ISOLATION AND BIOLOGICAL SCREENING OF SECONDARY METABOLITES FROM STEM BARK OF *ALSTONIA SCHOLARIS* (L.) R. Br.

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ABSTRACT

Alstonia scholaris (L.) R. Br. is commonly known as Saptaparni or Devil's tree used in various traditional medications for the cure of several human ailments. As a result of our investigation on the stem bark of *Alstonia scholaris,* twelve secondary metabolites have been isolated, out of which compounds **2**, **3**, **7** and **9** have been isolated first time from the genus *Alstonia.* Structures of the isolates were characterized with the help of spectroscopic techniques. All the isolates were subjected to biological screening but only two showed DPPH radical scavenging activity and inhibitory potential against the enzymes lipoxygenase and urease. Diospyrolide (**3**) and 19-20-*Z*-vallesamine (**1**) exhibited excellent urease inhibitory and antioxidant potential with the IC_{50} value 19.2 ± 0.10 (21.5 ± 0.47) and 24.9 ± 0.11 (44.2 ± 0.28), respectively.

Key-words: *Alstonia scholaris* stem bark, phytochemistry, antioxidant activity, lipoxygenase and urease inhibition.

INTRODUCTION

The family Apocynaceae is widely distributed in South and Southeast Asia and comprises of 250 genera and 2000 species mostly growing as trees, shrubs and vines. The most important genera of this family are *Rauwolfia* and *Alstonia* which together provide a large reservoir of alkaloids of immense therapeutic values (Manodeep *et al*., 2012; Chatterjee *et al*., 1956). *Alstonia* having of around 60 members which are commonly found almost in all parts of hemisphere, but are particularly abundant in Africa and Asia. One of these is *A. scholaris* (L.) R. Br. which is commonly known as Devil's tree. It was identified by Robert Brown, the famous Scottish Botanist (Srivastava *et al*., 2016). Although, lots of species of the *Alstonia* have been investigated for their alkaloidal constituents but *A. scholaris* has been found to be particularly rich in indole type alkaloids (Khyade *et al*., 2014). It is an evergreen fast-growing tree that grows up to 40 m tall. Bark is grayish brown, rough with white milky latex that flows rapidly when cut. Various parts are used by local population for the cure of various human ailments but the most important parts by medicinal point of view are the leaves and stem bark which have been used in the treatment of dysentery, malaria, asthma, epilepsy, skin disorders and chronic respiratory diseases (Bhogayata *et al*., 2016; Zhu *et al*., 2014).

Previous phytochemical studies revealed that this plant is good source of a wide range of natural products with diverse structures e.g. alkaloids, triterpenoids, coumarins, iridoids, saponins, tannins, steroids, flavonoids and phenolic compounds (Zhu *et al*., 2014). A series of phytochemical investigations showed that monoterpene indole alkaloids are the major components of this plant. These exhibit fascinating bio-diversity, such as cytotoxic, anti-inflammatory, antibacterial, and antifungal activities. Because of their complex structures and diverse bioactivities, such alkaloids have drawn great attention both from the synthetic chemists and pharmacologists (Zhu *et al*., 2019).

Medicinal utility of *Alstonia* prompted us to reinvestigate the chemical constituents of the stem bark. During this study twelve secondary metabolites have been isolated and their subsequent biological screening has also been carried out to ascertain their therapeutic utility.

MATERIALS AND METHODS

General experimental

Silica gel (Si 60, 70-230 mesh, E. Merck, Darmstadt, Germany) was used for column chromatography. I.R spectra were run on Shimadzu IR-460 spectrometer (Shimadzu Corporation, Tokyo, Japan). UV spectra were scanned on Thermo scientific spectrophotometer (Shimadzu Corporation, Tokyo, Japan). 1D and 2D-NMR spectra were performed on Bruker (Bruker, Fallendel, Switzerland) AM 600 and AM 400 NMR spectrometers with tetramethylsilane used as an internal standard. The chemical shifts (δ) are in ppm and coupling constant (J) in Hz.

EIMS were recorded on a JEOL JMS600H-1 spectrometer. HREIMS spectra were recorded on Thermo Finngal MAT-95 XP mass spectrometer. For optical rotation measurements, polatronic D Polarimeter was used. Purity of the compounds was check on TLC cards (Si-gel, Merck PF254, 0.25 mm thickness). Cerric sulphate was used as a spray reagent.

Collection of Stem Bark

The stem bark of the plant (5.12 kg) was collected from University of Karachi, in May, 2017. Identification of the plant was accompanied by Plant Taxonomic of the Department of Botany. A voucher specimen has been deposited in the Herbarium of the Department of Botany, University of Karachi (voucher no. G.H-94482).

