Two New Diterpenoids from *Ballota limbata*

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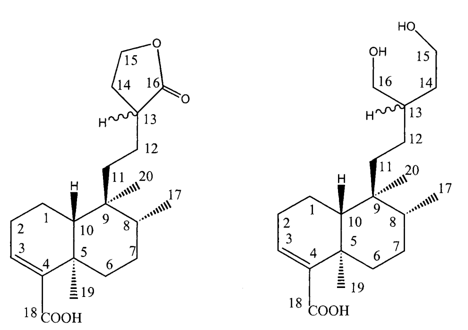
Two new clerodane-type diterpenoids trivially named as ballotenic acid (1) and ballodiolic acid (2) have been isolated from *Ballota limbata* along with three the known compounds; β-amyrin, oleanolic acid, and β-sitosterol. The structure elucidation of the new compounds was based primarily on two-dimensional (2D)-NMR techniques including correlation spectroscopy (COSY), heteronuclear multiple quantum coherence (HMQC), heteronuclear multiple bond correlation (HMBC), and nuclear Overhauser effect (NOE) experiments. Compounds 1 and 2 displayed inhibitory potential against lipoxygenase enzyme in a concentration-dependent fashion with IC_{50} values of 99.6 μM and 38.3 μM, respectively.

Key words *Ballota limbata*; clerodane diterpenoid; Lamiaceae; lipoxygenase inhibitory assay

The genus *Ballota* (Lamiaceae) is comprised of about 33 species which are mainly found in the Mediterranean region.[1] In Pakistan only two species are available, *Ballota aucheri* and *Ballota limbata* BOISS. (Syn. *Otostigia limbata*; labiatae). *Ballota limbata* is locally called "Bui" or "Phut kandu".[2] It is widely distributed in North West Frontier Province and lower hills of West Punjab in Pakistan, and traditionally it has been used in the treatment of gums of children and ophthalmia.[3] The leaves and the tops of *Ballota saxatilis* are used for colic, asthma, influenza, insomnia, and haemorrhoids. Infusions prepared from the leaves have been reported to possess antiulcer, antispasmodic, and sedative activities. Aerial parts, and their aqueous and hydroalcoholic extracts are widely used in European medicine for their neurosedative activities.[1] *Ballota larendana* and *Ballota nigra* have potent antidepressant activity, while *B. larendana* possesses anxiolytic activity.[4] A literature survey revealed that no significant work has been carried out so far on *B. limbata*. This prompted us to carry out a phytochemical investigation of this species. Here we report the isolation and structure elucidation of two new clerodane diterpenoids (Fig. 1), which have shown inhibitory potential against lipoxygenase.

Lipoxygenases (EC 1.13.11.12) constitute a family of nonheme iron containing dioxygenases that are widely distributed in animals and plants. This enzyme plays a key role in the biosynthesis of a variety of chemical mediators such as hydroxyeicosatetraenoic acids (HETEs), leukotrienes, lipoxins and hepoxylines in mammalian cells.[5] These mediators play a role in a variety of disorders such as bronchial asthma, inflammation,[6] and tumor angiogenesis.[7] Lipoxygenases are therefore a potential target for the rational drug design and discovery of mechanism-based inhibitors for the treatment of bronchial asthma, inflammation, cancer, and autoimmune diseases.



compound (1) compound (2)

Fig. 1. Structures of Compounds **1** and **2**

Results and Discussion

The $CHCl_3$ extract of the air dried whole plant of *Ballota limbata* was subjected to silica gel chromatography to give two new clerodane-type diterpenoids provisionally named ballotenic acid (**1**) and ballodiolic acid (**2**) along with three known compounds; β-amyrin, oleanolic acid, and β-sitosterol.

