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Drier Climatic Conditions Increase Withanolide Content of *Withania coagulans* Enhancing Its Inhibitory Potential Against Human Prostate Cancer Cells

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Abstract

Prostate cancer is one of the major causes of cancer-related deaths in men and there is a growing interest in identifying natural compounds for its management. We analyzed bioactive withanolides in *Withania coagulans* from 11 different sites in Pakistan and evaluated the antiprostate cancer activities of leaf extracts from two sites with the greatest amounts. Total withanolide concentration differed by ~ 17-fold between sites, ranging from 1.01 ± 0.01 mg/g dry weight (mean ± SE) at Jand to 16.83 ± 0.02 mg/g at Mohmand Agency. Different tissues varied in their total withanolide content with roots having the least (0.42 ± 0.07 mg/g dry weight) and leaves the most (2.45 ± 0.45 mg/g). We found strong inverse correlations between site annual precipitation versus withanolide amounts in fruits ($r = -0.84$, $P = 0.001$), leaves ($r = -0.88$, $P < 0.001$), roots ($r = -0.91$, $P < 0.001$), and total ($r = -0.89$, $P < 0.001$), but not stems ($r = -0.20$, $P = 0.556$). Extracts made from Mianwali and Mohmand Agency leaves possessed high anticancer activity in terms of increased induction of apoptosis and decreased cell viability, cell proliferation, invasion, and migration of different prostate cancer cell lines. These results are useful for the selection of withanolide-rich germplasm with potent anticancer properties.

Keywords Prostate cancer · *Withania coagulans* · Withanolides · Climatic conditions · Ecotypes · Anticancer properties

Abbreviations

MTT	3-(45-Dimethylthiazol-2-yl-2,5-diphenyl tetrazolium bromide
BrdU	5-Bromo-2′-deoxyuridine
ML	Mianwali leaf extract
AL	Mohmand Agency leaf extract
HPLC	High-performance liquid chromatography
UHPLC-MS	Ultra-high performance liquid chromatography-mass spectrometry

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Introduction

Withania coagulans is an important medicinal plant that holds a key place in folk medicine due to its extensive use in the treatment of many ailments [1, 2]. The fruit of *W. coagulans*, a small yellowish berry, is used for milk coagulation, as a blood purifier, and to treat chronic liver illness, respiratory, intestinal, and digestive disorders, and nausea [1, 3]. The fruits also have sedative, antidepressant, and anti-inflammatory properties [2] and are used as a carminative and to treat dyspepsia, while the leaves are used as a febrifuge [4]. An extensive literature survey revealed that *W. coagulans* possesses a wide variety of pharmacological activities, including antidiabetic activities [5, 6], with aqueous extracts of the fruits decreasing blood glucose levels by 52% [7]. It is widely used for treating sleeplessness, exhaustion, tuberculosis, insomnia, and impotence [8]. Furthermore, its flowers are effective against diabetes [9]. The twigs are used for teeth cleaning by chewing and the smoke of the plant is used for the relief of toothaches [10, 11]. In addition, *W. coagulans* is widely used to treat nervous fatigue, nervous disability, sleeplessness, failure of children to thrive, and impotence [12]. Roots are harvested in autumn and dried for later use [13]. Other properties including antimicrobial, anti-inflammatory, antitumor, hepatoprotective, antihyperglycemic, immunosuppressive, antitumor, free radical scavenging, and central nervous system-depressant activities have been attributed to the plant [14]. Furthermore, high antimutagenic potential of the extracts of *W. coagulans* fruits has also been reported [15].

The medicinal properties of *W. coagulans* are mainly attributed to the presence of withanolides [2, 16, 17], and the content of these compounds in plants has been shown to vary with geographical distribution [18], morphological differentiation [19], climatic [20], and genetic factors [18]. In Pakistan, the common habitat for this plant is relatively hot and dry areas, such as the districts of Mianwali, Sargodha, Karak, Kohat, and Mohmand Agency. Even though various tissue types of *W. somnifera* have previously been assessed for withanolide content [21], there is no information about which factors, such as geographical or climatic, may determine the concentration of these compounds in *W. coagulans*.

In this study, we investigated the relationship between the concentrations of different bioactive metabolites and the climatic factors influencing them in 11 accessions of *W. coagulans* from different areas of Pakistan. We then tested extracts of plants from accessions with the greatest withanolide content for their antiprostate cancer activity against human prostate cancer cell lines (DU145, PC3, and C4-2), the most frequently diagnosed cancer in men.

Materials and Methods

Collection of Plant Material

Withania coagulans Dunal (Solanaceae) was collected from 11 locations in Pakistan (Table 1), namely Ambiri Kala (Am); Jand (Ja); Karak (Ka); Kohat City, University Rd. (Kc); Khada (Kh); Lachi (La); Mianwali Musa khel (Mi); Mohmand Agency (Ag); Sargodha city (Sc); Soon Skaser valley (Sn); and Usterzai payan, Hangu Rd. (Up). The plants were identified by Prof. Dr. Rizwana Aleem Qureshi (taxonomist) in the Department of Plant Sciences, Quaid-i-Azam University, Islamabad.

Table 1 *Withania coagulans* collection sites throughout Pakistan and their coordinates, elevations, and climatic conditions (annual precipitation and June mean temperature)

Location (abbreviation)	Coordinates	Elevation (m)	Annual precipitation (mm)	Mean June temperature (°C)
Ambiri Kala (Am)	33° 03′ 13.77″ N 71° 00′ 56.35″ E	507	477	39.9
Jand (Ja)	33° 12′ 24.77″ N 73° 16′ 23.34″ E	479	638	40.0
Karak (Ka)	33° 06′ 44.17″ N 71° 06′ 08.92″ E	592	488	40.1
Khada (Kh)	33° 00′ 34.23″ N 71° 03′ 08.84″ E	508	477	40.2
Kohat City, University Rd. (Kc)	33° 35′ 05.29″ N 71° 26′ 34.96″ E	1001	438	42.0
Lachi (La)	33° 23′ 18.43″ N 71° 20′ 41.05″ E	455	481	33.7
Mianwali Musa Khel (Mi)	32° 38′ 04.83″ N 71° 45′ 02.98″ E	271	330	41.0
Mohmand Agency (Ag)	34° 33′ 53.94″ N 71° 28′ 51.94″ E	1024	100	41.1
Sargodha City (Sc)	32° 04′ 44.86″ N 72° 40′ 18.31″ E	189	410	41.0
Soon Skaser Valley (Sn)	32° 32′ 48.26″ N 71° 54′ 54.17″ E	700	600	42.1
Usterzai payan, Hangu Rd. (Up)	33° 36′ 11.02″ N 71° 15′ 33.32″ E	653	536	41.1

Plant Processing

After collection, the plant material was thoroughly rinsed with distilled water, followed by careful separation of different plant parts and subsequent air drying under shade. Fully dried plant material was subjected to vacuum drying under 0.1 bar pressure to ensure that it was completely moisture free. The different parts were separately homogenized to a fine powder (100 mesh) in a lab-scale grinder with short intervals under controlled milling temperature. The ground powder was sealed in air-tight bags and stored at 25 °C until further processing.

Withanolide Analysis of Various Plant Tissues by HPLC

Withanolides were extracted from the various dried and ground plant parts according to a standard procedure [22] with some modifications and then analyzed with high-performance liquid chromatography (HPLC) equipped with a photo diode array detector (PDA) following the method of Lan et al. [23], with slight modifications. A wavelength of 225 nm was used for the detection of withacoagulin H, withacoagulin G, withacoagulin I, coagulansin A, withanolide H, and withacoagulin E and 230 nm for coagulansin A and withanolide F.

