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Cyclooxygenase-2 enzyme inhibitory withanolides from *Withania somnifera* leaves

Bolleddula Jayaprakasam and Muraleedharan G. Nair*

Bioactive Natural Products and Phytoceuticals, Department of Horticulture and National Food Safety and Toxicology Center, Michigan State University, East Lansing, MI 48824, USA

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Abstract—Four novel withanolide glycosides and a withanolide have been isolated from the leaves of *Withania somnifera*. The structures of the novel compounds were elucidated as physagulin D (1→6)-β-D-glucopyranosyl-(1→4)-β-D-glucopyranoside (**1**), 27-*O*-β-D-glucopyranosyl physagulin D (**2**), 27-*O*-β-D-glucopyranosyl viscosalactone B (**3**), 4,16-dihydroxy-5β, 6β-epoxyphysagulin D (**4**), and 4-(1-hydroxy-2,2-dimethylcyclo-propanone)-2,3-dihydrowithaferin A (**5**) on the basis of 1D-, 2D NMR and MS spectral data. In addition, seven known withanolides withaferin A (**6**), 2,3-dihydrowithaferin A (**7**), viscosalactone B (**8**), 27-desoxy-24,25-dihydrowithaferin A (**9**), sitoindoside IX (**10**), physagulin D (**11**), and withanoside IV (**12**) were isolated. These withanolides were assayed to determine their ability to inhibit cycloxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) enzymes and lipid peroxidation. The withanolides tested, except compound **9**, showed selective COX-2 enzyme inhibition ranging from 9 to 40% at 100 μg/ml. Compounds **4**, **10** and **11** also inhibited lipid peroxidation by 40, 44 and 55%, respectively. The inhibition of COX-2 enzyme by withanolides is reported here for the first time.

1. Introduction

Withania somnifera (L) Dunal of solanaceae, is an erect evergreen shrub distributed throughout the drier parts of India. *W. somnifera*, known as Aswagandha, is well known for its use in Ayurvedic medicine. The Aswagandha root extract was reported as a folk remedy for adenopathy, arthritis, asthma, hypertension, inflammations, and rheumatism.[1] The leaves of *W. somnifera* were also used as a cure for several illnesses including tumors, inflammations, conjunctivitis and tuberculosis.[1] Currently, powdered roots or root extract of this plant are used as a dietary supplement in the United States.

The major chemical constituents reported from *W. somnifera* are called withanolides. These compounds are structurally diverse steroidal compounds with an ergosterol skeleton in which C-22 and C-26 are oxidized to form a δ-lactone.[2] The chemical investigations of the roots and leaves of *W. somnifera* resulted in the isolation and characterization of several withanolides.[3] The fruits of this plant are tiny orange berries and reported to contain saturated and unsaturated fatty acids.[4–6] However, leaves and fruits are not fully investigated for biological activities. The withanolides are classified according to their structural skeleton[2] and the structural variation is responsible for the wide array of pharmacological activities. Withanolides have been studied for their antinflammatory, antitumor, cytotoxic, immunomodulating activities and for the protection against CCl_4-induced hepatotoxicity.[2,7] They were also reported to induce phase-II enzymes in animal models, which is considered to be one of the mechanisms in cancer chemoprevention.[8,9]

Cyclooxygenase-1 (COX-1) and -2 (COX-2) enzymes are responsible for the conversion of arachidonic acid, a lipid present in the cell, to prostaglandins. Prostaglandins in turn cause inflammatory responses in the body. Inhibition of COX-1 enzyme may result in the formation of ulcers in many human and hence the selective inhibition of COX-2 enzyme by compounds has a major advantage over non-selective non-steroidal anti-inflammatory drugs (NSAIDs)[10] sold over the counter (OTC). It is important to note that over expression of COX-2 enzyme was observed not only in inflamed cells but also by various types of tumor cells.[11–13] Hence, COX-2 inhibitors with little or no COX-1 activity are of great interest for the chemoprevention of cancer.

Continuing our investigations on generally-regarded-as-safe (GRAS) plants for phytoceutical or nutraceutical applications, we have discovered that leaf extracts of *W. somnifera* possess excellent selective COX-2 inhibitory activity. In this study, we report the isolation and characterization of several novel withanolides and a number of known withanolides from *W. somnifera* leaf extracts. The

Keywords: withanolides; *Withania somnifera*; solanaceace; antioxidant; antiinflammatory; cyclooxygenase enzyme.

* Corresponding author. Tel.: +1-517-353-2915; fax: +1-517-432-2310; e-mail: nairm@msu.edu

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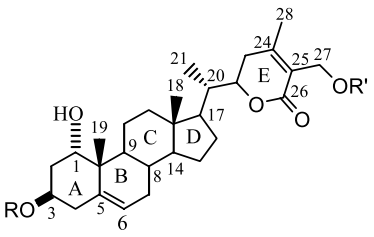
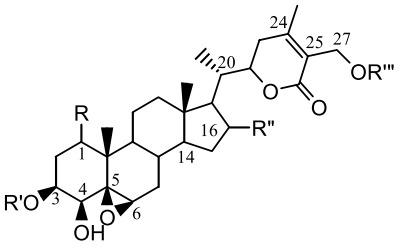
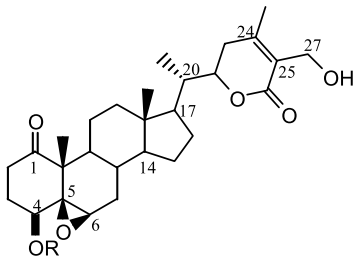
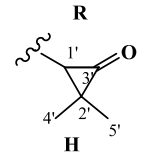


	R	R'
1	Glc-(1→6)-Glc-(1→4)-Glc	H
2	Glc	Glc
11	Glc	H
12	Glc-(1→6)-Glc	H

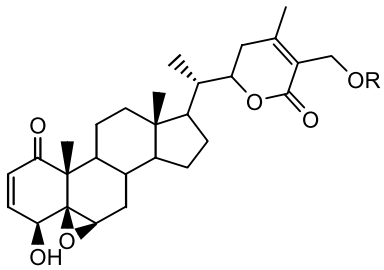


	R	R'	R"	R"'
3	=O	H	H	Glc
4	-OH	Glc	OH	H
8	=O	H	H	H

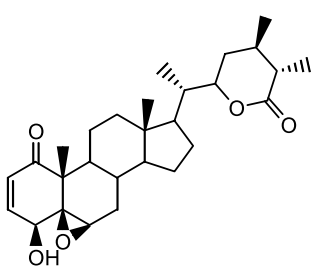




	R
5	
7	H



	R
6	H
10	Glc



inhibitory effects of withanolides isolated from the leaves on cyclooxygenase enzymes and their antioxidant activities are also presented.