Extraction and Isolation

Freshly collected stem bark was extracted twice with ethanol (EtOH) at room temperature. The crude extract was concentrated under vacuum. The resulting extract (161.6 g) was chromatographed on a silica gel column and successively eluted with hexane, combinations of hexane-dichloromethane, dichloromethane (DCM) and mixtures of DCM: methanol as mobile phase. As a result, fifteen major fractions were obtained. Fraction-3 which eluted with hexane-DCM (4:6-1:9) was further purified by repeated chromatography to obtain lupeol acetate (**4**, 20 mg). Elution with hexane-DCM (9.5:0.5) furnished stigmasterol (**12**, 22 mg). Further elution with hexane-DCM (3.0:7.0) afforded *β*-amyrin (**8**, 15.21 mg). Fraction-4 which obtained with DCM: MeOH (9.6:0.4) was purified using mixtures of hexane: EtOAc, leading to the isolation of betulin [hexane-EtOAc (9.8:0.2), **5**, 25 mg], betulinic acid [hexane-EtOAc (9.7:0.3), **6**, 30 mg] and diospyrolide [hexane-EtOAc (9.4:0.6), **3**, 20 mg]. The fraction-5 yielded with DCM: MeOH (9.5:0.5) was again chromatographed using hexane: EtOAc (9.7:0.3) to furnish top and tail fractions. The top semi pure sub-fraction could be further purified *via* Sephadex LH 20 followed by elution from methanol. It could be characterized as betulonic acid (**7**, 21 mg). The tail fractions also comprised of a semi pure compound which could be purified by HPLC (RP-18) using MeOH: H_2O (85:15), leading to 11-oxo-*β*-amyrin (**9**,15mg). Purification of fraction-6 which was obtained with the mobile phase DCM: MeOH (9.2:0.8), was re-purified through the elution with hexane: EtOAc (6.0:4.0), collecting 10 ml fractions. Fractions 1-30 provided a uniform compound which was subsequently identified as loganetin (**10**, 25 mg). The fractions 59-62 from the same column provided venoterpine (**2**, 36 mg). Fraction-7 which eluted with DCM: MeOH (9.1:0.9). In order to purify this fraction, a mixture of hexane-EtOAc (3:7) was used to afford vanillic acid (**11**, 12 mg). Elution with [EtOAc :MeOH (80:20)], 19,20-*Z*-vallesamine (**1**, 40 mg) was obtained.

Biological screening

Antioxidant activity

Antioxidant agents play an important role to prevent diseases like heart diseases and cancer due to formation of free radicals**.** The free radical scavenging activity was measured by 1,1-diphenyl-2-picryl-hydrazil (DPPH) using the method described by Gulcin *et al*. (2005). BHA was used as a standard. The IC_{50} values were calculated using the EZ-Fit Enzyme Kinetics Program (Perrella Scientific Inc., Amherst, USA) (Ahmad *et al*., 2006).

Enzyme inhibition studies

In this study, all the pure isolated compounds were screened against inhibitory assays against the enzymes lipoxygenase and urease.

Lipoxygenase inhibition activity

Lipoxygenase activity and the resultant fatty acid hydroperoxides initiate free radical carbon chains resulting modifications in proteins. The obtained constituents have been screened against lipoxygenase.

Urease inhibition activity

Urease is capable for the hydrolysis of urea. As a result, NH_3 and CO_2 are produced. It also promotes *Helicobacter pylori* by providing acidic medium in the stomach. This causes gastric cancer, peptic ulcer and urinary tract infection (Zahid *et al*., 2014). Our purified natural products were also studied in order to determine urease inhibitory potential. Indophenol method as described by Weatherburn (Weatherburn *et al*., 1967) performed for urease inhibition. Thiourea was used as a standard inhibitor of urease.

RESULTS

The ethanolic extract of the stem bark of *A. scholaris* was chemically analyzed to obtain twelve constituents of different classes including 19,20-*Z*-vallesamine (**1**) (Atta-ur-Rahman *et al*., 1987), venoterpine (**2**) (Authur *et al*.,

1967), diospyrolide (**3**) (Kuo and Chang 2000), lupeol acetate (**4**) (Jamal *et al*., 2008), betulin (**5**) (Wang *et al*., 2017), betulinic acid (**6**) (Wang *et al*., 2017), betulonic acid (**7**) (Ledeti *et al*., 2014), *β*-amyrin (**8**) (Wang *et al*., 2017), 11-oxo-*β*-amyrin (**9**) (Fingolo *et al*., 2013), loganetin (**10**) (Maurya *et al*., 2014), vanillic acid (**11**) (Costa *et al*., 2014) and stigmasterol (**12**) (Jamal *et al*., 2008). Among obtained constituents, venoterpine, diospyrolide, betulonic acid and 11-oxo-*β*-amyrin have been reported for the first time from the genus *Alstonia* and their NMR spectral data are given in Tables 1 and 2 and structures are shown in Fig.1.

