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Isolation and HPLC Estimation of Six Boswellic Acids from *Boswellia serrata* Extract†

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Boswellic acids are a complex mixture of pentacyclic triterpenic acids, consisting of six boswellic acids, namely, 11-keto-β-boswellic acid (**1**), 3-O-acetyl-11-keto-β-boswellic acid (**2**), α-boswellic acid (**3**), β-boswellic acid (**4**), 3-O-acetyl-α-boswellic acid (**5**), and 3-O-acetyl-β-boswellic acid (**6**). Six boswellic acids (**1–6**), antiinflammatory and anti-arthritic constituents of *Boswellia serrata*, have been isolated by a combination of column chromatography and preparative high performance liquid chromatography. The structures of these boswellic acids were characterized thoroughly by high resolution NMR and mass spectral data. The purity of the boswellic acids was analyzed by HPLC method. HPLC separation was performed on a C-18 column using mobile phase 0.1% v/v phosphoric acid in water and acetonitrile, with detection at 210 and 248 nm. Commercially available varieties of boswellic acids were analyzed to detect the percentage of these six boswelllic acids. The percentage of 11-keto-β-boswellic acid (**1**), 3-O-acetyl-11-keto-β-boswellic acid (**2**), α-boswellic acid (**3**), β-boswellic acid (**4**), 3-O-acetyl-α-boswellic acid (**5**), and 3-O-acetyl-β-boswellic acid (**6**) as estimated using their calibration curves were found to be 4.91 ± 0.066 to 5.66 ± 0.080, 1.13 ± 0.043 to 3.38 ± 0.062, 5.61 ± 0.021 to 7.29 ± 0.051, 15.80 ± 0.018 to 20.99 ± 0.028, 1.64 ± 0.032 to 3.57 ± 0.082 and 6.89 ± 0.010 to 12.92 ± 0.015 in five different samples. The total percentages of boswellic acids are 35.98 ± 0.190 to 53.68 ± 0.325.

Key Words: ***Boswellia serrata*, Boswellic acids, HPLC analysis.**

INTRODUCTION

The gum resin of *Boswellia serrata* Roxb. (Fam. Burseraceae) has been used for treatment of inflammatory disease in the traditional Ayurvedic medicine in India and other countries. Boswellic acids, a complex mixture of pentacyclic triterpene acids, obtained from the gum resin of *Boswellia serrata*[1–3], have been reported as inhibitors of 5-lipoxygenase, the key enzyme for leukotriene biosynthesis in inflammatory disorders[4–10]. These acids have also been shown to possess antitumour activity against human leukemia cell lines[11, 12] and immunomodulatory activity[13]. *Boswellia serrata* extract contains six different boswellic acids (Fig. 1). In view of their medicinal importance, a method has been developed for

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the isolation of boswellic acids (**1–6**) from *Boswellia serrata* extract by a combination of column chromatography and preparative HPLC and estimation of boswellic acids using HPLC separation technique.

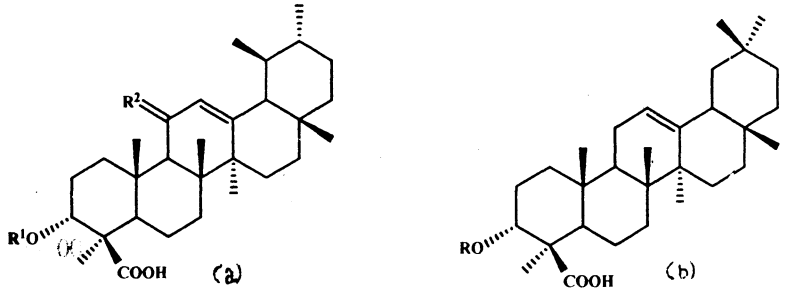

Fig. 1. Chemical structures of boswellic acid

1. R^1 = H; R^2 = O	11-Keto-β-boswellic acid	(Structure a)
2. R^1 = Ac; R^2 = O	3-O-Acetyl-11-keto-β-boswellic acid	(Structure a)
3. R = H	α-Boswellic acid	(Structure b)
4. R^1 = H; R^2 = 2H	β-Boswellic acid	(Structure a)
5. R = Ac	3-O-Acetyl-α-boswellic acid	(Structure b)
6. R^1 = Ac; R^2 = 2H	3-O-Acetyl-β-boswellic acid	(Structure a)

Usually total boswellic acids are estimated in *Boswellia serrata* extract by using a non-aqueous titration[14]. A few HPLC methods have also been described for estimation of boswellic acids in human plasma and *Boswellia serrata* extract[15, 16]. The present paper describes the isolation, identification and HPLC method of estimation of individual boswellic acids and total boswellic acids in the *Boswellia serrata* extract.