2. Results and discussion

The *W. somnifera* leaf extract purchased from PhytoMyco Research Corporation was compared to the methanol extract of fresh leaves harvested from *W. somnifera* plants grown in the greenhouses of Bioactive Natural Products and Phytoceuticals Laboratory at Michigan State University. Both extracts were identical in composition, as confirmed by TLC analysis. The extracts were purified by preparative TLC and by HPLC to yield pure withanolides **1**–**12**.

Compound **1** was isolated as an amorphous powder and its molecular formula was determined as $C_{46}H_{73}O_{20}$ by HRFABMS as indicated by an $[M+H]^+$ ion at *m/z* 945.4682 (calcd 945.4695). Compound **1** showed absorption bands in its IR spectrum at 3406 and 1698 cm^{-1}, respectively, corresponding to an –OH and an α,β-unsaturated lactone moieties. Three anomeric protons doublets at δ 4.22, 4.20 and 4.15 were correlated to three anomeric carbons at δ 103.0, 103.9 and 104.8, respectively, in its HMQC spectrum and suggested that compound **1** contained a triglycosidic moiety. Apart from the glycosidic signals, compound **1** exhibited signals for 28 carbons. The signals at 80.1, 32.8, 160.4, 123.6, and 168.6 ppm were

assigned to a six-membered ring α,β-unsaturated δ-lactone moiety in the molecule and the olefinic carbons at δ 139.0 and 125.5 were assigned to C-5 and C-6. The DEPT spectrum of compound **1** showed the presence of three methine carbons at δ 74.9, 73.6, 80.1 and a methylene carbon at δ 57.6 and were indicative of C-1, C-3, C-22 and C-27 oxygenated carbons.

The singlets at δ 0.60, 0.90, and 1.90 and a doublet at 0.92 ppm in its ^{1}H NMR were assigned to C-18, 19, 28 and 21, respectively. An olefinic proton at δ 5.50 was placed at C-6 as it showed correlations with this carbon at 125.5 ppm in its HMQC. The C-6′ and C-4″ of the sugar units in compound **1** appeared at 70.0 and 77.8 ppm, respectively. These carbons normally appear at around 62 and 71 ppm, respectively, in glucose that are not conjugated. The attachment of one the glucose units to C-3 of the aglycone was confirmed by the HMBC correlations (Fig. 1a) observed between H-1′ at δ 4.22 and C-3 at 73.6 ppm. Other HMBC correlations significant to glucose linkages in compound **1** (Fig. 1a) were H-1″ at δ 4.20 to C-6′ at δ 70.0 and H-1‴ at δ 4.15 to C-4″ at 77.8 ppm, respectively. Both ^{1}H and ^{13}C NMR spectral data confirmed that compound **1** possessed only D-glucose as sugar moieties. The TLC analysis of sugar obtained from the acid hydrolysis of compound **1** was identical to an authentic sample of D-glucose. The ^{1}H NMR spectral data of the aglycone was identical to the published spectral data of sominone.[14] Also, the ^{13}C NMR data of compound **1** was compared to sominone, obtained as a hydrolysis product of physagulin D (**11**) in our laboratory. Therefore, glucose linkages in compound **1** were established as [β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl-(1→4) β-D-glucopyranoside]. The mass spectral fragments obtained at *m/z* 783, 621 and 459 for compound **1** in its FABMS showed the successive loss of three glucose units which further confirmed the proposed structure of compound **1** as physagulin D (1→6)-β-D-glucopyranosyl-(1→4)-β-D-glucopyranoside.

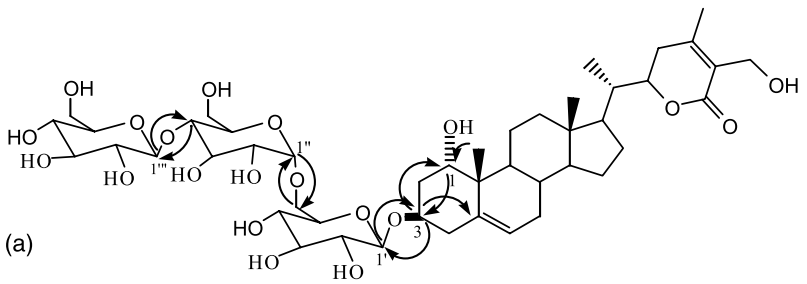
The IR spectrum of compound **2**, isolated as an amorphous powder, showed absorption bands at 3407, 1696 cm^{-1} were due to the hydroxyl and an α,β-unsaturated δ-lactone carbonyl functionalities. The mass spectrum of **2** displayed an $[M+H]^+$ ion at *m/z* 783.4168 (calcd 783.4188), which was consistent with the molecular formula as $C_{40}H_{63}O_{15}$. The ^{1}H NMR signals appeared as singlets at δ 0.76, 1.01 and 2.11, respectively, were assigned to three methyl groups in **2**. It also showed one methyl doublet at δ 1.22, C-27 methylene protons as doublets, integrated for one proton each, at δ 4.60 and 4.46, two oxymethine multiplets at δ 4.50 and 3.83 and an olefinic proton at δ 5.49. ^{13}C NMR of **2** exhibited signals for an α,β-unsaturated δ-lactone carbonyl at δ 168.6, two oxygenated methines at δ 74.9 and 73.6 and olefinic carbons at δ 139.1 and 125.4. The NMR spectra of compound **2** were similar to that of **1**, with a major difference due to the absence of one of the sugars as indicated by the lack of anomeric proton and carbon signals at δ 4.20 and 104.8, respectively. Acid hydrolysis of **2** gave D-glucose and sominone,[14] identified by comparing the TLC with hydrolysis products of **1**. Compound **2** gave a molecular ion at *m/z* 783, which is 162 amu less than that of **1**, suggested that its probable structure was simonene diglucoside. One of the glucose units in compound **2** was assigned at C-3 based on the HMBC correlations (Fig. 1b) observed between H-3 at δ 3.83 and C-1′ at δ 102.7. The second glucose unit in compound **2** was assigned at C-27 as this carbon at δ 63.5 was shifted to downfield by 5.9 ppm when compared to the chemical shift of similar carbon in **1** at δ 57.6. The linkage in compound **2** was further supported by its HMBC spectral correlations (Fig. 1b) observed between C-27 at δ 63.5 and H-1″ at δ 4.31. The placement of sugar units at C-3 and C-27 was further confirmed by comparison of ^{13}C NMR data of A-ring and lactone ring carbons in **2** with that of physagulin D (**11**) and sitoindoside IX (**10**). Therefore, structure of **2** was concluded as 27-*O*-β-D-glucopyranosyl physagulin D.