Analysis of Extracts with UHPLC-MS

Based on HPLC analysis, leaves of accessions from two locations [Mianwali Musa Khel (Mi) and Mohmand Agency (Ag)] with the highest withanolide content were selected to make extracts (designated as ML and AL) to be used in anticancer bioassays. The levels of

withanolides in these extracts were subsequently determined with ultra-high performance liquid chromatography (UHPLC) with mass spectrometry (MS) detection.

We used the following purified compounds—coagulansin A, withacoagulin G, withacoagulin H, withacoagulin I, withanolide F, withanolide H, and withacoagulin—as retention time standards for the LC-MS analysis (Fig. 1) [4, 17, 24, 25]. Quercetin (purchased as its dihydrate; Sigma-Aldrich) was used as a standard for quantification and all other reagents and solvents were of analytical grade. We lyophilized the *W. coagulans* leaf extracts until dry and then dissolved an accurately weighed portion of each in methanol with a combination of vortex mixing and 30 min of sonication in a water bath at ambient temperature. All samples were then centrifuged at 14,000×*g* for 10 min and the clear supernatant was transferred to glass LC vials. We injected 2 μm of the final solutions into a Waters UHPLC equipped with both a PDA and an electrospray ionization (ESI) single quadrapole mass spectrometer (MS). Compounds were separated with an Acquity UPLC HSS C18 column (100 × 2.1 mm, 1.8 μm particle size) held at 40 °C with a constant flow rate of 0.5 mL min^{-1} using a binary gradient of a 0.1% *v*/*v* aqueous solution of formic acid (mobile A) and acetonitrile with 0.1% *v*/*v* formic acid (mobile B), similar to that of Huang et al. [24]. The

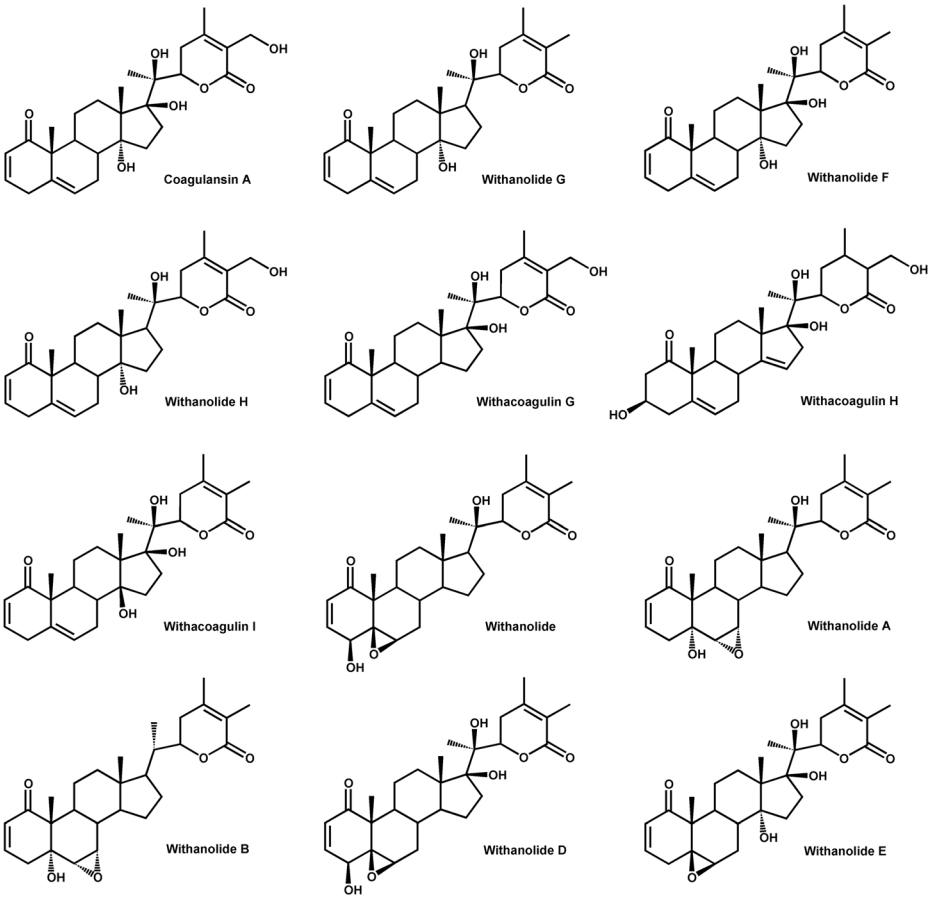

Fig. 1 Structures of withanolides found in either plant tissues or leaf extracts and other compounds (withanolide, withanolide A, B, D, and E) with the same exact masses as unknowns 1–5 from the ML and AL leaf extracts

gradient began with B at 10% followed by an immediate ramp to 30% B over 6 min, then increased to 35% at 10 min, 45% at 20 min, and then increased to 90% B over the next 5 min before returning to initial conditions of 10% B at 30 min. The PDA was set to collect UV data (210–450 nm) during each entire run. MS conditions consisted of source temperature at 150 °C, desolvation temperature at 250 °C, nebulization gas flow of 500 L h^{-1}, and capillary and cone voltages set at 3 kV (negative ionization mode) and 30 V, respectively. Mass spectra (*m*/*z* 200–1000) were acquired in centroid mode. We used a five-point standard curve of quercetin to calculate the amounts of the putative withanolides as milligrams of quercetin equivalents per gram of plant extract, using the summed integrated peak areas from extracted ion chromatograms (EIC) of both the deprotonated $(M-H)^-$ and formate adduct $(M-H+HCOOH)^-$ of each compound.

Correlations of Site Climatic Factors with Withanolide Concentrations

We performed correlation analyses on various site climatic factors (annual precipitation and mean June and January temperatures) versus amounts of withanolides in fruits, leaves, stems, roots, and total amounts. We also tested whether a correction existed between annual precipitation and elevation of the different sites.

Preparation of Plant Extracts for Bioassays

The plant extracts used for various bioassays were made by adding 10 mL of a mixture of methanol and chloroform (1:1 *v*/*v*) to 1 g of *W. coagulans* leaf powder. Mixtures were kept at room temperature overnight then concentrated with a rotatory flash evaporator, followed by freeze-drying. Prior to the bioassays, we dissolved a portion of the extract in Roswell Park Memorial Institute (RPMI) medium and made a series of dilutions from 10 to 250 μg/mL.

Cell Cultures

Immortalized normal human prostate epithelial cells (RWPE-1) and human prostate cancer cell lines C4-2 (androgen-independent), DU145, and PC3 (both androgen-sensitive) were obtained from ATCC (Manassas, VA). All cell lines were maintained as per the manufacturer's recommendations.

Cell Growth Assay

We performed a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay to study the effect of *W. coagulans* extracts on cell growth using PC3, C4-2, DU145, and RWPE-1 cell lines. Cells were plated (1×10^4 cells per well) in 1 mL of complete culture medium in 96-well microtiter plates containing a range of 10–250 μg/mL of *W. coagulans* extracts. After treatment in a humidified incubator for 24 h at 37 °C, 200 μL of MTT (5 mg/mL:1 XPBS) was added to each well and incubated for 2 h after which 200 μL of DMSO was added. The plates were then centrifuged (1800×*g* for 5 m at 4 °C) and the absorbance was read at 540 nm. The effect of *W. coagulans* extracts on growth inhibition was calculated as percent cell growth inhibition where DMSO-treated cells were used as a 100% control.

