Available online at www.sciencedirect.com
ScienceDirect
Phytochemistry 69 (2008) 1000–1004
PHYTOCHEMISTRY
www.elsevier.com/locate/phytochem

Withanolides from *Withania somnifera* roots

Laxminarain Misra [a,*], Priyanka Mishra [a], Archana Pandey [a], Rajender S. Sangwan [a], Neelam S. Sangwan [a], Rakesh Tuli [b]

[a] *Central Institute of Medicinal and Aromatic Plants, P.O.-CIMAP, Lucknow 226015, India*
[b] *National Botanical Research Institute, Lucknow 226001, India*

Received 17 August 2007; received in revised form 16 October 2007
Available online 3 December 2007

Abstract

Two new and seven known withanolides along with β-sitosterol, stigmasterol, β-sitosterol glucoside, stigmasterol glucoside, α + β glucose were isolated from the roots of *Withania somnifera*. Among the known compounds, Viscosa lactone B, stigmasterol, stigmasterol glucoside and α + β glucose are being reported from the roots of *W. somnifera* for the first time. One of the new compounds contained the rare 16β-acetoxy-17(20)-ene the other contained unusual 6α-hydroxy-5,7α-epoxy functional groups in the withasteroid skeleton. The structures were elucidated by spectroscopic methods and chemical transformations.

Keywords: *Withania somnifera* L.; Solanaceae; Ashwagandha; Withanolides; Withasteroids

1. Introduction

In continuation of our interest in the chemical investigation of Indian medicinal and aromatic plants (Misra and Wagner, 2004, 2007; Misra and Siddiqi, 2004, 2005), we have taken up *Withania somnifera* as one of the major plants for chemical and biological investigations (Sangwan et al., 2004). Our studies on the investigation of its leaves and fruits have been reported recently (Misra et al., 2005; Lal et al., 2006). Now, in the present paper we report the chemical investigation on the constituents of its roots.

2. Results and discussion

The roots of *W. somnifera,* which is popularly known as Ashwagandha- the Indian ginseng, are extensively used in most of the Indian herbal pharmaceuticals and nutraceuticals (Sangwan et al., 2004) and are well described in Ayurveda, the ancient Indian system of plant medicine for immuno-modulation and anti-ageing (Anonymous, 1962; Ray and Gupta, 1994). Our investigation has afforded 14 compounds from the methanol extract of roots, out of which two (**1, 2**) are new. Compound **3**, stigmasterol, stigmasterol glucoside and α + β glucose have been isolated for the first time from roots while the remaining 8 compounds were known. The structures of the known compounds were confirmed by the comparison of their spectral data available in the literature (Ray and Gupta, 1994; Su et al., 2002) and our library. The structures of new compounds were established by spectroscopic measurements, which are discussed below.

Compound **1** in its IR spectrum showed bands at 3449, 1718, 1702, 1686, 1243 and 1155 cm^{-1} indicative of the presence of hydroxyl, acetoxy, α,β-unsaturated lactone and carbonyl along with an epoxide group in the molecule. Its FAB HRMS showed $[M]^+$ at *m/z* 510.2954 corresponding to the molecular formula $C_{30}H_{38}O_7$. The ^1H NMR spectrum of **1** showed typical signals for a withasteroid (Experimental). The slightly upfield chemical shift of H-2 and H-3 at δ 5.85 and 6.60, respectively, suggested that the 2-en-1-one withasteroid belongs to the oxidations at C-5, C-6, C-7 which is typical of withanone type of steroids