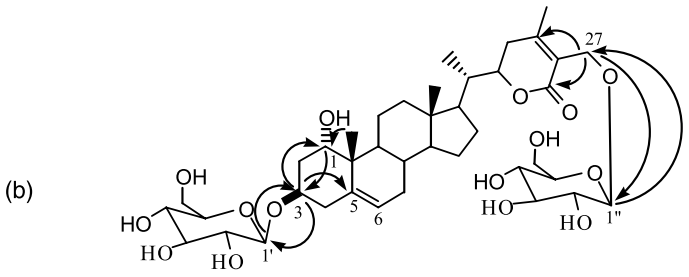
The HRFABMS of compound **3**, obtained as an amorphous powder, revealed an $[M+Na]^+$ peak at *m/z* 673.3200 (calcd 673.3224) and corresponded to a molecular formula of $C_{34}H_{50}O_{12}$. The IR spectrum of compound **3** indicated the presence of hydroxyl, α,β-unsaturated δ-lactone and a six-membered ring ketone in the molecule as indicated by absorption bands at 3425, 1700, 1652 cm^{-1}, respectively. The ^{1}H NMR spectrum of compound **3** displayed signals for three oxygenated methine protons at δ 3.66, 3.33 and 4.44, four methyls at δ 0.67, 0.98, 1.18, and 2.1 and oxymethylene protons at δ 4.59 and 4.45. It also showed a doublet for an anomeric proton at δ 4.31 and a broad singlet at 3.15 ppm, which correlated to the anomeric carbon at δ 102.7 and an epoxide carbon at δ 56.6 in its HMQC, respectively. The ^{13}C NMR spectrum showed signals due to carbonyl carbon of a keto group at δ 210.2; an epoxide moiety at δ 63.8 and 56.6 and for a six-membered ring α,β-unsaturated lactone moiety at δ 78.9, 29.6, 159.1, 122.5, and 167.4. The spectral data suggested that compound **3** was closely related to viscosalactone B (**8**).

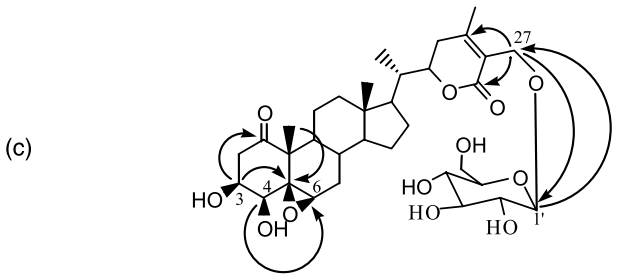
Hydrolysis of compound **3** gave viscosalactone B (**8**) and D-glucose as confirmed by spectral studies. This indicated that compound **3** was a viscosalactone glucoside. Analyses of its HMBC spectrum (Fig. 1c) suggested that the glucose moiety was attached to C-27 as this carbon at δ 61.6 correlated with the anomeric proton at 4.31 ppm. Linkage of one of the glucose units to C-27 was also supported by the downfield shift of C-27 by 5.3 ppm as compared to its aglycone viscosalactone B (**9**), which appeared at δ 57.1. The spectral evidence confirmed the structure of compound **3** as 27-*O*-β-D-glucopyranosyl viscosalactone B (**3**).

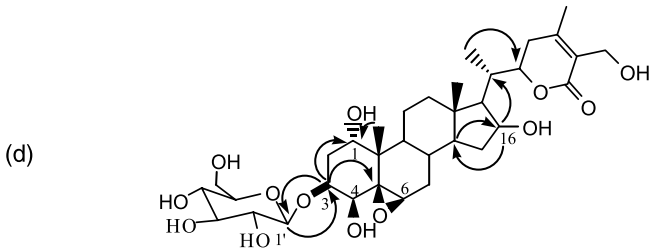
Compound **4** was obtained as an inseparable mixture with compound **12** and the ratio was about 2:1. The HRFABMS displayed an $[M+H]^+$ ion at *m/z* 669.3456 (calcd 669.3486) which corresponded to the molecular formula $C_{34}H_{53}O_{13}$. The base peak at *m/z* 507, produced by the loss of 162 amu from the molecular ion, indicated that compound **4** contained a mono glycoside. ^{1}H and ^{13}C NMR assignments for compound **4** were unambiguously assigned by supporting evidences from DEPT, HMQC and HMBC spectral studies.