Bromo-deoxyuridine Cell Proliferation Assay

We used the thymidine analog 5-bromo-2′-deoxyuridine (BrdU) to assess the antiproliferative potential of *W. coagulans* extracts using BrdU Cell Proliferation Assay Kit #6813 (Cell Signaling Technology, Danvers, MA), following the manufacturer's protocol. Cell lines DU145 and C4-2 were used and treated with *W. coagulans* extracts ML and AL at concentrations from 0 to 100 μg/mL. In a 96-well plate, ~100,000 cells were plated and incubated for 24 and 48 h, after which 1× BrdU was added to plate wells and cells were placed in an incubator for 1 h and then the medium was removed. To get a suspension of cells, the plates were centrifuged at 300×*g* for 10 min and then the medium was removed. Next, 100 μL/well of the fixing/denaturing solution was added, the plate was kept at room temperature for 30 min, and then the solution was removed. Then, 100 μL/well 1× detection antibody solution was added, the plate was kept at room temperature for 1 h, and the solution was removed and washed three times with 1× wash buffer. This was followed by the addition of 1× horseradish peroxidase (HRP)-conjugated secondary antibody solution and holding the plate at room temperature for 30 min, after which the solution was aspirated and washed three times with 1× wash buffer. Finally, 100 μL 3,3′,5,5′-tetramethylbenzidine (TMB) substrate was added and the plate was incubated for 30 min at room temperature.

Apoptosis by Flow Cytometry

DU145 and C4-2 cells treated with *W. coagulans* (25–50 μM, 24 h) in RPMI medium were trypsinized and fixed in 1% paraformaldehyde:1× PBS for an hour and washed twice with cold PBS and centrifuged. The pellet was suspended in chilled 70% ethanol and stored overnight. Next, the cells were centrifuged for 5 min at 100×*g*, and the pellet obtained was washed twice with cold PBS to remove ethanol. The cells were labeled with fluorescein isothiocyanate (FITC) and propidium iodide using the Apo-Direct Kit (BD Pharmagen, San Jose, CA) as per the manufacturer's protocol. Analysis was performed with a FACScan (Becton Dickinson, Franklin Lakes, NJ). About 10,000 cells per sample were collected and the DNA histograms were analyzed with ModFitLT software (Verity Software House, Topsham, ME).

Scratch Wound Assay

The effect of treatment with *W. coagulans* extracts on invasion of prostate cancer cells was determined using the scratch wound assay. Human prostate cancer cells (C4-2) were added to a 96-well plate at 5000 cells per well and when 100% confluency was obtained a sieve line was made in the cells with the help of the tip. The prepared cell plate was then treated with *W. coagulans* at 25 and 50 μM. Photos were taken at time 0 and 24 h following treatment.

Cell Migration Assay

An antimigration assay was performed using a Millipore ECM505 migration kit, as per the kit protocol. The effect of treatment with *W. coagulans* leaf extracts (ML and AL) on human prostate cancer cell (DU145 and C4-2) migration was determined using this assay.

Cell Harvesting

The procedure was optimized for this assay by using a cell migration kit. Cells were starved by incubating them for 18–24 h prior to the assay in serum-free medium (RPMI). We prepared a cell suspension containing 0.2–2.0 × 10^6 cells/mL in serum-free media and then added 250 μL of the counted cell suspension into each insert. Next, we added 400 μL of serum-free media in the presence of a chemoattractant (10% fetal bovine serum) to the lower chamber, ensuring that the bottom of the insert membrane contacted the media. Plates were incubated for 24 h at 37 °C in a CO_2 incubator (4–6% CO_2). We then removed the cells/media from the top side of the insert by pipetting out the remaining cell suspension. We then placed the migration insert into a clean well containing 225 μL of prewarmed cell detachment solution and incubated for 30 min at 37 °C. To dislodge cells completely from the underside, we gently tilted the migration insert back and forth several times during incubation. Finally, we removed the inserts from the wells and discarded them. We transferred 112 μL of the 225-μL cell detachment solution to a black-walled 96-well plate suitable for fluorescence measurement and 112 μL of the 400-μL solution from the feeder well into the same well of the 96-well plate used in the previous step. The 96-well plates were read with a fluorescence plate reader using a 480/520-nm filter set.

Statistical Analysis

All experiments were performed in triplicate. All statistical analyses were done with SAS software version 9.4 [26]. HPLC data were analyzed with two-way ANOVAs and all bioassays with Student's *t* tests. *P* values < 0.05 were considered significant.

Results

Variation of Total Withanolides by Location and Tissue Type

Biosynthesis of withanolides varied between both location ($F = 9367.1$, $P < 0.001$, Fig. 2) and tissue type ($F = 32{,}331.0$, $P < 0.001$, Fig. 2), with the highest total withanolide concentration in the leaves regardless of the site from which they were collected. We also found a statistical interaction between location and tissue type ($F = 1422.3$, $P < 0.001$, Fig. 2), mostly due to the much higher levels of withanolides in the fruits and leaves of plants from Ag compared to the other locations. The total withanolide concentration in the samples collected from the various sites differed by almost 17-fold in the following order (mean ± SE): Ja (1.01 ± 0.002 mg/g), Ka (1.82 ± 0.011 mg/g), La (2.21 ± 0.04 mg/g), Am (2.38 ± 0.04 mg/g), Sn (2.67 ± 0.03 mg/g), Kc (3.38 ± 0.02 mg/g), Kh (3.40 ± 0.01 mg/g), Sc (4.30 ± 0.02 mg/g), Up (4.40 ± 0.01 μg/g), Mi (5.15 ± 0.01 mg/g), and Ag (16.83 ± 0.02 mg/g). The order of withanolide concentration of all plants by tissue type was as follows (mean ± SE): roots (0.42 ± 0.07 mg/g dry weight), stems (0.58 ± 0.07 mg/g dry weight), fruit (0.87 ± 0.21 mg/g dry weight), and leaves (2.45 ± 0.45 mg/g dry weight).

Variation of Individual Withanolides by Location and Tissue Type

We observed different levels of individual withanolides not only between different tissues of the same plant but also between different locations, and these observations interacted between plant tissue and site for all the studied compounds (Fig. 3, Table 2). Higher amounts of single

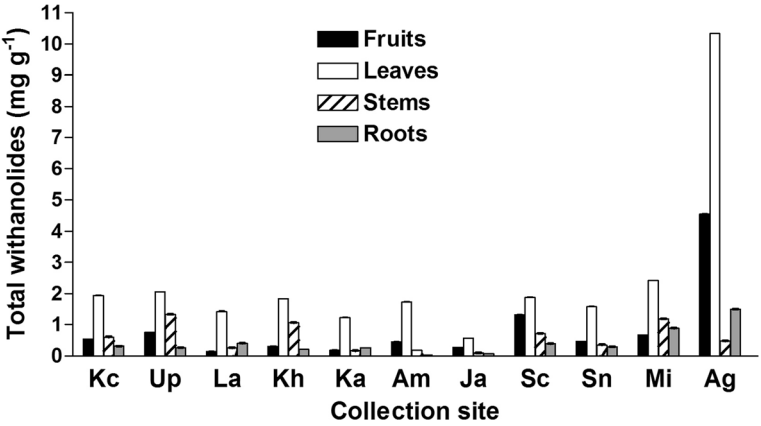

Fig. 2 Total withanolide concentration (mg/g dry weight ± SE) in different plant parts of *Withania coagulans* from 11 collection sites in Pakistan. Abbreviations: Kohat City, University Rd. (Kc); Usterzai payan, Hangu Rd. (Up); Lachi (La); Khada (Kh); Karak (Ka); Ambericala (Am); Jand (Ja); Sargodha city (Sc); Soon Skaser valley (Sn); Mianwali Musa Khel (Mi); and Mohmand Agency (Ag)

withanolides were found mostly in the leaves and fruits of the plants from Ag. Overall, we found that withanolide H was the most abundant withanolide, especially in the fruits, leaves, and roots, followed by withacoagulin E, coagulansin A, withacoagulin, withacoagulin I, withacoagulin H, and withacoagulin G (Fig. 1).