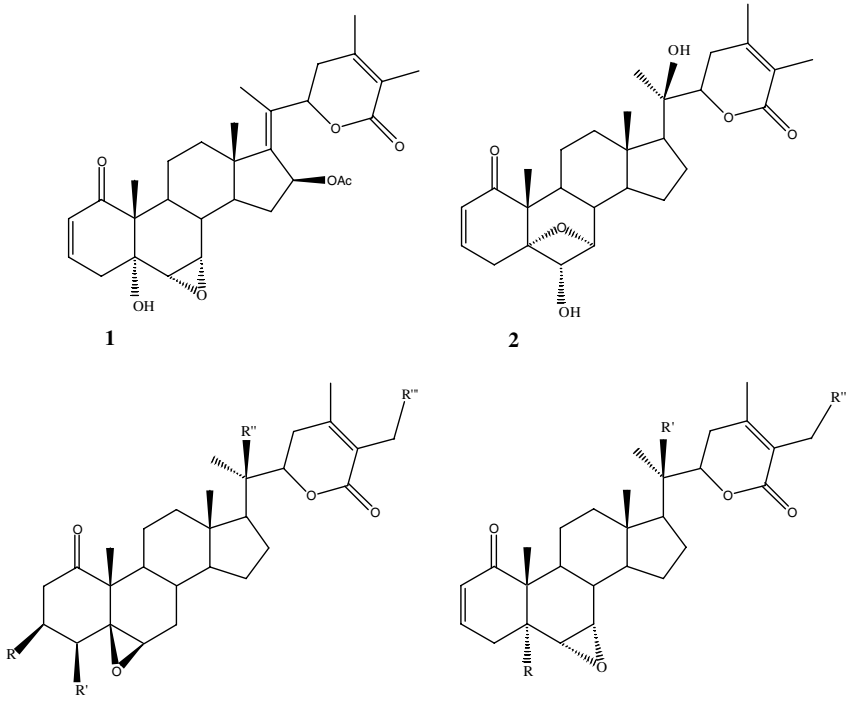
* Corresponding author. Tel.: +91 522 2717529; fax: +91 522 2342666.
E-mail address: laxmisra@hotmail.com (L. Misra).

0031-9422/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.phytochem.2007.10.024

and not withaferin A type (the two major steroids of *W. somnifera*). This was further supported by the multiplicity and coupling constant pattern of H-3 as *ddd* ($J = 10.0$, 5.0, 2.5 Hz) and epoxide signals at δ 3.06 (*d*, $J = 3.2$ Hz) and 3.30 (*dd*, $J = 3.2$, 2.1 Hz). The presence of an extra signals at δ 5.69 as a broad doublet ($J = 3.6$ Hz) and a singlet at δ 2.0 in the ^{1}H NMR spectrum and the signals at δ 21.6 (q), 170.18 (s) along with a doublet at δ 75.15 in the ^{13}C NMR spectrum clearly suggested that the molecule possesses an acetoxy group in withanone type of skeleton.

The downfield shift of (i) methyl at C-21 to δ 1.86 as singlet (ii) H-22 to δ 4.85 as *dd* (in place of typical ddd) suggested that a double bond is present at C-17 and C-20. This observation was also supported by the additional singlets at δ 131.7 and 146.3 in its ^{13}C NMR spectrum. The larger downfield chemical shift of C<u>H</u>-OAc at δ 5.69 also indicated that it is present in the vicinity of a double bond. It is quite probable that the acetoxy functional group is attached at C-16 in the withanone type of steroids with double bond at C-17–C-20. The acetoxy group at C-16 was supported by its downfield chemical shift at δ 75.15 in the ^{13}C NMR spectrum, also. In the ^{1}H^{1}H COSY spectrum the broad doublet ($J = 3.6$ Hz) at δ 5.69 showed correlations with H-15 at δ 1.86 only. The HMBC spectrum showed correlation of H-16 with C-14, C-15, C-17; H-21 with C-17 and C-16; H-18 with C-17 and C-20. Similarly, in the NOESY spectrum, H-16 showed correlation with H-22, H-18 and H-15α indicating the β-orientation of the acetoxy at C-16. The β-acetoxy at C-16 was further supported by the coupling constant of H-16 as 3.6 Hz. In case of α-acetoxy the coupling constant must be larger (9.0, 7.2 Hz) as reported in the case of a withasteroid earlier isolated from *Iochroma coccineum* by Alfonso et al. (1991) whose structure was confirmed by X-ray analysis. The structure of **1** was deduced from DEPT, ^{1}H^{1}H COSY, HSQC, HMBC, NOESY spectra (Experimental) and found to be consistent with 16β-acetoxy-6α,7α-epoxy-5α–hydroxy-1- oxowitha-2,17(20),24-trienolide (Fig. 1).