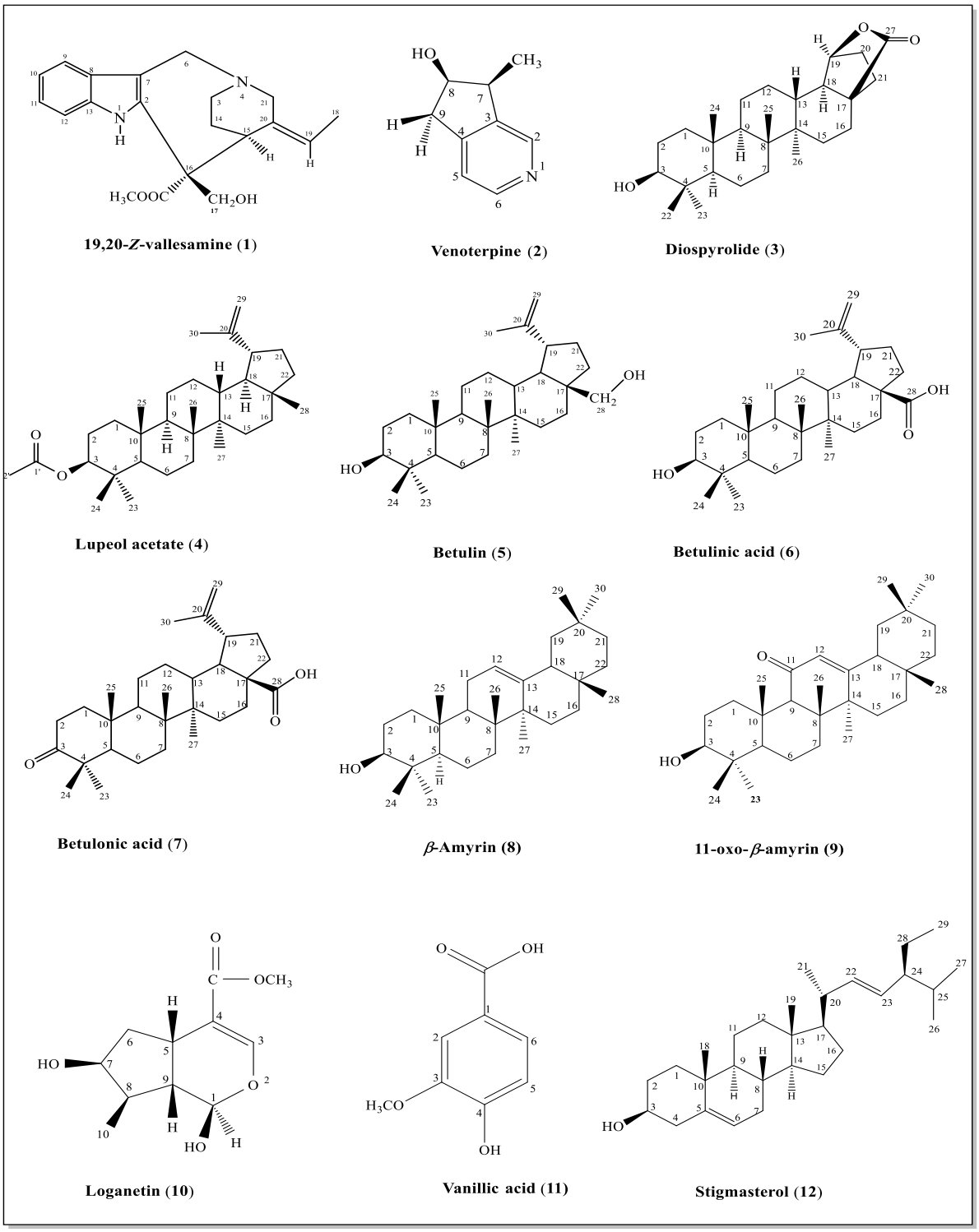

Fig.1. Chemical constituents from stem bark of *Alstonia scholaris.*

Table 1. ^{1}H-NMR Spectral Data of Isolates **2**, **3**, **7** and **9**.