Apart from the sugar carbons, ^{13}C NMR and DEPT spectra displayed signals for four methyl groups at δ 11.9, 13.6, 15.0 and 20.0, α,β-unsaturated δ-lactone carbonyl at δ 168.5 and oxygenated methines at δ 79.0, 75.6, 73.6, and 59.4. The molecular ion at *m/z* 669, 48 amu higher than that of









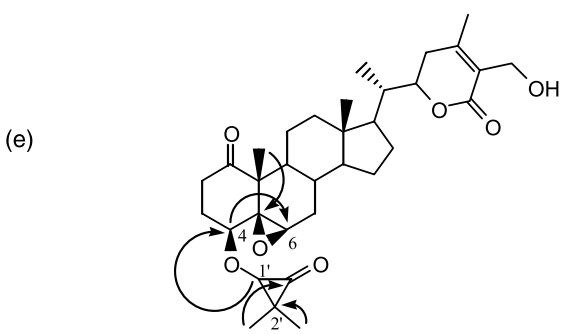


Figure 1. (a)–(e) Some of the significant HMBC (⟶) correlations oberved in compounds **1**–**5**.

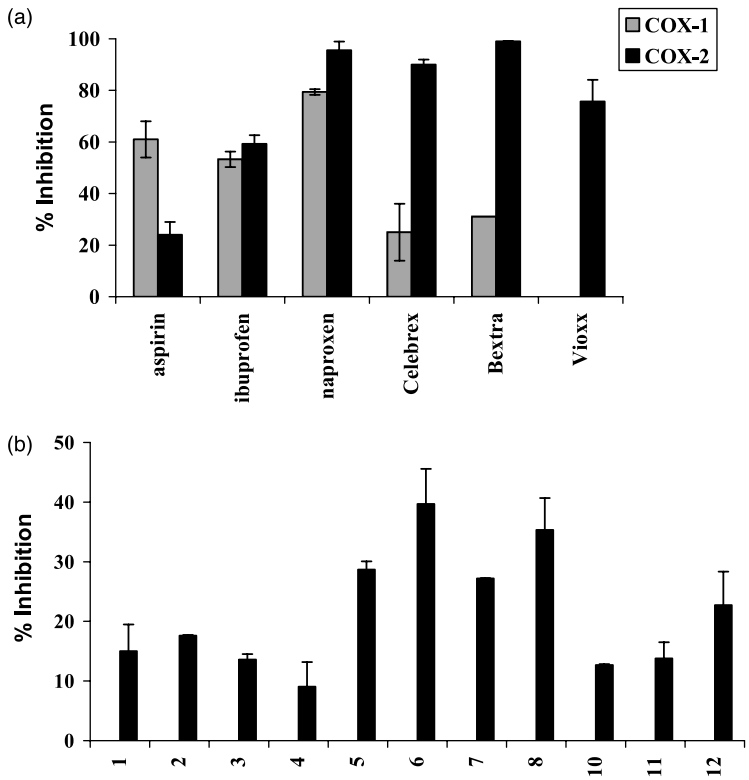


Figure 2. (a) Inhibition of COX-1 and -2 enzymes by commercial non-steroidal anti-inflammatory agents (NSAIDs). Aspirin, ibuprofen and naproxen were tested at 180, 2.1 and 2.5 μg/ml, respectively. Celebrex, Vioxx and Bextra were assayed at 1.67 μg/ml, respectively. DMSO solvent control did not inhibit COX enzymes. Data are represented as mean±one standard deviation (n=2). (b) COX-1 and -2 inhibitory activities of withanolides **1**–**12** at 100 μg/ml. DMSO solvent control did not inhibit COX enzymes. Vertical bars represent the standard deviation of each data point (n=2). Withanolides **1**–**12** did not inhibit COX-1 enzyme even at 500 μg/ml concentration.

physagulin D (*m/z* 621), indicated that compound **4** contained three additional oxygen functionalities in its structure. One such oxygen functionality, assigned as an epoxide at C-5 and C-6, resonated at δ 65.5 and 59.4, respectively in its ^{13}C NMR spectrum. A β-orientation of C-5 and C-6 epoxide moiety was assigned in compound **4** from its ^{1}H and ^{13}C NMR chemical shifts and by comparison of the spectral data of the known withanolides (**7**,**8**) isolated from the leaves in our laboratory. The second and third oxygen functionalities were determined as hydroxyl groups and were placed at C-4 and C-16 as confirmed by HMBC experiments (Fig. 1d). Comparison of ^{1}H and ^{13}C NMR data of **4** with physagulin D indicated that the sugar moiety was glucose and it was linked to C-3 of the molecule. The linkage of glucose unit to C-3 was also substantiated by HMBC correlations (Fig. 1d) observed between C-3 at δ 73.6 and H-1′ at δ 4.38. Therefore, the structure of **4** was confirmed as 4,17-dihydroxy-5β, 6β-epoxyphysagulin D.

The IR spectrum of compound **5**, isolated as an amorphous powder, showed absorption bands for an –OH and a δ-lactone carbonyl group at 3434 and 1704 cm^{-1}, respectively. The absorption peak at 1704 cm^{-1} was very broad (ranging from 1860 to 1700 cm^{-1}) and we report here only the peak maximum. It is surprising to note that the C=O absorption for the cyclopropanone moiety was not resolved. The HRFABMS gave an $[M+H]^+$ ion at *m/z* 555.3335, which analyzed for $C_{33}H_{47}O_7$ (calcd 555.3323). The ^{13}C NMR and DEPT spectra of compound **5** showed the presence of six methyl, nine methylene, nine methine and nine quaternary carbons. The ^{1}H NMR spectrum of **5** showed signals for four methyl groups at δ 0.69, 0.98, 1.18, and 2.07. Two doublets at δ 4.35 and 4.28 were assigned to two protons of a methylene at C-27. In addition, the proton signal appeared at δ 3.19 was assigned to H-4. The ^{13}C NMR spectrum of compound **5** was similar to the spectrum of dihydrowithaferin A (**7**) except that **5** had additional carbon signals at δ 210.1, 52.1, 72.8, and 25.0. The corresponding proton signals in **5** were at δ 1.35 (6H, s) and 3.70 (1H, s). The six protons singlet at δ 1.35 was assigned to the gem-dimethyl groups of a cyclopropanone moiety in compound **5**. The appearance of the *gem*-dimethyls in **5** as a singlet is in agreement with the literature values of cyclopropanone derivatives, 2,2-dimethyl cyclopropanone and 2,2,3,3-tetramethyl cyclopropanone.[15] One would expect the *gem*-dimethyls in compound **5** to appear as separate singlets but extensive NMR evidence from our studies on **5** indicate the presence of only a singlet integrated for 6 protons. Also, the singlet at 3.70 ppm was assigned to the 1′-proton of the cyclopropanone moiety in **5**. The gem-dimethyl carbons of the cyclopropanone moiety in **5** appeared as an intense overlapping singlet at 25.0 ppm in its ^{13}C NMR spectrum similar to the singlet observed for the gem-dimethyls in its proton spectrum. It was further supported by MS, HRMS and DEPT spectral data. Analyses of the HMBC spectrum revealed that 2,2-dimethylcyclopropanone moiety in **5** was linked via C-4 hydroxyl in dihydrowithaferin A (**7**) (Fig. 1e). The fragments at *m/z* 472 $[M+H-C_5H_7O]^+$ and 471 $[M+H-C_5H_8O]^+$ in its FABMS also supported the proposed structure for compound **5**.