The IR spectrum of **2** showed bands at 3433, 1702, 1686, and 1140 cm^{-1} indicative of the presence of hydroxyl, α,β-unsaturated lactone and ketone along with an epoxide group in the molecule. Its FAB HRMS showed $[M]^+$ at *m*/*z* 470.2960 corresponding to the molecular formula $C_{28}H_{38}O_6$. The ^{1}H NMR spectrum of **2** showed typical signals for a 2-en-1-one withasteroid with a slightly upfield chemical shift of H-2 and H-3 at δ 5.88 and 6.57, respectively, suggesting that the steroid has oxidations at C-5, C-6, C-7 as in case of compound **1**. With the coupling pattern of H-3 (*ddd*, $J = 10.0$, 5.0, 2.5 Hz) at δ 6.57, it was evident that C-4 was not oxygenated and **2** has a withanone type oxidation pattern in the ring A. However, in ring B the hydroxy and epoxy pattern appeared to be different in the ^{1}H NMR spectrum. In place of typical epoxide protons of H- 6 and H- 7 at δ 3.05 (*d*, $J = 3.2$ Hz) and 3.30 (*dd*, $J = 3.2$, 2.1 Hz), it showed a doublet ($J = 12.0$ Hz) at δ 3.66 and a triplet ($J = 12.0$ Hz) at δ 3.88. Its ^{13}C NMR also



1

2

3. R = R' = R''' = OH, R'' =H
4. 2-ene, R = R'' =H,R' = R''' = OH
5. 2-ene, R = R''' = H,R' = R'' =OH

6. R = OH, R' = R'' =H
7. R = R' = OH, R'' =H
8. R = R'' =OH, R'= H
9. R = R' = R''=OH

Fig. 1. Structure of Withanolides from *W. somnifera* roots.

showed signals at δ 76.22, 77.28, 62.5 in place of typical δ 73.0, 57.0 and 56.0 for C-5, C-6, C-7, respectively (see Table 1).

After acetylation, **2** yielded **2a** which showed almost negligible downfield shift for H-7 (δ 4.00) while H-6 shifted drastically to δ 5.23 with an additional methyl singlet at δ 2.18 in its ^{1}H NMR spectrum. It clearly indicated that the molecule contains one secondary hydroxyl group. In the ^{1}H^{1}H COSY the doublet at δ 5.23 showed correlation with the triplet at δ 4.00 which showed further correlation with H-8 at δ 2.29 clearly indicating that the epoxide linked C-5 and C-7 with a free hydroxyl at C-6. This type of oxidation pattern was further supported by the HMBC (Experimental). The α-stereochemistry suggested for 6-hydroxy-5,7-oxide was based on the larger coupling constant (12.0 Hz) among H-6, H-7 and H-8 (Bessalole et al., 1987). This stereochemistry was supported by the acidic conversion of **2** into withanolide A (**7**) having similar stereochemistry as C-5α, C-6α and C-7α. The proposed stereochemistry of **2** was further linked biogenetically to the major steroids of *W. somnifera* with 5α-hydroxy-6α,7α-epoxy type of functional groups.