Correlations of Site Climatic Factors with Withanolide Concentrations

In general, *W. coagulans* plants produced increasing amounts of withanolides as site precipitation decreased. Correlation analyses showed strong inverse relationships between collection

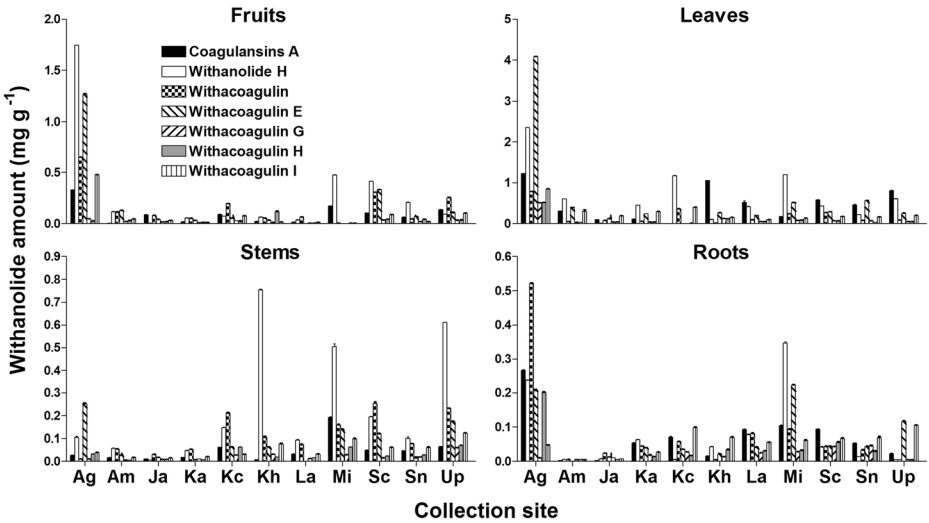

Fig. 3 Concentrations (mg/g dry weight ± SE) of individual withanolides in *Withania coagulans* from 11 sites in Pakistan. Abbreviations: Kohat City, University Rd. (Kc), Usterzai payan, Hangu Rd. (Up), Lachi (La), Khada (Kh), Karak (Ka), Ambiri Kala (Am), Jand (Ja), Sargodha City (Sc), Soon Skaser valley (Sn), Mianwali Musa Khel (Mi), and Mohmand Agency (Ag). See Table 2 for statistical results

Table 2 *F* ratios and *P* values from a 2-factor ANOVA assessing the effect of tissue, and collection site, and their interactions, on the amounts of seven individual withanolides in *Withania coagulans*. Corresponding data are shown in Fig. 2

Compound	Tissue		Site		Tissue × site	
	F	*P*	*F*	*P*	*F*	*P*
Coagulansin A	10,034.8	*< 0.001*	1802.2	*< 0.001*	889.1	*< 0.001*
Withanolide H	12,510.0	*< 0.001*	5126.9	*< 0.001*	1189.2	*< 0.001*
Withacoagulin	1752.5	*< 0.001*	1592.0	*< 0.001*	717.8	*< 0.001*
Withacoagulin E	563.5	*< 0.001*	349.0	*< 0.001*	128.6	*< 0.001*
Withacoagulin G	1670.8	*< 0.001*	305.4	*< 0.001*	323.6	*< 0.001*
Withacoagulin H	459.8	*< 0.001*	357.6	*< 0.001*	159.7	*< 0.001*
Withacoagulin I	3809.1	*< 0.001*	576.5	*< 0.001*	274.0	*< 0.001*

Italics indicates statistical significance at $P < 0.05$

site annual precipitation versus withanolide amounts in fruits ($r = -0.84$, $P = 0.001$), leaves ($r = -0.88$, $P < 0.001$), roots ($r = -0.91$, $P < 0.001$), and total ($r = -0.89$, $P < 0.001$), but not stems ($r = -0.20$, $P = 0.556$). There was no relationship between the amounts of withanolides in different tissues or the total versus average June temperature (all $P > 0.356$). Finally, elevation was not correlated with precipitation ($r = -0.33$, $P = 0.328$).

Analysis of Withanolides in *W. coagulans* Leaf Extracts ML and AL

We found seven known and five putative withanolides in the two *W. coagulans* extracts tested using UHPLC-MS analysis (ML and AL, Table 3). The unknown compounds had exact masses matching those of known withanolides (Table 4). For instance, the compound found at 10.80 min (unknown 1) has the same exact mass as coagulansin A, and the compound at 17.34 min (unknown 5) has the same exact mass as withanolide B. Three other unknowns [2–4] had the same exact mass (*m*/*z* 469.3) as several other known withanolides (Fig. 1, Table 4).

Total withanolide content of the AL extract was almost twice that of ML (Table 3). In addition, eight of the 12 compounds (coagulansin A, unknowns 1, 2, 4, and 5, withacoagulin I,

Table 3 Amounts (mg/g dry weight) of various withanolides and totals found in *W. coagulans* leaf extracts ML and AL. RT = retention time of UHPLC-MS-extracted ion chromatogram (EIC) peak. *m*/*z* values obtained in the negative ionization mode

Compound	RT (min)	ML	AL
Coagulansin A	9.70	6.86	9.07
Withanolide G	10.69	18.44	8.74
Unknown 1 (*m*/*z* 485.3)	10.80	6.70	19.97
Unknown 2 (*m*/*z* 469.3)	11.41	6.44	12.63
Unknown 3 (*m*/*z* 469.3)	11.76	3.02	0.71
Withacoagulin H	12.03	0.19	0.15
Withacoagulin I	12.53	0.48	1.01
Withacoagulin G	13.06	3.59	0.85
Withanolide F	15.48	2.00	8.96
Unknown 4 (*m*/*z* 469.3)	16.05	5.70	17.44
Withacoagulin E	16.73	1.60	16.06
Unknown 5 (*m*/*z* 453.3)	17.34	5.65	13.11
Total		60.67	108.70

Table 4 UHPLC-MS retention times, exact masses, and masses of both the deprotonated [(M-H)$^-$] and formate adducts [(M−H+HCOOH)$^-$] of available standards and related withanolides

Compound	RT (min)	Exact mass	(M−H)$^-$	(M−H+HCOOH)$^-$
Available standards				
Coagulansin A	9.70	486.2618	485.2545	531.2600
Withanolide H	10.69	470.2668	469.2596	515.2650
Withacoagulin G	13.06	470.2668	469.2596	515.2650
Withacoagulin I	12.53	470.2668	469.2596	515.2650
Withacoagulin H	12.03	468.2512	467.2439	513.2494
Withanolide F	15.48	470.2668	469.2596	515.2650
Withacoagulin	16.73	452.2563	451.2490	497.2545
Other withanolides with the same exact masses as the leaf extract unknowns				
Withanolide	Na	470.2668	469.2596	515.2650
Withanolide A	Na	470.2668	469.2596	515.2650
Withanolide B	Na	454.2719	453.2646	499.2701
Withanolide C	Na	522.2384	521.2311	567.2366
Withanolide D	Na	470.2668	469.2596	515.2650
Withanolide E	Na	486.2618	485.2545	531.2600

withanolide F, and withacoagulin E) were present in higher amounts in AL. The remaining four (withanolide G, unknown 3, withacoagulin H, and withacoagulin G) were more abundant in ML.