Position	**2** δ_H (*J* in Hz) CD_3OD 400 MHz	**3** δ_H (*J* in Hz) $CDCl_3$ 400 MHz	**7** δ_H (*J* in Hz) $CDCl_3$ 400 MHz	**9** δ_H (*J* in Hz) $CDCl_3$ 400 MHz
2	8.31 (1H, s)	1.13 (1H, m), 1.62 (1H, m)	2.36 (1H, m), 2.51 (1H, m)	1.59 (1H, m), 1.66 (1H, m)
3	-	3.17 (1H, br.dd, *J* = 10.8, 5.1)	-	3.18 (1H, dd, *J* = 12, 4.4)
5	7.31 (1H, d, *J* = 4.8)	0.67 (1H, d, *J* = 9.2)	1.42 (1H, d, *J* = 7.3)	0.82 (1H, d, *J* = 8.0)
6	8.29 (1H, d, *J* = 4.8)	1.39 (1H, m), 1.51(1H, m)	1.48 (2H, m)	1.54 (2H, m)
7	3.24 (m)	1.36 (1H, m), 1.43 (1H, m)	1.44 (2H, m)	1.50 (2H, m)
8	4.51 (1H, m)	-	-	-
9	2.90 (dd, *J* = 16.8, 2.4), 3.14 (dd, *J* = 16.8, 5.6)	1.29 (1H, m)	1.38 (1H, m)	2.43 (1H, s)
10	1.34 (3H, d, *J* = 7.2)	-	-	-
11	-	1.28 (1H, m), 1.47 (1H, m)	1.35 (1H, m), 1.44 (1H, m)	-
12	-	1.51 (1H, m), 2.04 (1H, m)	1.05 (1H, m), 1.72 (1H, m)	5.51 (1H, s)
19	-	4.60 (1H, .br. s)	3.02 (1H, td, *J* = 10.8, 4.8)	1.39 (2H, m)
22	-	0.95 (3H, s)	1.46 (1H, m), 1.91 (1H, m)	1.24(1H, m), 1.36 (1H, m)
23	-	0.73 (3H, s)	1.06 (3H, s)	0.81 (3H, s)
24	-	0.82 (3H, s)	1.01 (3H, s)	0.91 (3H, s)
25	-	0.92 (3H, s)	0.93 (3H, s)	1.13 (3H, s)
26	-	0.84 (3H, s)	1.00 (3H, s)	1.12 (3H, s)
27	-	-	1.05 (3H, s)	1.39 (3H, s)
28	-	-	-	0.97 (3H, s)
29	-	-	4.70 (1H, br.s), 4.69 (1H, br.s)	85 (3H, s)
30	-	-	1.68 (3H, s)	0.85 (3H, s)

Table 2. ^{13}C-NMR Spectral Data of Isolates **2**, **3**, **7** and **9**.

Position	**2** δ_C CD_3OD 100 MHz	**3** δ_C $CDCl_3$ 100 MHz	**7** δ_C $CDCl_3$ 100 MHz	**9** δ_C $CDCl_3$ 100 MHz	**Position**	**3** δ_C $CDCl_3$ 100 MHz	**7** δ_C $CDCl_3$ 100 MHz	**9** δ_C $CDCl_3$ 100 MHz
1	-	38.9	38.2	38.7	16	22.2	31.7	26.4
2	147.0	27.3	35.0	23.4	17	51.1	57.7	32.3
3	144.0	78.9	221.2	78.8	18	55.0	51.2	47.5
4	153.2	38.8	49.8	38.0	19	79.2	50.4	45.0
5	121.1	55.4	56.1	54.9	20	29.6	152.1	31.0
6	145.6	18.2	20.7	18.4	21	28.9	34.8	34.4
7	43.1	34.1	33.5	32.6	22	27.9	40.7	36.4
8	75.6	40.7	41.8	43.3	23	15.3	27.2	28.0
9	41.8	50.9	48.4	61.5	24	16.4	21.4	16.6
10	12.8	37.3	38.0	36.9	25	15.6	16.5	15.7
11	-	20.6	22.6	200.3	26	13.1	16.4	17.3
12	-	27.5	26.9	128.1	27	179.4	15.0	23.5
13	-	34.2	39.7	170.6	28	-	180.7	28.7
14	-	40.6	43.6	45.3	29	-	110.0	33.0
15	-	28.1	30.8	26.4	30	-	19.5	23.5

Bio-screening of pure isolates

Alstonia scholaris contains a number of chemical constituents of medicinal importance. The constituents obtained in the present study were assayed for the following activities:

DPPH Radical Scavenging Activity
Urease Inhibitory Potential
Lipoxygenase Inhibitory Potential

Antioxidant Activity

All the isolates showed anti-oxidant activity in DPPH radical-scavenging assay and inhibitory potential against the enzymes lipoxygenase and urease. 19, 20-*Z*-Vallesamine was found significantly active with an IC_{50} value of 24.9 ± 0.11 as compared to the standard BHA (butyl hydroxyanisole) (Table 3) while betulin and betulinic acid were moderately active.