EXPERIMENTAL

The commercially available extract of boswellic acids (85%) was supplied by M/s Laila Impex, Vijayawada. All the solvents and chemicals used were of either AR or HPLC grade.

^{1}H and ^{13}C NMR spectra were recorded at 400 and 100 MHz, respectively, on an AMX-400 FT NMR spectrometer and EIMS on Jeol D-300 spectrometer. Melting points were recorded on a V. Scientific melting point apparatus (MP-1) and are uncorrected. UV spectra were recorded on a PDA detector of Shimadzu HPLC; IR spectra on a Perkin-Elmer spectrum BX FTIR. The preparative HPLC (Shimadzu) conditions are: Supelcosil PLC-18, 250 × 21.2 mm, 12 μ, mobile phase 0.1% v/v phosphoric acid in water : acetonitrile (10 : 90), flow rate 20 mL/min and UV detector at λ_{max} 210 and 248 nm.

The HPLC (Shimadzu) system for estimation of boswellic acids equipped with Alltima C18, 5 μ (250 mm × 4.6 mm) column, LC-10AT pumps, SCL-10A system controller, SIL-10A autoinjector, SPD-M10 AVP photo diode array detector set

at a wavelength of 210 and 248 nm for detection and class M10A software were used. A Millipore Swinnex type filter (pore size = 0.45 μm) was used for filtration.

Isolation of boswellic acids 1 to 6: 20 g of commercially available extract of boswellic acids (85%) (B.No. L 001008) was subjected to column chromatography over silica gel (ACME 100–200 mesh) using mixtures of hexane and ethyl acetate in increasing polarity. Selected fractions were combined based on TLC and analytical HPLC into four fractions. Fractions 1 and 2, on recrystallization from a mixture of hexane-ethyl acetate gave 11-keto-β-boswellic acid **1** (1.3 g) and 3-O-acetyl-11-keto-β-boswellic acid **2** (810 mg) respectively. Preparative HPLC of fraction 3 gave α-boswellic acid **3** (150 mg) and β-boswellic acid **4** (1.3 g) and fraction 4 yielded 3-O-acetyl-α-boswellic acid **5** (300 mg) and 3-O-acetyl-β-boswellic acid **6** (1.15 g), respectively.

Identification of Compounds: The isolated boswellic acids **1** to **6** were identified by interpretation of NMR spectral data and comparison of IR, mass spectral data and melting points with those reported in literature[3, 11].

11-Keto-β-boswellic acid (1): m.p. 196–198°C, UV (methanol) 247 nm, IR (KBr) 3450, 1696 and 1660 cm^{-1}, EIMS: m/z 470 [M^+, 19%], 273 (100) and 232 (54). ^1H NMR (Table-1), ^{13}C NMR (Table-2).

3-O-Acetyl-11-keto-β-boswellic acid (2): m.p. 260–262°C, UV (methanol) 247 nm, IR (KBr) 3153, 1733, 1702 and 1659 cm^{-1}, EIMS: m/z 512 [M^+, 5%], 452 (4), 273 (20), and 232 (11), ^1H NMR (Table-1), ^{13}C NMR (Table-2).

α-Boswellic acid (3): m.p. 268–270°C, UV (methanol) 195 nm, IR (KBr) 3400, 1700 and 1381 cm^{-1}, EIMS: m/z 456 [M^+, 13%], 441 (6), 238 (26), 218 (100), 203 (47) and 189 (18). ^1H NMR (Table-1), ^{13}C (Table-2).

β-Boswellic acid (4): m.p. 234–236°C, UV (methanol) 194 nm, IR (KBr) 3417, 1697 and 1378 cm^{-1}, EIMS: m/z 456 [M^+, 100%], 441 (41), 238 (19) and 218 (38). ^1H NMR (Table-1), ^{13}C NMR (Table-2).