Compound **1** was isolated as a colourless oil. Its structure was established mainly by high field ^{1}H-NMR, high resolution mass spectroscopy and supported by its ^{13}C-NMR spectrum in which 20 carbon peaks could be detected. Its molecular formula, $C_{20}H_{30}O_4$, was deduced from accurate mass measurement of the highest peak at m/z 316.203 $[M-H_2O]^+$ corresponding to a molecular composition of $C_{20}H_{28}O_3$. The IR absorption of compound **1** indicated the presence of a five membered γ-lactone (1764 cm^{-1})[8] whereas α,β-unsaturated carbonyl absorption was observed at 1678 cm^{-1}. The UV spectrum of compound **1** showed absorption at λ_{max} 212 nm. In IR an intense and broad absorption centered at 2922 cm^{-1} suggested the presence of carboxylic acid. The ^{1}H-NMR spectrum of **1** exhibited typical signals for a tricyclic clerodane carbon skeleton supported by DEPT experiment on **1** by ^{13}C-NMR spectroscopy disclosed the presence of two tertiary and one secondary methyl carbons, two olefinic carbons and five quaternary carbons (which include two carbonyl carbons). The HMBC correlations of olefinic methane at C-3 δ_H 6.81 with C-18 δ_C 171.0 and C-4 δ_C 141.1 established the presence of α,β-unsaturated carboxylic acid. ^{1}H-, ^{13}C-NMR, HMQC, and HMBC spectra when recorded in $CDCl_3$, had close similarity to that of neocleroda-3,13-dine-15,16-olide-18-oic acid,[9] except for the lack of the usual double bond between C-13 and C-14. The ^{1}H-NMR signal of H-13 appeared at δ_H 2.40 and H-14 and H-15 at δ_H 1.26 and 4.8—4.36, respectively. In ^{13}C-NMR the chemical shift values of C-13 appeared at δ_C 39.6, C-14 δ_C 29.6, C-15 δ_C 66.4, and C-16 δ_C 172.8. In HMBC experiments it was shown to have ^{1}H–^{13}C correlations between the methine proton at H-13 δ_H 2.40

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with C-11 δ_C 36.0, C-12 δ_C 22.6, C-14 δ_C 29.6 and C-16 δ_C 172.8. The H-15 δ_H 4.18—4.36 protons were also correlated to C-16 δ_C 172.8, C-13 δ_C 39.6, and C-14 δ_C 29.6.

The stereochemistry of compound **1** was established from the combined evidence of its spectral data in comparison with those of known clerodane diterpenoids.[9,10] However the stereochemistry of C-13 could not be deduced spectroscopically.[8,11,12] The chemical shifts of H-17, H-19, and H-20 at δ_H 0.79, δ_H 1.23, and δ_H 0.72, respectively, showed that we were dealing with an A/B ring *trans* fused clerodane. Nuclear Overhauser effect (NOE) experiments carried out on compound **1** established NOEs between H_3-19 and H_3-20, and between H_3-17 and H_3-20, consistent with a *cis* relationship between these methyl groups. These results and the fact that the irradiation of H-10 did not cause any increase in the intensities of either the 19 or 20-methyl signals, confirmed the *trans* stereochemistry of A and B rings of the decalin system of **1**.[13] A *trans* A/B ring junction was also evident from the ^{13}C-NMR chemical shifts of the C-19 methyl carbon δ_C 20.5 and C-20 carbon δ_C 18.3. Hence, the methyls were disposed *cis*, *i.e.* in one plane.[9,10] In *cis*-clerodanes, C-19 carbon resonates at round about δ_C 25, whereas in *trans*-clerodane it appears at δ_C 11-19. Moreover, C-20 in *cis*-clerodanes resonates at a lower field δ_C 21—29 than that in *trans*-clerodanes δ_C 17—19.[14]

Compound **2**, another new bicyclic clerodane diterpene, was isolated from the chloroform fraction. It was found to have the molecular formula $C_{20}H_{34}O_4$ derived from accurate mass measurement of the molecular ion peak at *m/z* 320.2320 $[M-H_2O]^+$ accompanied by the loss of an H_2O molecule, like compound **1**. It lacked the five membered free γ-lactone ring attached to C-12 as in the previous compound. The side chain ($C_4H_9O_2$) including C-13 to C-16 attached to C-12 of compound **2** was characterized on the basis of IR, ^{1}H-, ^{13}C-NMR, HMQC, HMBC, and ^{1}H–^{1}H COSY experiments. Compound **2** contained free hydroxyl groups present at C-15 and 16 deduced from IR absorption. The IR spectrum of compound **2** showed free hydroxyl absorption at 3628 cm^{-1} and hydrogen bonded hydroxyl absorption at 3589 cm^{-1}, which corresponds closely to those (3636, 3477 cm^{-1}) of butane-1,4-diol,[15,16] whereas α,β-unsaturated carbonyl absorption was observed at 1686 cm^{-1}. The UV spectrum showed an absorption band at λ_{max} 214 nm. A broad peak at 2926 cm^{-1} indicated the presence of an OH moiety of α,β-unsaturated carboxylic acid. According to ^{1}H- and ^{13}C-NMR spectra, compound **2** contained two tertiary methyls, one secondary methyl (having chemical shift values similar to compound **1**), seven upfield methylenes, and two downfield methylenes attached with hydroxy moieties. Compound **2** was quite similar to **1**, suggesting a bicyclic clerodane series, however, in ^{1}H-NMR there were two free hydroxy methylene signals at δ_H 3.65—4.26 and δ_H 3.77 for H-15 and H-16, respectively.[15] The latter signal showed HMBC correlations with upfield C-13 δ_C 39.8 and C-12 δ_C 24.9 while the former signal H-15 δ_H 3.65—4.26 showed its HMBC correlation with C-13 δ_C 39.8 and C-14 δ_C 29.7. This connectivity was further enhanced by H-12 δ_H 1.61—1.69, which was correlated through HMBC interaction with C-9 δ_C 38.6, C-14 δ_C 29.7, and C-16 δ_C 61.1. Similarly H-14 δ_H 1.23—1.29 was connected to C-13 and C-15 through HMBC correlation. The positions of C-15 δ_C 66.3 and C-16 δ_C 61.1[17] having free hydroxyl groups were further supported by ^{1}H–^{1}H COSY correlation spectroscopy. The stereochemistry of H-13 at δ_H 1.58 could not be determined, as discussed for compound **1**.