The ^{1}H NMR showed two singlets at δ 0.94 and 1.25 for H-18 and H-19 and two more singlets at δ 1.88 and 1.97 for H-27 and H-28 with typical double bond at C-24 and C-25. Another singlet at δ 1.34 for H-21 indicated that it contains a hydroxyl group at C-20 which was further supported by the singlet at δ 77.71 in its ^{13}C NMR spectrum. The coupling pattern of H-22 (*dd*, $J = 13.0$, 4.0 Hz) and HMBC, HSQC and DEPT experiments (Experimental) also supported a C-20 hydroxyl group and **2** is 5,7α-epoxy-6α,20α–dihydroxy-1-oxowitha-2,24- dienolide (Fig. 1).

The configurations at C-17 and C-22 were followed as described in our earlier publications (Misra et al., 2005; Lal et al., 2006). In the case of **2**, the configurations were also supported by its conversion into **7** whose structure has been studied in detail (Subramanian et al., 1971). However, compound **1** with 16β-acetoxy group and **2** with 5,7α-epoxide group, are unusual oxidized/cyclized compounds. The readily conversion of **2** into a more thermostable withanolide A (**7**) suggests that it may be a biogenetic precursor of withanolide A as the latter is the major constituent of the roots of *W. somnifera*.

Table 1
^{13}C NMR spectral data (δH)[a] for compound **1, 2, 2a** (75 MHz, $CDCl_3$)

Carbon	1	2	2a
1	202.9, s	202.1, *s*	203.0, *s*
2	129.4, *d*	128.4, *d*	128.7, *d*
3	139.9, *d*	141.1, *d*	139.4, *d*
4	37.5, *t*	40.9, *t*	40.6, *t*
5	73.6, *s*	76.2, *s*	75.1, *s*
6	56.5, *d*	77.3, *d*	77.4, *d*
7	57.1, *d*	62.5, *d*	62.5, *d*
8	35.1, *d*	42.6, *d*	42.6, *d*
9	35.8, *d*	42.8, *d*	42.7, *d*
10	51.5, *s*	52.0, *s*	51.5, *s*
11	34.9, *t*	22.5, *t*	22.4, *t*
12	37.2, *t*	35.6, *t*	35.4, *t*
13	45.8, *s*	45.0, *s*	45.0, *s*
14	48.8, *d*	56.9, *d*	56.7, *d*
15	22.4, *t*	24.6, *t*	24.5, *t*
16	75.15, *d*	27.0, *d*	26.8, *d*
17	131.7, *s*	55.0, *s*	54.0, *s*
18	12.9, *q*	12.1, *q*	12.3, *q*
19	15.0, *q*	20.3, *q*	20.4, *q*
20	146.3, *s*	77.7, *s*	78.3, *s*
21	17.5, *q*	14.7, *q*	14.8, *q*
22	77.8, *d*	81.5, *d*	80.9, *d*
23	33.15, *t*	31.8, *t*	31.6, *t*
24	148.7, *s*	145.0, s	148.7, *s*
25	122.7, *s*	123.0, *s*	122.0, *s*
26	166.5, *s*	167.5, *s*	167.0, *s*
27	12.5, *q*	14.3, *q*	14.3, *q*
28	20.6, *q*	20.8, *q*	20.6, *q*
OAc	170.2, *s*	–	170.1, *s*
	21.6, *q*	–	20.9, *q*

[a] Values from proton noise decoupled spectra and supported by DEPT and HSQC.

3. Experimental

3.1. General

It has been followed as described in our previous paper (Misra et al., 2005).

3.2. Plant material

The *W. somnifera* (Ashwagandha) roots were collected from Hyderabad, India in October 2004. The specimen voucher of the material is deposited in our institute's herbarium and the crop is propagated in our experimental farm.