W. coagulans Shows Bioactivity Against Prostate Cancer Cells

Since withanolides in *W. coagulans* have proven bioactivity against cancer [34, 35], we focused on how leaf extracts (ML and AL) of *W. coagulans* would affect prostate cancer cell lines. We tested *W. coagulans* ML and AL extracts against cancer cell lines with several different bioassays and found their inhibition effects to be mostly proportional to withanolide content. In addition, the greater withanolide content of AL resulted in greater anticancer activity in all assays we performed (MTT, BrdU, antimigration, scratch wound, and apoptotic assays), as compared to ML.

W. coagulans Inhibits Growth and Proliferation of Prostate Cancer Cells

To assess growth and proliferation of prostate cancer cells, we performed a MTT assay using *W. coagulans* ML and AL leaf extracts at concentrations ranging from 10 to 250 μg/mL for 24 and 48 h against normal human prostate cells (RWPE-1) and androgen-sensitive (C4-2) and androgen-independent (PC3 and DU-145) prostate cancer cells (Fig. 4, Table 5). We found that both of these extracts had minimal effects on RWPE-1 cells, but showed strong cell growth inhibitory effects against C4-2, DU145, and PC3 human prostate cancer cell lines in a dose- and time-dependent manner. At the highest concentration, both extracts reduced cell growth by about 50 to 70%, depending on the cell line (Fig. 4). As expected, results showed that *W. coagulans* extract activity was overall more potent at 48 h as compared to 24 h exposure time, especially for the AL extract. In the case of C4-2, IC50 values for *W. coagulans* differed between the extracts from the two locations. AL extract had IC50 values of 46.55 and 23.47 μg/mL after 24 and 48 h, while ML extract had IC50 values of 101.3 μg/mL after 24 h and 75.7 μg/mL after 48 h. When DU145 cells were used for the experiment, the IC50 values of ML and AL *W. coagulans* extracts were 111.2 and 89.8 μg/mL, respectively, after 24 h, and 95.51 and 26.14 μg/mL, respectively, after 48 h.

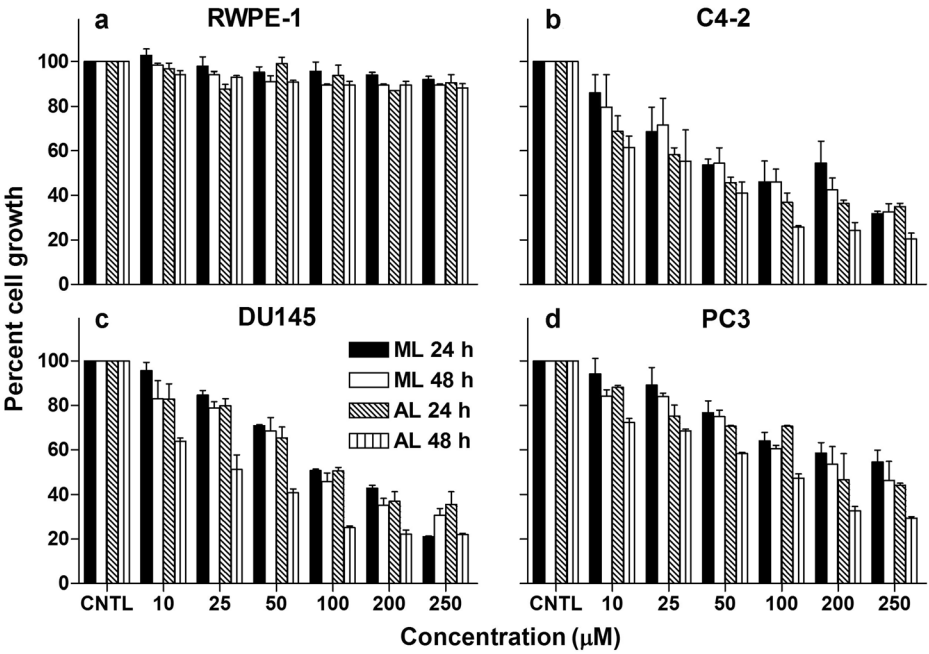

Fig. 4 Effect of *Withania coagulans* extracts ML and AL on the growth (percentage survival) of **a** normal RWPE-1 cells and **b**–**d** human prostate cancer cell lines **b** C4-2, **c** DU145, and **d** PC3 treated with AL and ML extracts at the indicated concentrations for 24 and 48 h. See Table 5 for statistical results

W. coagulans plant extracts ML and AL were also tested for their antiproliferative effects against C4-2 and DU145 cells using the BrdU assay (Fig. 5, Table 6). The C4-2 cell line treated with either extract and D145 cells treated with the AL extract responded in a similar way in the 24-h test with all three showing overall lower cell proliferation values than DU145 cells exposed to the ML extract (Fig. 5a). In the 48-h assay, C4-2 cells treated with the AL extract showed the least amount of proliferation at all concentrations, and once again, DU145 treated with ML proliferated the most (Fig. 5b).

W. coagulans Inhibits Migration of Prostate Cancer Cells

We used the BrdU assay to evaluate the antimigration activity of *W. coagulans* extracts against human prostate cancer cells C4-2 and DU145 (Fig. 6a, Table 7). We found no effect for either the ML or the AL extracts when tested with the C4-2 cell line when compared to the plus FBS control (Fig. 6a, Table 7). In contrast, both extracts showed strong antimigration activity against the DU145 cell line, with both concentrations of the ML extract showing about twice the values of the plus FBS control and the AL extract about 2.5 times higher (Fig. 6b, Table 7).

We also performed the scratch wound assay for the evaluation of the antimigratory effects of *W. coagulans*. DU145 and C4-2 cells were treated with different concentrations (25 and 50 μg/mL) of ML and AL extracts (Fig. 7). Observations made in the wound-healing assay supported antimigration assay results, i.e., the numbers of cells that migrated in the scratch wound were significantly less in the treated cells (C4-2; DU145) as compared to the untreated controls, suggesting that *W. coagulans* inhibited the migration of C4-2 and DU145 cells (Fig. 6).

Table 5 *t* test results for the effects of *Withania coagulans* extracts ML and AL on the growth (percentage survival) of normal RWPE-1 cells and human prostate cancer cell lines DU145, PC3, and C4-2 at the indicated concentrations for 24 and 48 h. Italics indicates statistical significance at $P < 0.05$. Corresponding data are shown in Fig. 4