From the *in vitro* quantitative inhibition study of lipoxygenase enzyme by compounds **1** and **2**, it was found that compound **2** had higher inhibitory potential (IC_{50} value 38.3$\pm$1.3) μM than compound **1** (IC_{50} value 99.6$\pm$2.0) μM.

This may be due to the presence of two hydroxy groups at C-15 and C-16 in compound **2**. These electron-donating groups convert the Fe^{3+} form of the enzyme to the Fe^{2+} form, which is inactive. The IC_{50} value of the positive control (Baicalein) was found to be (22.5$\pm$0.5) μM.

Table 1. ^{13}C-, ^{1}H-NMR, and HMBC Data of Compounds **1** and **2**

Position	1			2		
	^{13}C-NMR	^{1}H-NMR	HMBC	^{13}C-NMR	^{1}H-NMR	HMBC
1	17.4	0.76 m	10, 9, 2	17.5	0.80—0.89 m, ovlp	10, 9, 5
2	27.4	2.23—2.27 m	10	27.4	2.14—2.34 m	3, 10
3	140.5	6.81 t (1.8)	4, 5, 18	140.0	6.79 br s	18, 5, 1
4	141.1			141.3		
5	37.5			37.5		
6	35.7	2.20—2.44 m	5, 10, 8	35.6	2.34—2.38 m, ovlp	4, 5, 10, 7
7	27.2	1.37—1.46 m, ovlp	6, 8, 5	27.2	1.38—1.49 m	5, 8, 9
8	36.1	2.36 m	10, 17	36.1	1.46 m	10, 7, 9
9	38.7			38.6		
10	46.5	1.37 br d (7.3)	1, 2, 4, 5, 9	46.6	1.36 br d (10.4)	4, 9, 5, 2
11	36.0	1.29—1.44 m, ovlp	12, 20, 9, 10, 13	35.8	1.39—1.44 m, ovlp	10, 9, 13, 20
12	22.6	0.83—0.89 m	13, 14, 11	24.9	1.61—1.69 m	9, 14, 11, 16
13	39.6	2.40 m	14, 16, 12, 11	39.8	1.58 m, ovlp	14, 16
14	29.6	1.26 m	13, 12	29.7	1.23—1.29 m	15, 13
15	66.4	4.18—4.36 m	16, 13, 14	66.3	3.65—4.26 m	13, 14
16	172.8			61.1	3.77 m	12, 13
17	15.9	0.79 d (6)	7, 8, 9	15.9	0.75 d (5.3)	8, 9
18	171.0			172.0		
19	20.5	1.23 s	10, 5, 4	20.5	1.21 s	10, 4, 5
20	18.3	0.72 s	10, 9, 8, 11	18.4	0.70 s	10, 11, 9, 8

Experimental

General Experimental Procedure UV and IR spectra were recorded on Hitachi-UV-3200 and Jasco-320-A spectrophotometers, respectively. ^{1}H- and ^{13}C-NMR spectra were recorded on a Bruker AM-400 spectrometer with tetramethylsilane (TMS) as an external standard. 2D NMR spectra were recorded on a Bruker AMX 500 NMR spectrometer. Optical rotations were measured on a Jasco DIP-360 digital polarimeter using a 10 cm cell tube. Mass spectra (EI and HR-EI-MS) were measured in an electron impact mode on Finnigan MAT 12 or MAT 312 spectrometers and ions are given in *m/z* (%). Fast atom bombardments (FAB) MS were measured on a JEOL HX110 mass spectrometer. TLC was performed with pre-coated silica gel G-25-UV$_{254}$ plates and detection was done at 254 nm, and by ceric sulphate in 10% H_2SO_4. Silica gel (E. Merck, 230—400 mesh) was used for column chromatography.