3.3. Extraction and isolation of compounds

The shade dried roots (4.028 kg) were ground and defatted three times with *n*-hexane by keeping at RT overnight. The marc was further extracted with MeOH (3×4 l) at RT overnight. A 1/3 of methanol extract (38 g) was chromatographed over a column of silica gel (700 g) with *n*-hexane as mobile phase and then elution was carried out in *n*-hexane and EtOAc with solvent gradient. The polarity was increased by sequentially adding 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% ethyl acetate, pure ethyl acetate and finally 2.5%, 5%, 10%, 15%, 20% methanol was added in the ethyl acetate. The fractions (150 ml each) of CC were collected and pooled into thirteen major fractions based on their TLC pattern. Fr. 1 yielded stearic acid (24.0 mg) while fr. 2 contained β-sitosterol (30.5 mg) and stigmasterol (16.0 mg). Fr 3 contained withanolide B (**6**, 4.0 mg). Fr. 4 and 5 yielded withanolide B (**6**, 14.5 mg) and withanolide A (**7**, 620.6 mg) respectively. Fr.6 on further CC and crystallization yielded withanolide A (**7**, 2.5 mg), and a mixture of two compounds which after preparative TLC ($CHCl_3$:

EtOAc:MeOH:C_6H_6 70:8:2:20) yielded compound **1** (14.0 mg, Rf 0.75) and withanolide D (**5**, 5.0 mg). Fr.7 on further CC and crystallization yielded withanolide A (**7**, 160.5 mg) and withanolide B (**6**, 13.0 mg). Fr.8 after CC afforded withanolide B (**6**, 5.0 mg), withanolide A (**7**, 620.0 mg) and a mixture of three compounds which after preparative TLC yielded 27-hydroxy withanolide B (**8**, 38.8 mg), withaferin A (**4**, 51.1 mg) and β-sitosterol glucoside (12.2 mg). Fr.9 after further CC afforded compound **2** (12.5 mg, Rf 0.37, $CHCl_3$:EtOAc:MeOH:C_6H_6 70:8:2:20). Fr. 10 after further CC yielded β-sitosterol glucoside (70.5 mg) and an unidentified compound (130.0 mg). Fr. 11 on further CC yielded 27-hydroxy withanolide B (**8**, 8.8 mg), withaferin A (**4**, 51.3 mg), β-sitosterol glucoside (131.3 mg) and a mixture which after preparative TLC yielded 27-hydroxy withanolide A (**9**, 3.4 mg) and β-sitosterol glucoside (128.0 mg) and stigmasterol glucosides (60.0 mg). Fr.12 on further CC and preparative TLC ($CHCl_3$:EtOAc:MeOH 82:12:6) afforded compound **3** (10.5 mg, Rf 0.42). Fr. 13 gave the enantiomeric mixture of α- and β-glucose (13.0 mg).

*3.3.1. 16β-Acetoxy-6,7α-epoxy-5α–hydroxy-1-oxowitha-2,17(20),24-trienolide (**1**)*

M.p.: 238-40°C; $[\alpha]_D^{30}$: +0.97° MeOH, $c = 0.24$; IR (KBr) cm^{-1}: 3449(OH), 1718 (acetate), 1702 (α,β-unsaturated lactone), 1686 (α,β-unsaturated ketone), 1243 and 1155 (epoxide); FAB HRMS: 510.2954 (Calc. for $C_{30}H_{38}O_7$ 510.2960) *m/z* (rel. int.): 510 (4) $[M]^+$, 450 (5) $[M- AcOH]^+$, 432 (5) $[450- H_2O]^+$, 387 (24), 285 (22), 125 (100); ^1H NMR spectral data (300 MHz, $CDCl_3$) δH: 5.85 (1H, *dd* ($J = 10.0$, 3.0 Hz), H-2), 6.60 (1H, *ddd* ($J = 10.0$, 5.0, 2.5 Hz), H-3), 2.58 (1H, *m*, H-4), 2.67 (1H, *ddd* ($J = 22.0$, 5.0, 2.5 Hz) H-4′), 3.12 (1H, *s*, C-5OH), 3.06 (1H, *d* ($J = 3.2$ Hz), H-6), 3.30 (1H, *dd* ($J = 3.2$, 2.1 Hz), H-7), 1.86 (2H, *m*, H-15), 5.69 (1H, *d br* ($J = 3.6$ Hz), H-16), 4.85 (1H, *dd* ($J = 13.0$, 3.3 Hz), H-22), 2.72 (1H, *m*, H-23), 2.18 (1H, *m*, H-23′), 1.91 (3H, *s*, H-27), 2.03 (3H, *s*, H-28), 1.86 (3H, *s*, H-21), 0.97 (3H, *s*, H-18), 1.19 (3H, *s*, H-19), 2.00 (3H, *s*, OAc) (interpretation supported by $^1H^1H$ COSY, HSQC); HMBC: H-3 → C-1, C-2, C-4, C-5, H-16 → C-14, C-15, C-17, H-21 → C-17, C-16, C-22, H-18 → C-17, C-20, OCO<u>CH</u>$_3$ → O<u>CO</u>CH$_3$; NOESY: H-16 → H-15, H-18, H-22, H-22 → H-23, H-21, H-16, H-21 → H-16, H-22.

