)	109.31 (CH_2)	12.05 (CH_3)
24.	-	-	29.20 (CH_3)
25.	15.72 (CH_3)	15.72 (CH_3)	18.04 (CH_3)
26.	17.48 (CH_3)	17.48 (CH_3)	19.04 (CH_3)
27.	28.06 (CH_3)	28.06 (CH_3)	31.49 (CH_3)
28.	28.74 (CH_3)	150.96 (C)	170.97 (C)
29.	23.53 (CH_3)	23.53 (CH_3)	19.39 (CH_3)
30.	28.11 (CH_3)	28.11 (CH_3)	31.87 (CH_3)
31.	-	105.93 (CH_2)	20.00 (CH_3)
32.	-	19.74(CH_3)	170.96(C)
33	21.29 (CH_3)	-	21.15 (CH_3)

♣ Multiplicity assignments based on DEPT experiment
One-bond heteronuclear correlations determined by HMQC experiment

3β-Hydroxy-24-nor-urs-4, 12, 28-triene triterpene (2)

White amorphous substance, $[\alpha]^{29}_{D}$ = 40 ($CHCl_3$), UV: (MeOH) λ_{max} nm: only terminal absorbtion, IR ($CHCl_3$) ν_{max} cm^{-1} hydroxyl (OH, 3415 cm^{-1}) and C = C (1630 cm^{-1}) groups, HREI MS: m/z 192.1513 (calcd. 192.1514, $C_{13}H_{20}O$) and m/z 244.2191 (calcd. 244.2190, $C_{18}H_{28}$), 174.1406 (calcd. 174.1408, $C_{13}H_{18}$ fragment c); F. D. 436; MS: m/z 436; ^{1}H-NMR: ($CDCl_3$, 500 MHz), ^{13}C-NMR (CD_3OD, 100MHz) δ; data is presented in Tables 1-2.

3,28-β-Diacetoxy-5-olea-triterpene (3)

White amorphous compound; $[\alpha]_D^{29}$ = 64.5° ($CHCl_3$); UV: (MeOH) λ_{max} nm: only terminal absorbtion, IR ($CHCl_3$) ν_{max} cm^{-1} (C=O, 1735 cm^{-1}), (C=C, 1630 cm^{-1}); EIMS: m/z 530, HREI MS: m/z 208.1462 (calcd. 208.1463), 304.2403 (calcd. 304.2402), 148.1252 (calcd. 148.1251) and 244.2191 (calcd. 244.2190); ^{1}H-NMR (500MHz, $CDCl_3$); δ: ^{13}C-NMR (100MHz, CD_3OD) data is presented in Tables 1-2.

α-Amyrin acetate (4)

White amorphous solid; M. P. = 166-170°C, $[\alpha]_D^{29}$ = + 83.00° (CHCl3); UV λ_{max} (MeOH) nm, only terminal absorption; IR ($CHCl_3$) cm^{-1}; 2850 (C-H), 1630 (C=C),1715 (C=O), 1136 and 1369 (C-O) cm^{-1} EI-MS gave m/z at 469 ($M^{\cdot}$ + H, $C_{32}H_{52}O_2$), 454 ($M^{\cdot}$ + H -CH_3), 409 (42%), 394 (28%), 357 (15%), 298 (11%), 273 (39%), 267 (38%), 249 (68%), 232 (24%), 218 (100%), 203 (73%), 189 (75%), 175 (66%), ^{1}H-NMR showed signals at δ_{ppm}= 0.78 (s, 1 x CH_3), 0.86 (s, 2 x CH_3), 0.87 (s, 2 x CH_3), 0.97 (s, 1 x CH_3), 1.00 (s, 1 x CH_3), 1.06 (s, 1 x CH_3), 2.02 (s, $COCH_3$), 4.50 (m, 1-3β-H), 5.11 (t, J= 3.6 Hz, C-12H), ^{13}C-NMR showed signals at 171.0, 139.71,124.41, 81.02, 59.16, 55.35, 47.73,42.82, 39.73, 39.72, 39.62, 38.56, 36.89, 34.82, 33.36, 31.32, 28.79, 28.45, 28.18, 26.68, 23.68, 23.44, 23.29, 23.29, 21.34, 18.32, 17.55, 16.94, 16.79, 15.77, ^{1}H-NMR ($CDCl_3$, 500MHz) δ: ^{13}C-NMR ($CDCl_3$, 125MHz) [31-33].

Ursolic acid (5)

Greenish white powder, $[\alpha]_D^{29}$ = 59 (c = 0.3, pyridine); M. P. 283-285 °C, UV λ_{max} (MeOH) nm; only terminal absorption, EIMS m/z: 456.4 $[M]^{+\cdot}$, 438.4, 423.4, 300.3, 248.3, 203.2, 133.1; HRMS m/z: 456.3603 ($C_{30}H_{48}O_3$), IR ($CHCl_3$) ν_{max}; ^{1}H-NMR ($CDCl_3$, 400MHz); ^{13}C-NMR ($CDCl_3$, 125MHz) [29].