TABLE-1.
SELECTED ^1H NMR (400 MHz, $CDCl_3$, δ) SPECTRAL DATA OF **1–6**

Proton	1	2	3	4*	5	6
H-3	4.07 (br s)	5.30 (br s)	4.08 (br s)	4.08 (br s)	5.30 (br s)	5.30 (br s)
H-12	5.54 (s)	5.55 (s)	5.19 (br s)	5.14 (br s)	5.19 (br s)	5.15 (br s)
CH_3-23	1.34 (s)	1.34 (s)	1.35 (s)	1.35 (s)	1.24 (s)	1.24 (s)
CH_3-25	1.30 (s)	1.23 (s)	0.89 (s)	0.91 (s)	0.89 (s)	0.91 (s)
CH_3-26	1.13 (s)	1.14 (s)	1.00 (s)	1.04 (s)	1.00 (s)	1.05 (s)
CH_3-27	1.18 (s)	1.19 (s)	1.15 (s)	1.09 (s)	1.18 (s)	1.12 (s)
CH_3-28	0.82 (s)	0.82 (s)	0.83 (s)	0.80 (s)	0.84 (s)	0.81 (s)
CH_3-29	0.79 (d, 6.3 Hz)	0.80 (d, 7.2 Hz)	0.87 (s)	0.79 (d, 5.6 Hz)	0.87 (s)	0.80 (d, 6.0 Hz)
CH_3-30	0.94 (br s)	0.94 (br s)	1.25 (s)	0.92 (d, 5.3 Hz)	1.24 (s)	0.91 (brs)
CH_3COO-3	—	2.08 (s)	—	—	2.09 (s)	2.10 (s)

*Assignments are supported by ^1H-^{13}C COSY data.

3-O-Acetyl-α-boswellic acid (5): m.p. 230–232°C, UV (methanol) 194 nm, IR (KBr) 3455, 1744, 1697 and 1379 cm^{-1}, EIMS: m/z 498 [M^+, 7%], 483 (4), 437 (4), 203 (46) and 218 (100). ^1H NMR (Table-1), ^{13}C NMR (Table-2).

3-O-Acetyl-β-boswellic acid (6): m.p. 272–274°C, UV (methanol) 194 nm, IR (KBr) 3294, 1747, 1697 and 1378 cm^{-1}. EIMS: m/z 498 [M^+, 27%] 483 (8), 438 (5), 218 (100) and 203 (27). ^1H NMR (Table-1), ^{13}C NMR (Table-2).

TABLE -2
^{13}C NMR (100 MHz, $CDCl_3$, δ) SPECTRAL DATA OF **1–6**

Carbon	1	2	3	4*	5	6
1	33.9	34.6	33.6	33.9	34.3	34.5
2	26.3	23.6	25.9	26.2	23.7	23.6
3	70.5	73.1	70.7	70.7	73.3	73.2
4	47.3	46.5	46.8	47.4	46.7	46.7
5	48.9	50.4	49.1	49.1	50.6	50.6
6	18.4	18.4	19.7	19.7	19.7	19.6
7	33.0	32.9	32.8	33.1	32.8	33.1
8	45.2	45.1	39.9	40.1	39.8	40.1
9	60.5	60.4	47.3	46.9	46.8	46.8
10	37.6	37.4	37.6	37.5	37.5	37.4
11	199.6	199.2	24.1	23.4	23.7	23.4
12	130.5	130.5	121.8	124.5	121.9	124.5
13	165.2	164.9	145.1	139.6	145.1	139.5
14	43.9	43.8	41.9	42.3	42.0	42.3
15	28.9	28.9	26.2	28.1	26.2	28.1
16	27.2	27.3	27.0	26.5	27.0	26.6
17	33.9	34.0	32.5	33.8	32.6	33.8
18	59.1	59.1	47.3	59.2	47.4	59.2
19	39.3	39.4	46.8	39.6	46.9	39.6
20	39.3	39.4	31.0	39.7	31.1	39.8
21	31.0	30.9	34.7	31.3	34.8	31.2
22	41.0	40.9	37.1	41.5	37.2	41.5
23	24.4	23.8	23.5	24.1	23.6	23.6
24	182.5	181.0	182.0	182.9	182.0	181.0
25	13.3	13.2	13.1	13.3	13.2	13.3
26	18.9	18.8	16.7	16.9	16.8	16.9
27	20.6	20.6	26.1	23.2	26.0	23.2
28	27.6	27.6	28.4	28.8	28.5	28.7
29	17.5	17.4	33.3	17.4	33.4	17.4
30	21.2	21.1	23.6	21.3	23.6	21.2
3-$OCOCH_3$	—	17.02 &	—	—	170.4 &	170.2 &
	—	21.3	—	—	21.4	21.3