*3.3.2. 5,7α-Epoxy-6α,20α-dihydroxy-1-oxowitha-2,24-dienolide (**2**)*

M.p.: 242°C; $[\alpha]_D^{30}$: +12.73° MeOH, $c = 0.14$; IR (KBr) cm^{-1}: 3433(OH), 1702 (α,β unsaturated lactone), 1686 (α,β unsaturated ketone), 1140 (epoxide); FAB HRMS: 470.2960 (Calc. for $C_{28}H_{38}O_6$ 470.2966) *m/z* (rel. int.): 470 (8) $[M]^+$, 434 (5) $[M- 2H_2O]^+$, 416 (5) $[M- H_2O]^+$, 341 (35), 285 (22), 125 (100); ^1H NMR spectral data (300 MHz, $CDCl_3$) δH: 5.88 (1H, *dd* ($J = 10.0$, 3.0 Hz), H-2), 6.57 (1H, *ddd* ($J = 10.0$, 5.0, 2.5 Hz), H-3), 2.57 (1H, *m*, H-4), 2.10 (1H, *ddd* ($J = 22.0$, 5.0, 2.5 Hz) H-4′), 3.66 (1H, *d* ($J = 12.0$ Hz), H-6), 3.88 (1H, *t* ($J = 12.0$ Hz), H-7), 2.22 (1H, *m*, H-8), 4.23 (1H, *dd* ($J = 13.0$, 4.0 Hz), H-22), 2.51 (1H, *m*, H-23), 2.17 (1H, *m*, H-23'), 1.88 (3H, *s*, H-27), 1.97 (3H, *s*, H- 28), 1.30 (3H, *s*, H-21), 0.94 (3H, *s*, H-18), 1.20 (3H, *s*, H-19) (interpretation supported by $^1H^1H$ COSY, HSQC); HMBC: H-3 → C-1, C-2, C-4, C-5, H-6 → C-5, C-7, H-7 → C-5, C-6, C-8, H-21 → C-17, C-22, H-18 → C-17, C-20.

*Acetylation of **2***. Compound **2** (5 mg) in acetic anhydride (1 ml) in the presence of pyridine gave **2a** (5 mg). ^1H NMR spectral data (300 MHz, $CDCl_3$) δH: 5.89 (1H, *dd* ($J = 10.0$, 3.0 Hz), H-2), 6.51 (1H, *ddd* ($J = 10.0$, 5.0, 2.5 Hz), H-3), 2.63 (1H, *m*, H-4), 2.17 (1H, *ddd* ($J = 22.0$, 5.0, 2.5 Hz) H-4′), 5.23 (1H, *d* ($J = 12.0$ Hz), H-6), 4.00 (1H, *t* ($J = 12.0$ Hz), H-7), 2.29 (1H, *m*, H-8), 4.20 (1H, *dd* ($J = 13.0$, 4.0 Hz), H-22), 2.51 (1H, *m*, H-23), 2.17 (1H, *m*, H-23′), 1.88 (3H, *s*, H-27), 1.96 (3H, *s*, H-28), 1.30 (3H, *s*, H-21), 0.94 (3H, *s*, H-18), 1.25 (3H, *s*, H-19) 2.17 (3H, *s*, OAc) (interpretation supported by $^1H^1H$ COSY, HSQC).