Cell line and comparison	ML 24 h		ML 48 h		AL 24 h		AL 48 h	
	t	*P*	*t*	*P*	*t*	*P*	*t*	*P*
RWPE-1								
Control vs. 10 μM	0.0	0.326	0.4	0.725	0.7	0.535	0.4	0.723
Control vs. 25 μM	0.5	0.672	2.4	0.079	2.7	0.056	1.9	0.132
Control vs. 50 μM	2.2	0.094	2.6	0.060	0.3	0.753	1.8	0.144
Control vs. 100 μM	0.7	0.522	2.6	0.058	1.6	0.178	2.8	0.050
Control vs. 200 μM	1.7	0.157	2.2	0.096	1.4	0.231	2.8	0.050
Control vs. 250 μM	4.0	*0.016*	4.7	*0.009*	2.6	0.061	2.4	0.075
DU145								
Control vs. 10 μM	1.3	0.278	1.9	0.138	2.7	0.056	17.8	*< 0.001*
Control vs. 25 μM	8.7	*0.001*	1.9	0.133	3.0	*0.039*	12.0	*< 0.002*
Control vs. 50 μM	23.2	*< 0.001*	3.8	*0.020*	5.4	*0.006*	32.4	*< 0.001*
Control vs. 100 μM	36.7	*< 0.001*	4.9	*0.008*	7.4	*0.002*	44.0	*< 0.001*
Control vs. 200 μM	47.2	*< 0.001*	7.6	*0.002*	9.1	*0.001*	41.2	*< 0.001*
Control vs. 250 μM	83.3	*< 0.001*	8.1	*0.001*	8.6	*0.001*	48.4	*< 0.001*
PC3								
Control vs. 10 μM	1.2	0.316	4.7	*0.009*	4.9	*0.008*	12.5	*< 0.001*
Control vs. 25 μM	1.9	0.125	5.7	*0.005*	5.6	*0.005*	33.4	*< 0.001*
Control vs. 50 μM	2.8	*0.050*	8.4	*0.001*	10.8	*0.002*	63.9	*< 0.001*
Control vs. 100 μM	6.8	*0.002*	11.9	*< 0.001*	10.8	*0.002*	28.6	*< 0.001*
Control vs. 200 μM	7.8	*0.002*	6.1	*0.004*	5.0	*0.007*	32.9	*< 0.001*
Control vs. 250 μM	7.5	*0.002*	7.0	*0.002*	28.3	*< 0.001*	75.1	*< 0.001*
C4-2								
Control vs. 10 μM	2.2	0.092	1.6	0.176	4.6	*0.010*	3.7	*0.021*
Control vs. 25 μM	3.3	*0.029*	2.1	0.100	12.9	*< 0.001*	3.3	*0.029*
Control vs. 50 μM	10.1	*0.001*	3.2	*0.032*	18.4	*< 0.001*	5.7	*0.005*
Control vs. 100 μM	6.4	*0.003*	3.8	*0.020*	14.3	*< 0.001*	7.0	*0.002*
Control vs. 200 μM	5.0	*0.008*	4.0	*0.016*	39.6	*< 0.001*	7.0	*0.002*
Control vs. 250 μM	26.6	*< 0.001*	4.6	*0.010*	45.2	*< 0.001*	7.7	*0.002*

W. coagulans Extract Induces Cell Apoptosis of Prostate Cancer Cells

We evaluated the efficacy of *W. coagulans* extract in inducing apoptosis in prostate cancer cells. *W. coagulans* extract-treated cells showed dose- and time-dependent increase in the percentage of apoptosis and necrosis as compared to untreated controls (Fig. 8). More apoptotic cells were observed at 48 h treatment as compared to 24 h.

Discussion

We collected samples of *W. coagulans* from 11 locations in Pakistan and used HPLC to measure the levels of withanolides in different plant parts. We then selected plants with the highest amounts of withanolides from two locations [Mianwali (Mi) and Mohmand Agency (Ag)] and made leaf extracts (ML and AL, respectively), which were analyzed with UHPLC-MS. These extracts were further tested against various prostate cancer cell lines to determine their anticancer potential.

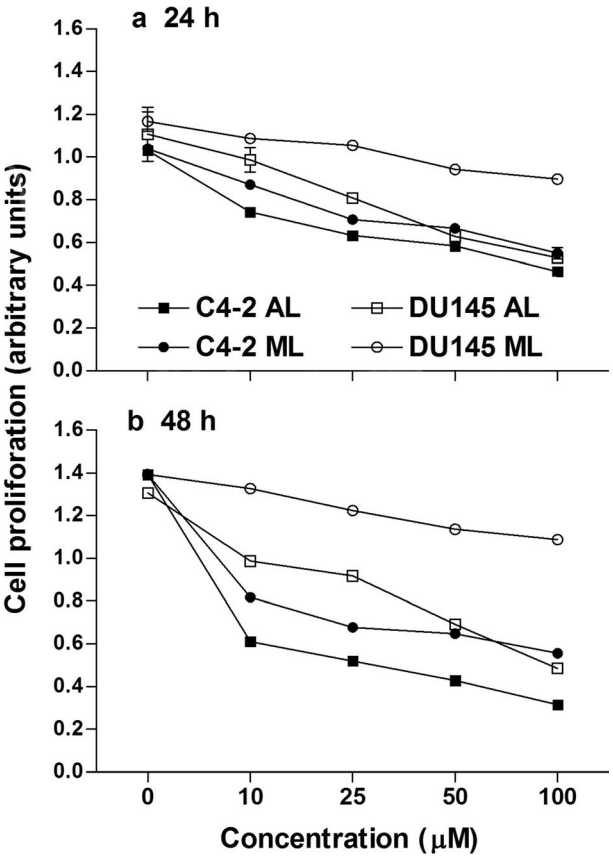

Fig. 5 Effect of *Withania coagulans* extracts ML and AL at the indicated concentrations on the proliferation of human prostate cancer cell lines DU145 and C4-2 after **a** 24 and **b** 48 h. See Table 6 for statistical results

Table 6 *t* test results for the effects of *Withania coagulans* extracts ML and AL on the proliferation of human prostate cancer cell lines DU145 and C4-2 at the indicated concentrations. Italics indicates statistical significance at $P < 0.05$. Corresponding data are shown in Fig. 5

Cell line and comparison	ML 24 h		ML 48 h		AL 24 h		AL 48 h	
	t	*P*	*t*	*P*	*t*	*P*	*t*	*P*
DU145								
Control vs. 10 μM	3.0	0.157	521.07	*< 0.001*	0.8	0.424	440.69	*< 0.001*
Control vs. 25 μM	6.0	0.070	2612.61	*< 0.001*	5.6	0.077	203.99	*< 0.001*
Control vs. 50 μM	24.0	*0.008*	5959.84	*< 0.001*	13.5	*0.021*	1861.92	*< 0.001*
Control vs. 100 μM	34.6	*0.004*	11,040.2	*< 0.001*	20.5	*0.011*	3360.02	*< 0.001*
C4-2								
Control vs. 10 μM	99,867.3	*< 0.001*	31,692.3	*< 0.001*	151.0	*< 0.001*	56,112.5	*< 0.001*
Control vs. 25 μM	43,010.0	*< 0.001*	70,785.0	*< 0.001*	10,236.2	*< 0.001*	52,682.2	*< 0.001*
Control vs. 50 μM	19,303.5	*< 0.001*	14,411.2	*< 0.001*	13,874.2	*< 0.001*	136,151.0	*< 0.001*
Control vs. 100 μM	342.7	*< 0.001*	128,471.0	*< 0.001*	36,539.3	*< 0.001*	105,438.0	*< 0.001*

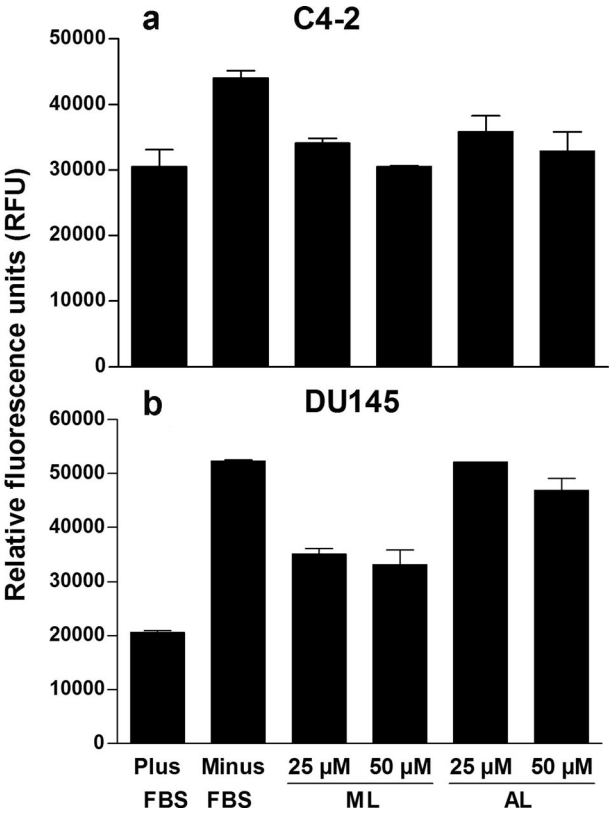

Fig. 6 Antimigration effects of *Withania coagulans* extracts ML and AL on **a** C4-2 and **b** DU145 cells. See Table 7 for statistical results