* Assignments are supported by ^1H-^{13}C COSY data

Preparation of Standard solution of Boswellic acids for HPLC: Methanolic standard solutions of 11-keto-β-boswellic acid (**1**), 3-O-acetyl-11-keto-β-boswellic acid (**2**), α-boswellic acid (**3**), β-boswellic acid 4, 3-O-acetyl-α-boswellic acid (**5**) and 3-O-acetyl-β-boswellic acid (**6**) were prepared separately at a concentration of 100 μg mL^{-1}.

Sample preparation of *Boswellia serrata* extract: About 50 mg of test sample was weighed, dissolved in methanol and made up to 25 mL with methanol.

Sample preparation of *Boswellia serrata* crude gum: 10 g of *Boswellia serrata* crude gum was taken into a 250 mL round-bottom flask, extracted with methanol (4 × 75 mL) under reflux on a water bath, each extraction for about 30 min to ensure the complete extraction of boswellic acids. The combined extracts were filtered and concentrated to a soft residue. The residue was dried at 105°C for 1 h. Accurately weighed about 50 mg of the dry powder into a 25 mL volumetric flask, dissolved and made up to volume with methanol.

Chromatographic conditions: The elution was carried out with gradient solvent system with a flow rate of 1 mL/ min. at ambient temperature. The mobile phase consisted of Pump A: 0.1% v/v phosphoric acid in water and Pump B: acetonitrile. Quantitation levels of boswellic acids were determined using the above solvents programmed linearly: 95% acetonitrile in B for 0–5 min, 100% acetonitrile in B for 10–12 min and 95% acetonitrile in B for 15–35 min. The compounds were quantified using class-M10A software.

Validation of HPLC Method: The linearity of the method was evaluated by analyzing a series of standard boswellic acids. 20 μL of each of the six working standard solutions containing 0.05–20 μg of standard boswellic acids **1** to **6** were injected into the HPLC. The elution was carried out as described above. Standard calibration curves were obtained by plotting the concentration of standard boswellic acids *vs.* peak area (average of three runs).

The calibration range was chosen to reflect normal boswellic acids in *Boswellia serrata* extract samples. This range included concentration from the lower limit of quantification to the upper limit of quantification. The limit of quantification (LOQ) was defined as the lowest standard boswellic acid concentration, which can be determined with an accuracy and precision of < 2%.

Determination of boswellic acids 1 to 6: The sample volume was 20 μL. Boswellic acid concentrations were calculated on the basis of linear calibration functions and with regard to the dilution factor. The contents of boswellic acids **1–6** were expressed as g per 100 g of extract or plant material.

RESULTS AND DISCUSSION

All the boswellic acids **1–6** were isolated from commercially available extracts using column chromatography followed by preparative HPLC. The boswellic acids obtained were characterized by comparison of IR, mass spectral data and melting points with those reported in literature cited[3, 11]. The NMR spectral data of **1**, **2**, **4** and **6** have been interpreted by comparison with those of other ursane triterpenoids (Table-1 and Table-2). Assignment of NMR data of compound **4** was further supported by ^{1}H-^{13}C COSY experiments. The data of **3** and **5** (Table-1 and Table-2) were interpreted by comparison with those of oleananes[17, 18]. These

acids were purified by preparative HPLC using 0.1% v/v phosphoric acid in water and acetonitrile solvents (10 : 90) as mobile phase. HPLC analysis of compounds **1–6** showed single peaks at retention times of 7.02 ± 0.06, 9.63 ± 0.11, 16.34 ± 0.24, 18.18 ± 0.28, 25.40 ± 0.46 and 29.57 ± 0.57 min, respectively. The typical chromatograms of six boswellic acids at 210 and 248 nm have been shown in Fig. 2.