Determination of DPPH Radical Scavenging Activity

The free radical scavenging activity was measured by 1,1-diphenyl-2-picryl-hydrazil (DPPH) [30]. The solution of DPPH of 0.3mM was prepared in ethanol. Five microlitres of each sample of different concentration (62.5µg - 500µg) was mixed with 95µl of DPPH solution in ethanol. The mixture was dispersed in 96 well plate and incubated at 37°C for 30min. The absorbance at 515nm was measured by microtitre plate reader (Spectramax plus 384 Molecular Device, USA) and percent radical scavenging activity was determined in comparison with the methanol treated control. BHA is used as standard.

DPPH scavenging effect (%) = Ac - As/Ac × 100

Where, Ac = Absorbance of Control (DMSO treated)
As = Absorbance of Sample

Urease Assay and Inhibition

Reaction mixtures comprising 25µL of enzyme (Jack bean Urease) solution and 55µL of buffers containing 100mM urea were incubated with 5µL of test compounds (1mM concentration) at 30°C for 15 min in

96-well plates. Urease activity was determined by measuring ammonia production using the indophenol method as described by Weatherburn [34]. Briefly, 45µL each of phenol reagent (1% w/v phenol and 0.005% w/v sodium nitroprusside) and 70µL of alkali reagent (0.5% w/v NaOH and 0.1% active chloride NaOCl) were added to each well. The increasing absorbance at 630nm was measured after 50 min, using a microplate reader (Molecular Device, USA). All reactions were performed in triplicate in a final volume of 200µL. The results (change in absorbance per min) were processed by using SoftMax Pro software (Molecular Device, USA). All the assays were performed at pH 8.2 (0.01M $K_2HPO_4.3H_2O$, 1mM EDTA and 0.01M $LiCl_2$). Percentage inhibitions were calculated from the formula 100-(ODtestwell/ODcontrol) x100. Thiourea was used as the standard inhibitor of urease.

Acknowledgement:

The authors wish to thank the Ministry of Science and Technology, government of Pakistan for providing the spectroscopic facilities for the current study.

Bibliography

1. Kirtikar, K. R. & Basu, B. D. (1980). Indian Medicinal Plants, Vol. II, Bhushen Singh and Mahendra Pal Singh, Dehradun, 111.

2. Nadkarni, A. K. & Nadkarni, K. M. (1976). Indian Materia Medica. Vol. I, Popular Prakashan, Bombay, 80.

3. The Wealth of India (2004). Raw Materials, Vol. I, CSIR, New Delhi,s 50.

4. Dhar, D. N., Suri, S. C. & Dwivedi, P. (1977). Chemical examination of the flowers of *Alstonia scholaris*. *Planta Medica, 31*(1), 33-34.

5. Patil, R. S., Juvekar, A. R., Joglekar, S. N., Shamkuwar, P. B. & Nimbkar, S. R. (1999). Study of antidiarrhoeal activity of *Alstonia scholaris* bark. *Indian Drugs, 36*(7), 463.

6. Lin, C. C., Lin, Y. H., Supriyatna, S. S. & Pan, S. L. (1996). The protective effect of *Alstonia scholaris* R. Br. on hepatotoxin-induced acute liver damage. *Am. J. Clin. Med., 24*(2), 153.

7. Gandhi, M. & Vinayak, V. K. (1990). Preliminary evaluation of extracts of *Alstonia scholaris* bark for in vivo antimalarial activity in mice. *J. Ethnopharmacol., 29*(1), 51-57.

8. Keawpradub, N., Kirby, G. C., Steele, J. C. P. & Houghton, P. J. (1999). Antiplasmodial activity of extracts and alkaloids of three Alstonia species from Thailand. *Planta Medica, 65*(8), 690-694.

9. Khan, M. R., Omoloso, A. D. & Kihara, M. (2003). Antibacterial activity of *Alstonia scholaris* and Leea tetramera. *Fitoterpia, 74*(7-8), 736-740.

10. Sultana, N., Khan, M. A., Ali, Y. & Afza, N. (2005). *Pak. J. Sci. Ind. Res., 48*(3), 180-183.

11. Bendal, M. A. & Pegg, D. T. (1983). Complete accurate editing of decoupled 13C spectra using DEPT and a quaternary-only sequence. *J. Mag. Reson., 53*(2), 272-296.

12. Atta-ur-Rahman (1986). *Nuclear Magnetic Resonance Spectroscopy.* Basic Principles, Springer-Verlag, New York, 227.

13. Atta-ur-Rahman (1989). *One-and Two-Dimentional NMR Spectroscopy.* Elsevier Science Publishers, Amsterdam, 406.

14. Sultana, N., Atta-ur-Rahman & Khalid, A. (2008). A new fatty ester and a new triterpene from Skimmia laureola. *Nat. Prod. Res., 22*(1), 37-47.