Table 7 *t* test results for the effects of *Withania coagulans* extracts ML and AL on the migration of human prostate cancer cell lines DU145 and C4-2 at the indicated concentrations versus plus FBS (fetal bovine serum). Italics indicates statistical significance at $P < 0.05$. Corresponding data are shown in Fig. 6

Cell line	*t*	*P*
C4-2		
Plus FBS vs. minus FBS	22.7	*0.041*
Plus FBS vs. 25 µM ML	2.2	0.275
Plus FBS vs. 50 µM ML	0.4	0.611
Plus FBS vs. 25 µM AL	1.8	0.315
Plus FBS vs. 50 µM AL	0.0	0.993
DU145		
Plus FBS vs. minus FBS	7615.0	*< 0.001*
Plus FBS vs. 25 µM ML	10,756.7	*< 0.001*
Plus FBS vs. 50 µM ML	136.5	*0.007*
Plus FBS vs. 25 µM AL	199.8	*0.005*
Plus FBS vs. 50 µM AL	20.6	*0.045*

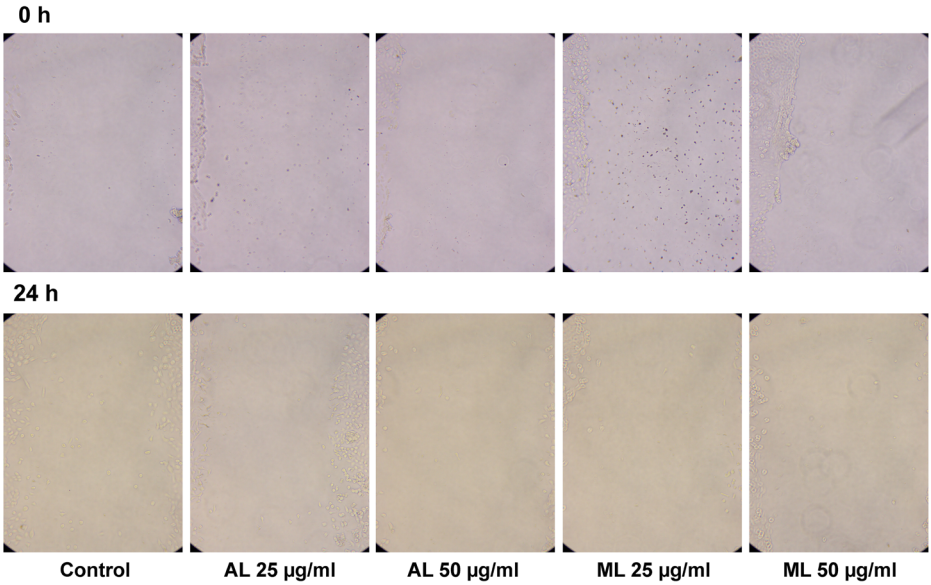

Fig. 7 Photomicrographs of scratch wound assay in prostate cancer cells C4-2 with *Withania coagulans* extracts ML and AL at the indicated concentrations for 0 and 24 h

Effect of Location and Tissue Type on Total Withanolide Content

One important finding of this study was the large variation in the amounts of withanolides among *W. coagulans* from various locations in Pakistan and between different tissue types within plants. Plants from the Ag site contained the greatest total amount of these secondary metabolites, followed by Mi. The Ag samples averaged more than twice the amount of withanolides compared to Mi and almost 17-fold more than the lowest concentration site (Ja). Besides having the overall highest concentration of withanolides, samples from Ag also contained the greatest amounts of individual withanolides, especially withacoagulin E. Our correlation analyses clearly showed that as site precipitation decreased the levels of withanolides in most tissues and the total increased. The Ag samples contained the highest amounts of withanolides and were collected from a barren mountainous region with extreme climate and scanty rainfall (100 mm annually), as compared to the other collection sites (all > 330 mm annually). Various biotic and abiotic stresses are known to cause changes in plant metabolism and possibly alter secondary metabolite production [27, 28]. For instance, Jochum et al. [29] found the production of high concentrations of secondary defense compounds in *Panax quinquefolius* when stressed by temperature. Pratt et al. [30] reported higher amounts of terpenoids in *Artemisia californica* plants that originated from the hotter and drier part of its range in southern California compared to those from cooler and wetter regions in the northern part of the state. In another report, variation in withanolide content in the roots of *W. somnifera* collected from different regions was explained by climatic conditions, moisture levels, soil texture, and fertility [31].

Across all *W. coagulans* plants tested, we found the highest withanolide concentration in the leaves, which contained almost three times the amount measured in fruits, and about four and six times greater levels than in the stems and roots, respectively. The relatively high levels of withanolides that we found in *W. coagulans* leaf tissue, compared to other plant parts, were

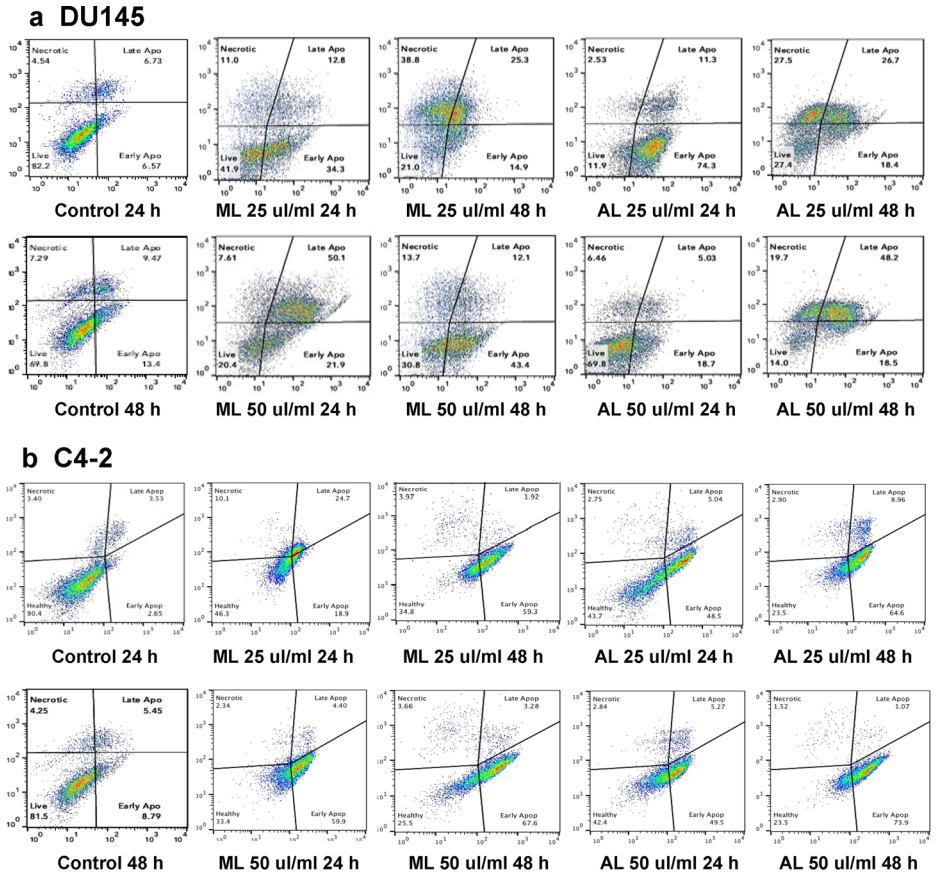

Fig. 8 Apoptosis of human prostate cancer cells **a** DU145 and **b** C4-2 treated with *Withania coagulans* extracts ML and AL at concentrations of 25 and 50 μg/mL for 24 and 48 h. Results shown are representative of three different experiments with similar results

also reported for *W. somnifera* [32]. The same study also found that roots biosynthesize withanolides by showing the production of withanolide A in both cultured and natural roots of *W. somnifera* [32]. This agrees with our finding that the roots of *W. coagulans* also contain withanolides.