*Conversion of **2** into withanolide A (**7**)*. Compound **2** (4 mg) was taken in acetic acid and heated at 80 °C for 12 h and reaction mixture was dried completely under reduced pressure. The residue was purified by TLC to afford withanolide A (**7**, 2 mg).

Acknowledgements

CSIR, New Delhi is thanked for the research grant under NMITLI and Director, CIMAP, Lucknow for the research facilities. Dr. Vijayakumar, IICT, Hyderabad, India is thanked for his assistance in the collection and identification of the plant material.

References

Alfonso, D., Kapetanidis, I., Bernardinelli, G., 1991. Iochromolide: A new acetylated withanolide from *Iochroma coccineum*. J. Nat. Prod. 54, 1576–1582.

Anonymous, 1962. The Wealth of India, PID, CSIR, New Delhi, vol. 8, pp. 37–38.

Bessalole, R., Lavie, D., Frolow, F., 1987. Withanolide Y, a withanolide from a hybrid of *Withania somnifera*. Phytochemistry 26, 1797–1800.

Lal, P., Misra, L.N., Sangwan, R.S., Tuli, R., 2006. New withanolides from fresh berries of *Withania somnifera*. Z. Naturforsch. B 61b, 1143–1147.

Misra, L.N., Siddiqi, S.A., 2004. Dhaincha (*Sesbania bispinosa*) leaves: A good source of antidiabetic (+)-Pinitol. Curr. Sci. 87, 1507.

Misra, L.N., Siddiqi, S.A., 2005. Biologically active inositol, sterols and lipid derivatives from *Sesbania bispinosa*. Indian J. Chem. 44B, 1915–1921.

Misra, L.N., Wagner, H., 2004. Alkaloidal constituents of *Mucuna pruriens* seeds. Phytochemistry 65, 2565–2567.

Misra, L.N., Wagner, H., 2007. Extraction of bioactive principles from *Mucuna pruriens* seeds. Indian J. Biochem. Biophys. 44, 56–60.

Misra, L.N., Lal, P., Sangwan, R.S., Sangwan, N.S., Uniyal, G.C., Tuli, R., 2005. Unusually sulfated and oxygenated steroids from *Withania somnifera* leaves. Phytochemistry 66, 2702–2707.

Ray, A.B., Gupta, M., 1994. Withasteroids, a growing group of naturally occurring steroidal lactones. Prog. in the Chem. Org. Nat. Prod, vol. 63. Springer-Verlag, New York, pp. 1–106.

Sangwan, R.S., Chaurasia, N.D., Misra, L.N., Lal, P., Uniyal, G.C., Sharma, R., Sangwan, N.S., Suri, K.A., Qazi, G.N., Tuli, R., 2004. Phytochemical variability in commercial herbal products and preparations of *Withania somnifera* (Ashwagandha). Curr. Sci. 86, 461–465.

Subramanian, S.S., Sethi, P.D., Glotter, E., Kirson, I., Lavie, D., 1971. 5,20α-dihydroxy-6α,7α-epoxy-1-oxo- (5α)witha-2,24-dienolide, a new steroidal lactone from *Withania coagulans*. Phytochemistry 10, 685–688.

Su, B.N., Misico, R., Park, E.J., Santarsiero, B.D., Mesecar, A.D., Fong, H.H.S., Pezzuto, J.M., Kinghorn, A.D., 2002. Isolation and characterization of bioactive principles of the leaves and stems of *Physalis philadelphica*. Tetrahedron 58, 3453–3466.