Enzyme inhibition studies

Lipoxygenase inhibition activity

Stigmasterol and betulin showed moderate inhibitory potential (Table 4). Lipoxygenase inhibiting activity was measured by modifying the spectrophotometric method developed by Tappel (Tappel, 1962). Baicalein was used as a standard inhibitor of lipoxygenase.

Table 3. Results of Antioxidant Activity of Pure Isolates.

Compound No.	Compound	IC_{50} (µM)
1	19-20-*Z*-Vallesamine	24.9 ± 0.11
2	Venoterpine	77.7 ± 0.17
3	Diospyrolide	96.7 ± 0.65
4	Lupeol acetate	60.8 ± 0.42
5	Betulin	59.8 ± 0.44
6	Betulinic acid	54.4 ± 0.12
7	Betulonic acid	89.6 ± 0.65
8	*β*-Amyrin	86.3± 0.21
9	11-Oxo-*β*-amyrin	Nil
10	Loganetin	85.5 ± 0.28
11	Vanillic acid	Nil
12	Stigmasterol	81.7 ± 0.48
Standard	**BHA**	**44.2 ± 0.28**

Table 4. Lipoxygenase Inhibition Activity of Pure Isolates.

Compound No.	Compound	IC_{50} (µM)
1	19-20-*Z*-Vallesamine	65.9 ± 0.33
2	Venoterpine	< 200
3	Diospyrolide	< 200
4	Lupeol acetate	40.8± 0.22
5	Betulin	38.9 ± 0.28
6	Betulinic acid	62.3 ± 0.36
7	Betulonic acid	< 200
8	*β*-Amyrin	NIL
9	11-Oxo-*β*-amyrin	< 200
10	Loganetin	< 200
11	Vanillic acid	< 200
12	Stigmasterol	26.5 ± 0.38
Standard	**Baicalein**	**22.6 ± 0.11**

Urease inhibition activity

Diospyrolide revealed significant urease inhibitory activity while stigmasterol and *β*-amyrin also showed potent activity. Other compounds were moderately active while betulin, betulinic acid and lupeol acetate were inactive. The results are demonstrated in Table 5.

Table 5. Urease Inhibition Activity of Pure Isolates.

Compound No.	Compound	IC_{50} (µM)
1	19,20-*Z*-Vallesamine	32.6 ± 0.62
2	Venoterpine	39.6 ± 0.48
3	Diospyrolide	19.2 ± 0.10
4	Lupeol acetate	41.5 ± 0.66
5	Betulin	84.6 ± 0.29
6	Betulinic acid	85.6 ± 0.73
7	Betulonic acid	28.6 ± 0.36
8	*β* -Amyrin	26.3 ± 0.44
9	11-Oxo-*β*-amyrin	33.5 ± 0.73
10	Loganetin	38.6 ± 0.55
11	Vanillic acid	34.1 ± 0.35
12	Stigmasterol	25.5 ± 0.28
Standard	**Thiourea**	**21.5 ± 0.47**

DISCUSSION

In the light of medicinal importance and utility of *Alstonia*, pharmacochemical studies have been undertaken on the stem bark of one of its species namely *A. scholaris*. As a result of standard isolation protocol, twelve known secondary metabolites have been isolated and characterized with the help of obtained data. Out of these, compounds venoterpine (**2**), diospyrolide (**3**), betulonic acid (**7**) and 11-oxo-*β*-amyrin (**9**) are herein reported for the first time from the genus *Alstonia* and their molecular weights are 149, 414, 454 and 440 a.m.u. respectively, depicted with the aid of electron impact mass spectra. Compounds **3**, **7** and **9** were melted at 265-267°C, 291-293°C and 230-232° C, respectively, while **2** was obtained as a grey oil.

All the isolates displayed antioxidant and inhibitory potential against urease and lipoxygenase. Diospyrolide (**3**) displayed excellent urease inhibitory potential while compounds **1, 2** and **7**-**12** were moderately active. On the other hand, compounds **5** and **12** showed moderate inhibitory activity against the enzyme lipoxygenase, while other compounds were inactive. The 19,20-*Z*-vallesamine (**1**) was showed significant antioxidant activity while betulin and betulinic acid were moderately active.

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