15. Sultana, N. Atta-ur-Rahman & Khalid, A. (2006). New natural cholinesterase inhibiting and calcium channel blocking quinoline alkaloids. *J. Enzym. Inhib. Med. Chem., 21*(6), 703-710.

16. Atta-ur-Rahman, Sultana, N., Choudhary, M. I., Shah, P. M. & Khan, M. R. (1998). Isolation and structural studies on the chemical constituents of skimmialaureola. *J. Nat. Prod., 61*(6), 713-717.

17. Sultana, N., Khan, A. & Choudhary, M. I. (2009). Protein glycation inhibitory activities of *Lawsonia inermis* and its active principles. *J. Enzym. Inhib. Med. Chem., 24*(1), 257-261.

18. Choudhary, M. I., Batool, I., Sultana, N. & Atta-ur-Rahman (2008). Microbial transformation of oleanolic acid by Fusarium lini and alpha-glucosidase inhibitory activity of its transformed products. *Nat. Prod. Res., 22*(6), 489-494.

19. Atta-ur-Rahman, Sultana, N., Jahan, S. & Choudhary, M. I. (1998). Phytochemical Studies on *Skimmia Laureola*. *Nat. Prod. Lett., 12*(3), 223-229.

20. Sultana, N. Atta-ur-Rahman & Khan, T. H. (2005). Tyrosinase Inhibitor Fatty Ester and a Quinoline Alkaloid from *Skimmia laureola. Z. Naturforsch. B., 60b,* 1186-1191.

21. Atta-ur-Rahman, Sultana, N., Jahan, S. & Choudhary, M. I. (2005). Studies on the Constituents of *Commiphora mukul. Z. Naturforsch., 60b,* 1202-1206.

22. Fatima, K. & Sultana, N. (2003). Studies on Bioassay Directed Antifungal Activity of Medicinal Plants Calotropis procera, Skimmia laureola, Peltophorum pterocarpum and two pure Natural compounds ulopterol and 4-methoxy-l-methyl- 3-(2 'S-hydroxy-3 '-ene butyl)-2-quinolone. *J. Chem. Soc. Pak., 25*(4), 328-330.

23. Fatima, K. & Sultana, N. (2003). Biological Studies on Fruit Pulp and Seeds of Annona Squamosa. *J. Chem. Soc. Pak., 25*(4), 331-334.

24. Atta-ur-Rahman, Sultana, N. & Choudhary, M. I. (2002). Triterpene and coumarins from Skimmia laureola. *Nat. Prod. Lett., 16*(5), 305-313.

25. Sultana, N. (2000). Phytochemical and structural studies on the chemical constituents of Adhatoda vasica, Sarcococca saligna and Skimmia laureola. Ph. D. Dissertation, HEJ.

26. Atta-ur-Rahman, Sultana, N., Choudhary, M. I., Shah, P. M. & Khan, M. R. (1998). Isolation and structural studies on the chemical constituents of skimmialaureola. *J. Nat. Prod., 61*(6), 713-717.

27. Ituka, A. & Itokama, H. (1986). Triterpenoids of Akebia quinata callus tissue. *Phytochemistry, 25*(7), 1625-1628.

28. Sultana, N. & Khalid, A. (2010). Phytochemical and enzyme inhibitory studies on indigenous medicinal plant *Rhazya stricta. J. Enzym. Inhib. Med. Chem., 24*(4), 305-314.

29. Brieskornch & Hofmannh (1962). [On the quantitative determination of ursolic acid and oleanolic acid by means of the Liebermann-Burchard reaction]. *Arch Pharm,* 295/67, 505-509.

30. Lee, S. K., Zakazia, H., Chung, H., Lovergi, L., Ganez, E. J. C., Mehta R. J., et al. (1998). Evaluation of the antioxidant potential of natural products. *Combinatorial Chemistry and High Theayhput Screening, 1*(1), 35-46.

31. Mahato, S. B. & Sen, S. (1997). Advances in triterpenoid research, 1990-1994, *Phytochemistry, 44,* 1185-1236.

32. Gallegos, R. S. & Roque, N. E. (1990). Analyse the mixture of triterpenoids for ^{13}C-NMR, Quimica. *Nova, 13,* 278-281.

33. Nusrat, J., Wasim, A. & Malik, A. (1995). New Steroidal Glycosides from Mimusops elengi. *J. Nat. Products., 58*(8), 1244-1247.

34. Khan, K. M., *et al.* (2004). Biscoumarin: new class of urease inhibitors; economical synthesis and activity. *Bioorg. Med. Chem., 12*(8), 1963-1968.