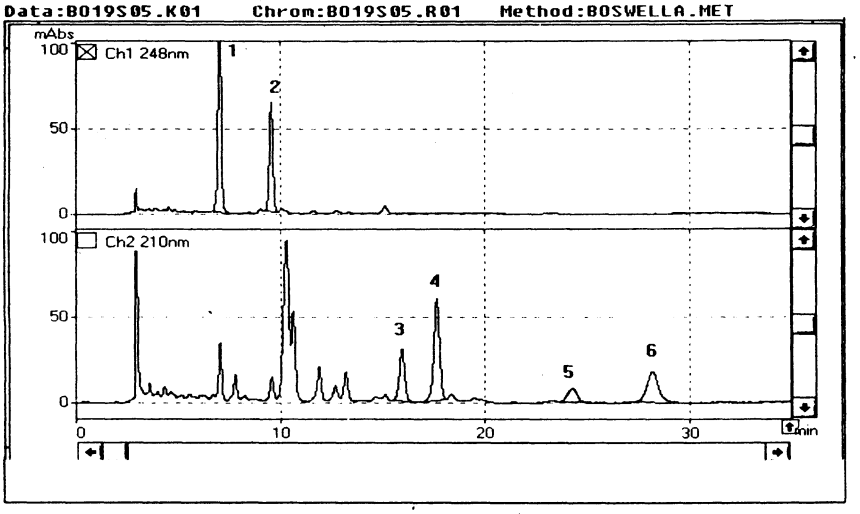

Fig. 2. HPLC chromatogram of *Boswellia* extract

1. 11-Keto-β-boswellic acid
2. 3-O-Acetyl-11-keto-β-boswellic acid
3. α-Boswellic acid
4. β-Boswellic acid
5. 3-O-Acetyl-α-boswellic acid
6. 3-O-Acetyl-β-boswellic acid

Calibration graphs were prepared to determine the boswellic acid content of different *Boswellia* samples. Calibration curves were derived from three independent injections of six concentrations of 11-keto-β-boswellic acid (**1**), 3-O-acetyl-11-keto-β-boswellic acid (**2**), α-boswellic acid (**3**), β-boswellic acid (**4**), 3-O-acetyl-α-boswellic acid (**5**) and 3-O-acetyl-β-boswellic acid (**6**) *vs.* the peak areas. Linearity was found in the concentration range between 0.1 and 10 μg, 0.05 and 10 μg, 0.2 and 10 μg, 0.3 and 20 μg, 0.1 and 5 μg and 0.5 and 10 μg for boswellic acids **1–6**, respectively, with high reproducibility and accuracy (Figs. 3–8).

The regression analysis of the experimental data shows that the linear relationship with correlation coefficients of boswellic acids **1–6** are 0.9999, 0.9999, 0.9999, 0.9999, 0.9997 and 0.9998, respectively. Accuracy of the method verified by the recovery studies is listed in Table-3. Using this method we have estimated the six boswellic acids in different grades of *Bosweliia serrata* extracts and *Boswelfia serrata* crude gum. The results are listed in Table-4. The individual peaks in all of the samples analyzed with no interference from other compounds. The identity of each peak was confirmed by determination of retention times and by spiking with standards.

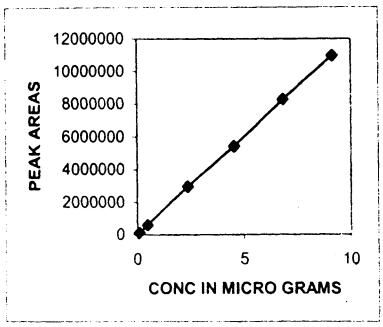

Figure 3. Linear relation ship between Peak area response and concentration of 11-keto-β-boswellic acid