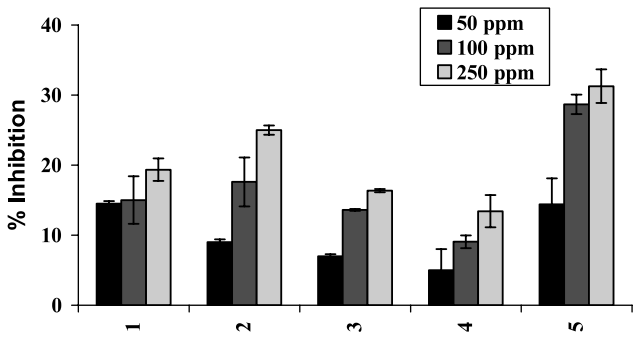


Figure 3. Dose dependent inhibition of COX-2 enzyme by compounds **1**–**5** at 50, 100 and 250 μg/ml. Vertical bars represent the standard deviation of each data point (n=2).

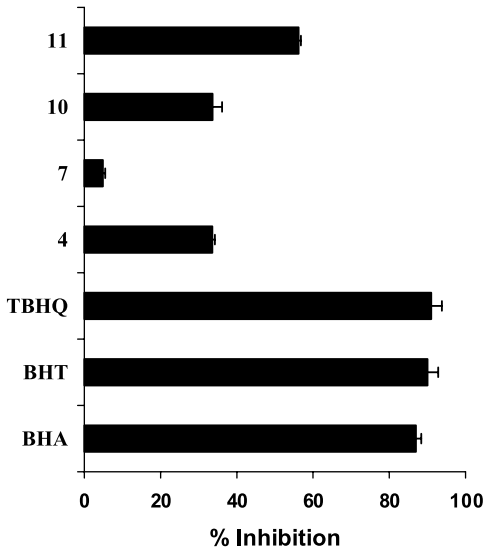


Figure 4. Inhibition of lipid peroxidation at 20 min by compounds **4**, **7**, **10**, **11** at 100 ppm and synthetic antioxidants BHA, BHT and TBHQ at 10 ppm concentration. DMSO, used as a solvent control, did not show activity. Similarly, withanolides **1**–**3**, **5**,**6**, **8**,**9**, and **12** did not show activity at 100 ppm concentration. Data are represented as mean±one standard deviation (n=2).

The withanolides isolated from the leaves were evaluated for their cyclooxygenase (COX) enzyme inhibitory activity using prostaglandin endoperoxide synthase isozymes-1 (COX-1) and PGHS-2 (COX-2). Aspirin, ibuprofen, naproxen, Celebrex, and Bextra were used as positive controls and they showed 61, 53, 79, 23 and 25% of COX-1; 7, 59, 95, 98 and 99% of COX-2 inhibition, respectively (Fig. 2a). Vioxx inhibited COX-2 enzyme by 80% and had no COX-1 enzyme inhibition. The novel withanolides **1**–**5** were tested at 50, 100 and 250 μg/ml and all other withanolides isolated from the leaves were tested at 100 μg/ml concentration. Compounds **6**,**7**, **8**, **10**, **11**, and **12** gave 39, 27, 35, 13, 14 and 23%, respectively, of COX-2 enzyme inhibition at 100 μg/ml (Fig. 2b). A dose dependent inhibition of COX-2 (Fig. 3) was observed for compounds **1**–**5** and the activity varied considerably among withanolides at concentrations tested. The COX-2 activity exhibited by compounds **1**–**5** were 15, 9, 7, 5 and 15%, respectively, at 50 μg/ml (Fig. 3). It is important to note that the withanolides tested did not inhibit COX-1 enzyme even at 500 μg/ml concentration. However, the activity remained the same for all compounds when the concentration was increased from 100 to 250 μg/ml in COX-2 assays. The lack of increased COX-2 activity at higher concentrations is probably due to the solubility of these withanolides under assay conditions. The reduced COX-2 activity of compounds **3** and **10** as compared to **8** and **6** might be due to the glycosylation at C-27 in **3** and **10**. Compound **9**, which is lacking a double bond between C-24 and C-25, showed neither COX-1 nor COX-2 activities. This indicated that the double bond in α,β-unsaturated δ-lactone moiety is critical for the COX-2 inhibitory activity.

The ability of withanolides to inhibit lipid peroxidation in a model system was used to determine whether they could act as antioxidants. The assay was conducted by using large unilamellar vesicles and peroxidation was initiated by adding Fe^{2+}. Except for compounds **4**, **7**, **10** and **11**, other withanolides tested did not inhibit the lipid peroxidation (Fig. 4). The monoglycosides **4**, **7**, **10**, and **11** inhibited 40, 5, 44 and 55%, respectively, of lipid peroxidation in our assay system.