Variation of Individual Withanolides by Location and Tissue Type

We observed different levels of individual withanolides both between different plant tissues and between plants collected from different locations, and these observations interacted between plant tissue and site for all the studied compounds. This statistical interaction was due to the much higher amounts of individual withanolides measured in the leaves and fruits of plants from Ag, compared to all the other sites. Overall, withanolide H was the most abundant withanolide, especially in fruits, leaves, and roots, followed by withacoagulin E, coagulansin A, withacoagulin, withacoagulin I, withacoagulin H, and withacoagulin G. Variations in the amounts of withanolides have been reported previously in other *Withania* species where fluctuation in the quantity of withaferien A and withanone varied in samples of

W. somnifera from different areas of India [31]. Our expanded survey of withanolides in *W. coagulans* found similar patterns to those from earlier work. For example, even though withacoagulin H, G, and I were previously reported in methanolic extracts of whole plants [17], we observed all of these withanolides in each tissue, but in different quantities. These seven compounds are strong antioxidants, as they inhibited nitric oxide production in lipopolysaccharide-activated murine macrophage RAW 264.7 cells [17]. They are also antitumor in nature, as they inhibited tumor necrosis factor-α (TNF-α)-induced nuclear factor-kappa B (NF-κB) activation with IC50 values in the range of 1.6–12.4 μM [17]. Coagulansin A was also reported in whole plant extracts [33]. However, we found the greatest amounts in the leaves and much lower levels in other tissues of plants from most locations. Withacoagulin E was previously reported in aerial parts of *W. coagulans* [25], but we also found that it is present in root tissue, although at comparatively lower amounts. Withacoagulin E also has demonstrated anticancer activity (IC50 < 20 mM) [34].

Analysis of Withanolides in *W. coagulans* Extracts

Our UHPLC-MS analysis of the ML and AL *W. coagulans* extracts showed an even higher diversity of withanolides than previously thought from the initial HPLC plant tissue data. Following the same pattern as the leaves, the total withanolide content of the AL extract was almost twice than that of ML. In addition, both extracts contained several putative compounds that appear to be isomers of existing withanolides.

W. coagulans Shows Bioactivity Against Prostate Cancer Cells

Since withanolides in *W. coagulans* have proven bioactivity against cancer [35, 36], we focused on how extracts (ML and AL) of *W. coagulans* would affect prostate cancer cell lines. We tested *W. coagulans* ML and AL extracts against cancer cell lines with several different bioassays and found their inhibition effects to be mostly proportional to withanolide content. In addition, the greater withanolide content of AL resulted in greater anticancer activity in most of the assays we performed (MTT, BrdU, antimigration, scratch wound, and apoptotic assays), as compared to ML. These comparison studies support the idea that plants containing higher withanolide content possess higher antiviability, antiproliferative, and apoptotic activities against cancer cells.

W. coagulans Inhibits Growth and Proliferation of Prostate Cancer Cells

We found strong cell growth inhibitory effects of both ML and AL *W. coagulans* extracts against PC3, DU145, and C4-2 human prostate cancer cell lines. We performed the MTT assay using the extracts against normal human prostate cells (RWPE-1) and androgen-sensitive (C4-2) and androgen-independent (PC3 and DU-145) prostate cancer cells. Prostate cancer cells were treated with the extract of *W. coagulans* (10–250 μg/mL for 24 and 48 h), and we observed significant inhibition of cell growth in a dose- and time-dependent manner. In the case of C4-2, IC50 values for *W. coagulans* differed between the extracts from the two locations. These observations corroborate findings from other laboratories using the foliage of *W. somnifera* [37].

W. coagulans plant extracts AL and ML were also tested for their antiproliferative effects against DU145 and C4-2 cells using the BrdU assay. AL exhibited greater antiproliferative

activity than ML at both 24 and 48 h. Our results showed that androgen-sensitive C4-2 cells had slightly more susceptibility to *W. coagulans* extract treatment than the androgen-independent cell line DU145. The higher levels of withanolides in AL, compared to ML, are most likely the reason for its greater antiproliferative activity against selected prostate cancer cell lines; however, the extracts also varied in their withanolide profile which could be another reason for differential performance.

W. coagulans Inhibits Migration of Prostate Cancer Cells

W. coagulans extracts showed antimigration activity against human prostate cancer cells DU145 (androgen-sensitive) but not C4-2 (androgen-independent). Once again, we found that overall the AL extract exhibited more pronounced inhibitory effects on migration of prostate cancer cells as compared to the ML extract, perhaps consistent with its higher levels of withanolides. We also performed scratch wound assay for the evaluation of antimigratory effects of *W. coagulans*. DU145 and C4-2 cells were treated with different concentrations (25 and 50 μg/mL) of ML and AL extracts. Observations made in the wound-healing assay supported the DU145 antimigration assay results, i.e., the numbers of cells that migrated in the scratch wound were significantly less in treated cells (C4-2; DU145) as compared to the untreated controls, suggesting that *W. coagulans* inhibited the migration of cells. We observed that C4-2 cells were more sensitive than DU145 to treatment, suggesting that *W. coagulans* might be more effective against aggressive prostate cancers.

W. coagulans Extract Induces Cell Apoptosis of Prostate Cancer Cells

We evaluated the efficacy of *W. coagulans* extract in inducing apoptosis in prostate cancer cells. *W. coagulans* extract-treated cells showed dose- and time-dependent increase in the percentage of apoptosis and necrosis as compared to untreated controls. More apoptotic cells were observed at 48 h treatment as compared to 24 h. The apoptotic activity of *W. coagulans* was previously assessed using methanolic extracts against the HeLa cell line, and potent activities were reported using both in vitro and in vivo models [38]. This is the first time two different accessions of *W. coagulans* have been compared and assessed for apoptotic activity on prostate cancer cell lines (C4-2 and DU145). Furthermore, we observed that AL showed potent apoptotic activity, and interestingly, in some cases, its apoptotic activity was better than the positive control. Although both accessions of *W. coagulans* induced apoptosis against prostate cancer cell lines, the AL extract showed more promising anticancer activity compared to ML.

Conclusion

In the present study, we analyzed the leaves, fruits, stems, and roots of *W. coagulans* from 11 locations in Pakistan by liquid chromatography for qualitative and quantitative distribution of seven withanolides. The amounts of these compounds showed substantial variation between different geographic areas and tissue types and tended to increase with site aridity. Samples from the Mohmand Agency (Ag) site contained the greatest amounts of foliar withanolides, followed by Mianwali Musa Khel (Mi). Bioassays using extracts made from Ag (AL) and Mi (ML) leaf material against prostate cancer cells showed strong anticancer activity, including

induced cell apoptosis and inhibited proliferation, cell migration, and invasion. Overall, these effects increased both in a dose-dependent manner and with time. Our results showed that extracts made from *W. coagulans* accessions high in withanolides show potent inhibitory effects against human prostate cancer cell lines, suggesting the plant is a promising candidate for anticancer research and isolation of anticancer compounds.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflicts of interest.

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