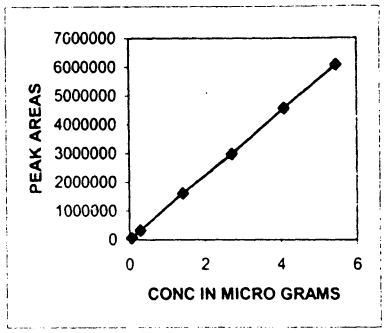

Figure 4. Linear relation ship between Peak area response and concentration of 3-O-acetyl-11-keto-β-boswellic acid

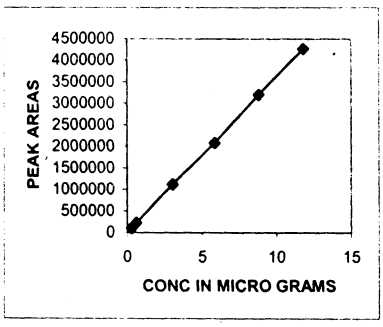

Figure 5. Linear relation ship between Peak area response and concentration of α-boswellic acid

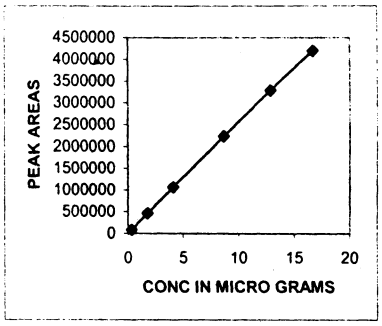

Figure 6. Linear relation ship between Peak area response and concentration of β-boswellic acid

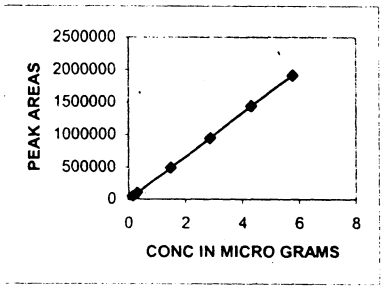

Figure 7. Linear relation ship between Peak area response and concentration of 3-O-acetyl-α-boswellic acid

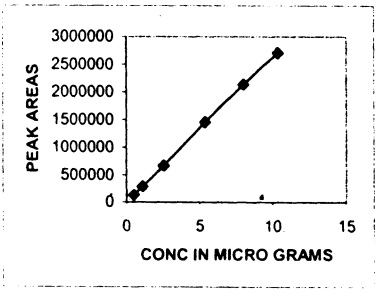

Figure 8. Linear relation ship between Peak area response and concentration of 3-O-acetyl-β-boswellic acid

TABLE-3
RECOVERY DATA

Compound	Amount added (mg)	Amount recovered (mg)	% Recovery*
11-Keto-β-boswellic acid (1)	0.130	0.1305	100.38
	0.260	0.2575	99.04
	0.520	0.5208	100.15
3-O-Acetyl-11-keto-β-boswellic acid (2)	0.130	0.1294	99.54
	0.260	0.2590	99.61
	0.520	0.5148	99.00
α-Boswellic acid (3)	0.145	0.1453	100.21
	0.290	0.2899	99.97
	0.580	0.5779	99.64
β-Boswellic acid (4)	0.120	0.1202	100.17
	0.240	0.2396	99.83
	0.480	0.4804	100.08
3-O-Acetyl-α-boswellic acid (5)	0.125	0.1240	99.20
	0.250	0.2498	99.92
	0.500	0.4982	99.64
3-O-Acetyl-β-boswellic acid (6)	0.120	0.1192	99.33
	0.240	0.2391	99.63
	0.480	0.4793	99.85

*Average of three determinations.

TABLE-4
PERCENTAGE (w/w) OF COMPOSITION OF BOSWELLIC ACIDS IN FIVE DIFFERENT GRADES OF EXTRACTS AND TWO DIFFERENT VARIETIES OF BOSWELLIA GUM BY HPLC*