Our in vitro results on the COX-2 enzyme inhibitory activities of withanolides provided scientific support for the use of *W. somnifera* leaf preparation as a folk remedy for the treatment of inflammation.[1] This study also represents the first report of the COX-2 enzyme inhibitory activity for this group of compounds. Over expression of COX-2 enzyme was observed in tumor cells and hence selective COX-2 inhibitors have a great potential to prevent tumor progression. Anecdotal reports indicate that the withanolides exhibit anticancer activity.[1] Therefore, these compounds might be useful as templates for the development of therapeutics for cancer chemoprevention. Since both roots and leaves of *W. somnifera* contain similar withanolides, consumption of *W. somnifera* root powder or leaf extract as a dietary supplement might decrease the inflammatory pain, the risk of cancer formation and progression of tumors by suppressing the COX-2 enzyme.

3. Experimental

3.1. General

^{1}H NMR spectra were recorded on a 500 MHz VRX spectrometer. ^{13}C NMR spectra were obtained at 125 MHz. Chemical shifts were recorded in either $CDCl_3$ or CD_3OD. HMBC was optimized for J=8.0 Hz. The silica gel used for MPLC was Merck Silica gel 60 (35–70 μm particle size). HRFAB and FAB mass spectra were acquired on JEOL HX-110 double focusing mass spectrometer operating in the positive mode. Preparative HPLC was performed on a recycling preparative HPLC (Japan Analytical Industry Co. model LC-20) with tandem C_{18} column (JAIGEL, 10 μm, 20×250 mm) at the flow rate of 3 ml/min. All organic solvents and standards used were ACS reagent grade. Yields of the withanolides are expressed in percentage dry weight of the leaves.

3.2. Plant material

The extract (H-341) was purchased from PhytoMyco Research Corporation, Greenville, NC and was prepared

as follows: The shade dried and ground leaves of *W. somnifera* were extracted sequentially with a mixture of dichloromethane and methanol (1:1, v/v), methanol and water to obtain three fractions. All fractions were then pooled, filtered and dried under reduced pressure. The resulting extract was stored at −20°C till use.

3.3. Isolation of withanolides

The combined crude extract from PhytoMyco Research Corporation (4 g) was stirred with *n*-hexane (500 ml) and filtered. The hexane insoluble portion (3.2 g) was chromatographed on MPLC using $CHCl_3$ and MeOH (v/v) under gradient condition. The fractions collected were I (600 mg, $CHCl_3$/MeOH, 9:1), II (500 mg, $CHCl_3$/MeOH, 8:2), III (1.2 g, $CHCl_3$/MeOH, 7:3), and IV (200 mg, $CHCl_3$/MeOH, 1:1). Repeated MPLC of fraction I using hexane/EtOAc (1:1, v/v) yielded pure compounds **6** (120 mg, 0.078%), **7** (50 mg, 0.032%) and a fraction 1 (4.0 mg). This fraction was further purified by PTLC (hexane/EtOAc, 6:4, v/v) to yield the pure compound **9** (2.1 mg, 0.0014%). Purification of fraction II by preparative HPLC using MeOH/H_2O (1:1, v/v) afforded compound **10** (20 mg, 0.013%) at 19.5 min and fraction 2 (50 mg) at 24.2–28.1 min. It was further purified on HPLC by using MeOH/H_2O (4:6, v/v) and gave compound **11** (40 mg, 0.026%) at 32.96 min. Similarly, fraction III was purified by Prep. HPLC (MeOH-H_2O, 6:4, v/v) and yielded pure compounds **12** (15 mg, 0.0097%) at 112 min and fractions 3 (950 mg) at 20–45 min and 4 (16 mg) at 140–160 min. Fraction 3 was further purified by HPLC (MeOH/H_2O, 6:4, v/v) to afford compounds **8** (200 mg, 0.13%) and **5** (10 mg, 0.0065%) at 66.8 and 78.6 min, respectively, and 700 mg of sucrose at 132 min. Fraction 4 was subjected to HPLC (MeOH/H_2O, 7:3, v/v) yielded compound **2** (12 mg, 0.0078%) at 81 min. The fraction 4 was purified by HPLC (MeOH/H_2O, 7:3) and collected fractions 5 (13 mg) at 20–30 min and fraction 6 (30 mg) at 30.1–66 min. The fraction 6 was purified by HPLC (MeOH/H_2O, 7:3, v/v) to yield compound **3** (8.5 mg, 0.0055%) at 57.9 min. Fraction 5 was further purified by HPLC using MeOH/H_2O (6:4, v/v) and afforded pure compound **1** (10.5 mg, 0.0068%) at 59.3 min and a mixture of compounds **4** and **12** at 96.4 min (5.0 mg, 0.0032%).

3.3.1. Compound 1. Colorless, amorphous powder; IR ν_{max} (KBr) 3406, 1698, 1650, 1383, 1076, 1042 cm^{-1}; ^1H NMR (CD_3OD) δ 0.60 (3H, s, Me-18), 0.90 (3H, s, Me-19), 0.92 (3H, d, J=7.0 Hz, Me-21), 1.90 (3H, s, Me-28), 3.96 (1H, m, H-3), 4.15 (1H, d, J=8.0 Hz, H-1‴), 4.20 (1H, d, J=8.0 Hz, H-1″), 4.22 (1H, d, J=8.0 Hz, H-1′), 4.30 (1H, t, J=2.3 Hz, H-1), 4.35 (2H, brs, H-27), 4.48 (1H, dt, J=13.0, 3.4 Hz, H-22), 5.50 (1H, brd, J=5.5 Hz, H-6); ^{13}C NMR data (Table 1); FABMS m/z 945 ($M+H^+$), 783 ($M+H^+$–glucose), 621 ($M+H^+$–2×glucose), 459 (aglycone); HRFABMS m/z 945.4682 ($M+H^+$; calcd for $C_{46}H_{73}O_{20}$, 945.4695).