Plant Material The plant *Ballota limbata* (Lamiaceae) was collected from Abbottabad, Pakistan, in June 2001, and identified by Dr. Manzoor Ahmad (Taxonomist) at the Department of Botany, Post Graduate-College, Abbottabad, Pakistan. A voucher specimen (# 6872) has been deposited in the herbarium of the Botany Department of Post-Graduate College, Abbottabad, Pakistan.

Extraction and Purification The air dried whole plant (35 kg) was exhaustively extracted with methanol (40 l×3) at room temperature. The extract was evaporated to yield the residue (315 g), which was partitioned between hexane (47 g), chloroform (95 g), ethyl acetate (69 g), butanol (33 g) and water (59 g). The chloroform extract was subjected to silica gel chromatography using hexane with a gradient of $CHCl_3$ up to 100% and followed by methanol. Twelve fractions were collected. Fraction no. 9 of the first column was loaded on silica gel (flash silica 230—400) and eluted with EtOAc : hexane (18 : 82) to purify compound **1** (11.6 mg). Similarly, fraction no. 10 was subjected to column chromatography and eluted with EtOAc : hexane (30 : 70) to purify compound **2** (10.8 mg) (ballodiolic acid) as a new bicyclic clerodane diterpene. Fractions no. 3 and 4 were loaded on silica gel (flash silica 230-mesh) and eluted with EtOAc : hexane (20 : 80) to purify three known compounds; β-amyrin, oleanolic acid, and β-sitosterol. The purity of the compounds was checked on TLC and HPTLC plates.

Compound **1**: Colorless oil; ^{1}H- (in $CDCl_3$; 500 MHz) and ^{13}C-NMR (in $CDCl_3$, 125 MHz) see Table 1. IR ($CDCl_3$) ν_{max} 2922, 1764, 1678 cm^{-1}. UV (MeOH) λ_{max}: 212 nm (4.1). EI-MS *m/z* (rel. int) 316 $[M-H_2O]^+$ (100), 301 (4), 273 (3.80), 221 (7.5), 203 (28), 175 (21). HR-EI-MS Found *m/z* 316.2038 (Calcd 316.4443 for $C_{20}H_{30}O_4$-H_2O), $[\alpha]_D^{23}$ $-0.50°$ (c=0.104, $CHCl_3$).

Compound **2**: Colorless oil, ^{1}H- (in $CDCl_3$; 500 MHz) and ^{13}C-NMR (in $CDCl_3$, 125 MHz) see Table 1. IR ($CDCl_3$) ν_{max} 3628, 3589, 2926, 1686 cm^{-1}. UV (MeOH) λ_{max}: 214 nm (3.7). EI-MS *m/z* (rel. int.) 320 $[M-H_2O]^+$ (30), 302 (3), 287 (1), 221 (17), 203 (30), 151 (12), 137 (27), 125 (80). HR-EI-MS: Found *m/z* 320.2320. (Calcd 320.4762 for $C_{20}H_{34}O_4$-H_2O), $[\alpha]_D^{23}$ $-19.7°$ (c=0.172, $CHCl_3$).

***In Vitro* Lipoxygenase Inhibitory Assay** Lipoxygenase inhibitory activity was conveniently measured by slightly modifying the spectrometric method developed by Tappel.[18] Lipoxygenase (1.13.11.12) type I-B and linoleic acid were purchased from Sigma (St. Louis, MO, U.S.A.). All other chemicals were of analytical grade. Reaction mixtures containing 160 μl (100 mM) sodium phosphate buffer (pH 8.0), 10 μl of test-compound solution, and 20 μl of lipoxygenase solution were mixed and incubated for 10 min at 25 °C. The reaction was then initiated by the addition of 10 μl linoleic acid (substrate) solution, with the formation of (9*Z*,11*E*)-(13*S*)-13-hydroperoxy-octadeca-9,11-dienoate, and the change of absorbance at 234 nm was followed for 6 min. Test compounds and the control were dissolved in methanol. All reactions were performed in triplicate in 96-well micro-plates in SpectraMax 340 (Molecular Devices, U.S.A.). The IC$_{50}$ values were then calculated using the EZ-Fit Enzyme kinetics program (Perrella Scientific Inc., Amherst, U.S.A.). The percentage (%) inhibition was calculated as follows $(E-S)/E\times100$, where E is the activity of the enzyme without test compound and S is the activity of enzyme with test compound.

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