Compound	Boswellia Extracts					Gum	
	I 109468	I 109470	I 90481	I 109516	A 007075	AR-757	AR-805
11-Keto-β-boswellic acid (1)	4.91±0.066	5.13±0.061	6.11±0.043	5.22±0.038	5.66±0.080	1.72±0.032	1.80±0.028
3-O-Acetyl-11-keto-β-boswellic acid (2)	1.13±0.043	1.14±0.058	1.59±0.059	1.26±0.060	3.38±0.062	0.86±0.009	1.07±0.010
α-Boswellic acid (3)	5.61±0.021	5.90±0.021	7.40±0.060	5.85±0.037	7.29±0.051	2.22±0.010	1.74±0.008
β-Boswellic acid (4)	5.80±0.018	16.86±0.012	20.99±0.028	16.58±0.018	20.86±0.035	5.89±0.019	4.47±0.017
3-O-Acetyl-α-boswellic acid (5)	1.64±0.032	1.84±0.033	2.99±0.078	1.82±0.069	3.57±0.082	0.70±0.020	1.01±0.018
3-O-Acetyl-β-boswellic acid (6)	6.89±0.010	7.60±0.011	9.66±0.018	7.31±0.017	12.92±0.015	2.70±0.018	2.87±0.017
Total boswellic acids (1-6)	35.98±0.190	38.47±0.196	48.74±0.286	38.04±0.239	53.68±0.325	14.09±0.108	12.96±0.098

Average of six determinations.

In the present study in *Bosweliia serrata* the active principles have been identified as 11-keto-β-boswellic acid (**1**), 3-O-acetyl-11-keto-β-boswellic acid (**2**), α-boswellic acid (**3**), β-boswellic acid (**4**), 3-O-acetyl-α-boswellic acid (**5**) and 3-O-acetyl-β-boswellic acid (**6**). We have developed simple analytical HPLC procedures for their determination with minimal sample preparation.

The method described is suitable for the routine analysis of large number of commercial samples of *Boswellia serrata* extracts.

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REFERENCES

1. J.L. Beton, T.G. Halsall and E.R.H. Jones, *J. Chem. Soc.*, **27**, 2904 (1956).
2. G.G. Allan, *Phytochemistry*, **7**, 963 (1968).
3. R.S. Pardhy and S.C. Bhattacharyya, *Indian J. Chem.*, **16B**, 176 (1978).
4. H. Safayhi, T. Mack, J. Sabieraj, M.I. Anazodo, LR. Subramanian and H.P.T. Ammon, *J. Pharmacol. Exp. Ther.*, **261**, 1143 (1992).
5. G.B. Singh, S. Singh and S. Bani, *Drugs of the Future*, **18**, 307 (1993).
6. E.R. Sailer, L.R. Subramanian, B. Rall, R.F. Hoernlein, H.P.T. Ammon and H. Safayhi, *Br. J. Pharmacol.*, **117**, 615 (1996).
7. H. Safayhi and E.R. Sailer, *Planta Med.*, **63**, 487 (1997).
8. E.R. Sailer, S. Schweizer, S.E. Boden, H.P.T. Ammon and H. Safayhi, *Eur. J. Biochem.*, **256**, 364 (1998).
9. S. Schweizer, A.F. von Brocke, S.E. Boden, E. Bayer, H.P.T. Ammon and H. Safayhi, *J. Nat. Prod..*, 63, 1058 (2000).
10. H. Safayhi, S.E. Boden, S. Schweizer and H.P.T. Ammon, *Planta Med.*, **66**, 110 (2000).
11. Y. Shao, C.T. Ho, C.K. Chin, V. Badmaev, W. Ma and M. Huang, *Planta Med.*, **64**, 328 (1998).
12. Y. Jing, S. Nakajo, L. Xia, K. Nakaya, Q. Fang, S. Waxman and R. Han, *Leukemia Res.*, **23**, 43 (1999).
13. M.C. Sharma, A. Kaul, A. Khajuria, S.S. Singh and G.B. Singh, *Phytotherapy Res.*, **10**, 107 (1996).
14. V. Rajagopal, Standardization of Botanicals, Eastern Publishers, New Delhi, Vol. 1, p. 51 (2003).
15. B.Buchele and T. Simmet, *J. Chromatogr. B.*, **795**, 355 (2003).
16. M. Ganzera and I.A. Khan, *Planta Med.*, **67**, 778 (2001).
17. M. Shamma, R.E. Glick and R.O. Mumma, *J. Org. Chem.*, **27**, 4512 (1962).
18. S.B. Mahato and A.P. Kundu, *Phytochemistry*, **37**, 1517 (1994).

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