3.3.2. Compound 2. Colorless, amorphous powder; IR ν_{max} (KBr) 3407, 1696, 1652, 1398, 1040 cm^{-1}; ^1H NMR (CD_3OD) δ 0.76 (3H, s, Me-18), 1.01 (3H, s, Me-19), 1.22 (3H, d, J=7.0 Hz, Me-21), 2.11 (3H, s, Me-28), 3.83 (1H, m, H-3), 4.31 (1H, d, J=8.0 Hz, H-1″), 4.36 (1H, d, J=8.0 Hz, H-1′), 4.46 (1H, d, J=11.5 Hz, H-27b), 4.48 (1H, dt, J=13.0, 3.4 Hz, H-22), 4.50 (1H, t, J=2.3 Hz, H-1), 4.60 (1H, d, J=11.5 Hz, H-27a), 5.49 (1H, brd, J=5.5 Hz, H-6); ^{13}C NMR data (Table 1); FABMS m/z 783 ($M+H^+$); HRFABMS m/z 783.4168 ($M+H^+$; calcd for $C_{40}H_{63}O_{15}$, 783.4188).

3.3.3. Compound 3. Colorless, amorphous powder; IR ν_{max} (KBr) 3425, 1700, 1652, 1400, 1259, 1058 cm^{-1}; ^1H NMR (CD_3OD) δ 0.67 (3H, s, Me-18), 0.98 (3H, d, J=7.0 Hz, Me-21), 1.18 (3H, s, Me-19), 2.10 (3H, s, Me-28), 3.15 (1H, brs, H-6), 3.33 (1H, d, J=8.0 Hz, H-4), 3.66 (dd, 1H, J=11.0, 3.0 Hz, H-3), 4.31 (1H, d, J=7.5 Hz, H-1′), 4.44 (1H, dt, J=13.5, 3.5 Hz, H-22), 4.45 (1H, d, J=11.5 Hz, H-27b), 4.59 (1H, d, J=11.5 Hz, H-27a); ^{13}C NMR data (Table 1); FABMS m/z 673 ($[M+Na]^+$). HRFABMS m/z 673.3200 ($[M+Na]^+$; calcd for $C_{34}H_{50}O_{12}$, 673.3224).

3.3.4. Compound 4. Colorless, amorphous powder; IR ν_{max} (KBr) 3424, 1697, 1652, 1398, 1384, 1076, 1042 cm^{-1}; ^1H NMR (CD_3OD) δ 0.69 (3H, s, Me-18), 1.0 (3H, d, J=7.0 Hz, Me-21), 1.22 (3H, s, Me-19), 2.08 (3H, s, Me-28), 3.14 (1H, brs, H-6), 3.41 (brt, J=7.5 Hz, H-16), 3.43 (1H, d, J=8.0 Hz, H-4), 3.60 (1H, m, H-3), 4.19 (1H, brs, H-27), 4.38 (1H, d, J=7.5 Hz, H-1′), 4.30 (1H, dt, J=13.5, 3.4 Hz, H-22), 4.46 (1H, t, J=2.3 Hz, H-1); ^{13}C NMR data (Table 1); FABMS m/z 669 ($M+H^+$), 507 (aglycone); HRFABMS m/z 669.3456 ($M+H^+$; calcd for $C_{34}H_{53}O_{13}$, 669.3486).

3.3.5. Compound 5. Colorless, amorphous powder; IR ν_{max} (KBr) 3434, 1704 (br), 1634, 1397, 1382, 1256, 1236, 1058, 999 cm^{-1}; ^1H NMR (CD_3OD) δ 0.69 (3H, s, Me-18), 0.98 (3H, d, J=6.5 Hz, Me-21), 1.18 (3H, s, Me-19), 1.35 (6H, s, Me-4′ and 5′), 2.07 (3H, s, Me-28), 3.15 (1H, brs, H-6), 3.19 (1H, t, J=2.3 Hz, H-4), 3.70 (1H, s, Hz, H-1′), 4.28 (1H, d, J=12.0 Hz, H-27), 4.35 (1H, d, J=12.0 Hz, H-27), 4.42 (1H, dt, J=13.0, 3.0 Hz, H-22); ^{13}C NMR data (Table 1); FABMS m/z 555 ($M+H^+$), 472 [$M+H-C_5H_7O]^+$, 471 [$M+H-C_5H_8O]^+$; HRFABMS m/z 555.3335 ($M+H^+$; calcd for $C_{34}H_{50}O_{12}$, 555.3323).

3.3.6. Acid hydrolysis. Compounds **1**–**3** each 1 mg and **11** (5 mg) were dissolved separately in 6% HCl in water and heated under reflux for 3 h. This solution was neutralized with 1N aqueous NaOH and extracted with EtOAc. The EtOAc extract was then concentrated and the aglycone was characterized by ^1H and ^{13}C NMR experiments. The aqueous solution was concentrated and analyzed for sugars. It was determined that only D-glucose was present in the aqueous portion as indicated by TLC analysis using $CHCl_3$/MeOH (1:1, v/v) as the mobile phase on silica gel plates and by comparison with an authentic sample of D-glucose.

3.3.7. Compounds 6–12. The structures of compounds **6**–**12** were derived as withaferin A (**6**);[16] 2, 3-dihydrowithaferin A (**7**);[16] viscosalactone B (**8**);[17] 27-desoxy-24,25-dihydrowithaferin A (**9**);[18] sitoindoside IX (**10**);[19] physagulin D (**11**);[20] and withanoside IV (**12**)[3] by detailed ^1H and ^{13}C NMR spectral experiments. The spectral data of these compounds were identical to their respective published spectral data.

Table 1. ^{13}C NMR chemical shifts of withanolides **1–5**

Carbon number	1	2	3	4	5
1	74.9 d	74.9 d	210.2 s	75.0 d	210.6 s
2	37.7 t	37.7 t	42.6 t	37.8 t	52.2 t
3	73.6 d	73.6 d	70.4 d	73.6 d	40.5 t
4	39.2 t	39.1 t	73.8 d	75.6 d	72.8 d
5	139.0 s	139.1 s	63.8 s	65.5 s	64.2 s
6	125.5 d	125.4 d	56.6 d	59.4 d	57.1 d
7	30.7 t	30.8 t	29.8 t	30.7 t	30.0 t
8	33.2 d	33.2 d	31.0 d	33.2 d	31.5 d
9	42.7 d	42.7 d	42.5 d	42.7 d	43.0 d
10	42.5 s	42.6 s	49.1 s	53.0 s	49.5 s
11	21.3 t	21.3 t	20.7 t	22.3 t	21.1 t
12	40.4 t	40.3 t	27.1 t	28.2 t	27.5 t
13	43.9 s	43.9 s	42.6 s	44.1 s	42.9 s
14	57.2 d	57.6 s	56.1 d	51.7 d	55.7 d
15	25.5 t	25.5 t	24.2 t	39.2 t	24.6 t
16	28.3 t	28.3 t	39.1 t	79.1 d	39.4 t
17	53.2 d	53.2 d	51.8 d	57.2 d	50.1 d
18	12.1 q	12.1 q	10.7 q	11.9 q	11.1 q
19	20.0 q	19.9 q	13.6 q	15.0 q	14.0 q
20	40.8 d	40.8 d	39.0 d	40.9 d	39.6 d
21	13.7 q	13.7 q	12.5 q	13.6 q	12.9 q
22	80.1 d	80.2 d	78.9 d	80.2 d	79.4 d
23	32.8 t	32.8 t	29.6 t	32.5 t	30.2 t
24	160.4 s	160.2 s	159.1 s	157.8 s	157.0 s
25	123.6 s	123.6 s	122.5 s	125.4 s	125.6 s
26	168.6 s	168.6 s	167.4 s	168.5 s	167.7 s
27	57.6 t	63.5 t	61.6 t	57.6 t	56.6 t
28	20.7 q	20.7 q	19.5 q	20.0 q	19.5 q
1′	103.0 d	102.7 d	102.7 d	103.0 d	72.8 d
2′	75.1 d	75.1 d	75.6 d	75.6 d	52.1 s
3′	78.0 d	78.1 d	76.9 d	78.0 d	210.1 s
4′	71.5 d	71.7 d	72.4 d	71.7 d	25.4 q (4′ and 5′-Me)
5′	77.9 d	78.0 d	76.8 d	77.9 d	
6′	70.0 t	62.9 t	62.4 t	62.7 t	
1″	103.9 d	103.9 d			
2″	75.0 d	75.0 d			
3″	78.0 d	78.0 d			
4″	77.8 d	71.6 d			
5″	77.9 d	77.9 d			
6″	62.7 t	62.8 t			
1‴	104.8 d				
2‴	75.0 d				
3‴	78.0 d				
4‴	71.5 d				
5‴	77.9 d				
6‴	63.5 t				

Data recorded in CD_3OD at 125 MHz at 25°C. Multiplicities were determined by DEPT experiments and confirmed by analysis of HMQC spectra.

3.3.8. CD analysis. CD spectra for compounds **1–5** were recorded on a JASCO, model J-710, CD-ORD spectrometer in MeOH under the following conditions: scan mode (wave length), band width (0.5 nm), sensitivity (50 mdeg), response (1 s), wave length range (200–400 nm), step resolution (1 nm), scan speed (100 nm min^{-1}), and accumulation (1). The CD maximum or minimum ($\Delta\varepsilon$) observed for compounds **1–5** were: **1** (*c* 0.001, MeOH) $\Delta\varepsilon$+73.7 (257); **2** (*c* 0.0005, MeOH) $\Delta\varepsilon$+15.4 (262.5); **3** (*c* 0.001, MeOH) $\Delta\varepsilon$+4.7 (261); **4** (*c* 0.0005, MeOH) $\Delta\varepsilon$+61.6 (260); and **5** (*c* 0.001, MeOH) $\Delta\varepsilon$+9.8 (260).

3.4. Cyclooxygenase enzyme inhibitory assay

COX-1 enzyme was prepared from ram seminal vesicles and COX-2 enzyme was isolated from insect cells cloned with human PGHS-2 enzyme. The inhibitory effects of test compounds on COX-1 and -2 were measured by monitoring the initial rate of O_2 uptake using an oxygen electrode (Instech laboratories, Plymouth Meeting, PA) attached to a biological oxygen monitor (Yellow Spring Instrument, Inc., Yellow Spring, OH) at 37°C. The enzymes were diluted (1:1) with Tris buffer (pH 7, 10–15 μl) and the test compounds (100 μg/ml, 10 μl) dissolved in DMSO were added to the assay mixture composed of 3 ml of 0.1 M Tris HCl, pH 7, 1 mmol phenol and 85 μg of hemoglobin. The mixture was incubated for 2–3 min and reaction was initiated by the addition of arachidonic acid (10 μl of 1.64 μM solution). The instantaneous inhibition was measured by using Quick Log Data acquisition and control computer software (Strawberry tree Inc., Sunnyvale, CA, USA). Positive controls aspirin, ibuprofen, naproxen were tested at 180, 2.1 and 2.5 μg/ml, respectively, and Celebrex, Vioxx and Bextra were tested at 1.67 μg/ml. DMSO was used as solvent control.[21]

3.5. Antioxidant activity

Large Unilamellar Vesicles (Liposome suspension) were prepared according to the published procedure.[22] The final assay volume was 2 ml, consisting of 100 μl HEPES buffer (50 mM HEPES and 50 mM TRIS), 200 μl 1 M NaCl, 1.64 ml of N_2-sparged water, 20 μl of test sample or DMSO and 20 μl of liposome suspension. The peroxidation was initiated by the addition of 20 μl of $FeCl_2{\cdot}4H_2O$ (0.5 mM). The flourescence was monitored at 0, 1, 3 and every 3 min up to 21 min using a Turner Model 450 Digital Fluorometer. The decrease of relative fluorescence intensity over the time indicated the rate of peroxidation. The percentage of inhibition was calculated with respect to DMSO control. All compounds were tested at 100 μg/ml and the positive controls BHA, BHT and TBHQ were tested at 10 μM.

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