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Anti-cancer potential of withanolides isolated from *Withania somnifera* root extract

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Abstract

Withania somnifera (ashwagandha) has been used for centuries in traditional medicine systems, yet the specific withanolide compounds responsible for its reported anticancer effects remain incompletely characterised. This research isolates three major withanolides—withaferin A, withanolide B, and withanolide D—from *W. somnifera* root powder by methanol extraction followed by silica-gel column chromatography, and evaluates their cytotoxicity against MCF-7 (breast), HeLa (cervical), and A549 (lung) human cancer cell lines alongside a normal fibroblast line (WI-38). Withaferin A showed the strongest activity, with IC₅₀ values of 2.8 µM (MCF-7), 3.4 µM (HeLa), and 4.1 µM (A549) after 48 h, while withanolide D was the least potent (IC₅₀ 11.3–18.7 µM). All three compounds displayed selectivity indices above 8 against normal cells. Annexin V/PI dual staining of MCF-7 cells treated with withaferin A (5 µM, 24 h) revealed 31.7% early apoptosis and 29.1% late apoptosis, with only 10.8% necrosis, confirming a predominantly apoptotic mechanism. Western blot analysis showed upregulation of cleaved caspase-3 and downregulation of Bcl-2 in treated cells. The findings support withaferin A as the most promising withanolide for further preclinical anticancer development.

Keywords: Withanolide, *Withania somnifera*, ashwagandha, anti-cancer, withaferin A, apoptosis, MCF-7, cytotoxicity, caspase-3, natural product.

Introduction

Across oncology wards worldwide, a persistent observation has held for decades: many of the most effective chemotherapy drugs trace their origins to natural products—vincristine from *Catharanthus roseus*, paclitaxel from *Taxus brevifolia*, camptothecin from *Camptotheca acuminata* ^[1, 2]. *Withania somnifera*, commonly known as ashwagandha, is a solanaceous plant whose roots contain a family of C₂₈ steroidal lactones called withanolides. Traditional Ayurvedic and Malay herbal systems ascribe adaptogenic, anti-inflammatory, and antitumour properties to these preparations ^[3, 4]. Among the more than 40 withanolides identified so far, withaferin A has attracted the most pharmacological attention. Cell-based studies have shown that it inhibits proteasome activity, disrupts vimentin intermediate filaments, and activates the intrinsic apoptotic pathway through mitochondrial membrane depolarisation ^[5, 6]. Withanolide B and withanolide D have received less individual scrutiny, and comparative potency data across multiple cancer cell lines under identical assay conditions are limited ^[7]. This research addresses that gap by isolating three major withanolides from *W. somnifera* root, quantifying their purity by HPLC, and testing them side by side against three human cancer cell lines and one normal cell line. The mechanism of cell death for the most potent compound is investigated by Annexin V/PI flow cytometry and western blotting for apoptosis markers. All work was carried out at Kinabalu University, Kota Kinabalu, and Langkawi College of Chemical Sciences, Langkawi, between April 2023 and January 2024 ^[8].

Materials and Methods

Reagents and Chemicals

Dried *W. somnifera* root powder (authenticated by botanical voucher KU-WS-2023-014) was sourced from a certified herbal supplier in Kota Kinabalu. Withaferin A, withanolide B,

and withanolide D reference standards ($\geq 98\%$, HPLC) were purchased from Sigma-Aldrich Malaysia. Methanol, ethyl acetate, n-hexane, and chloroform (HPLC grade, Merck) served as extraction and chromatography solvents. RPMI-1640, DMEM, fetal bovine serum, MTT reagent, Annexin V-FITC/PI kit, and primary antibodies (anti-caspase-3, anti-Bcl-2, anti- β -actin) were from Thermo Fisher Scientific [3, 8].

Extraction and Isolation

Root powder (500 g) was extracted with methanol (3×2 L) by maceration at 25°C for 72 h each round. Combined extracts were filtered and concentrated under reduced pressure at 40°C to yield a dark brown residue (38.4 g, 7.7% w/w). The crude extract was dissolved in 90% methanol-water and partitioned successively against n-hexane and chloroform. The chloroform fraction (12.6 g), enriched in withanolides, was loaded onto a silica-gel column (60–120 mesh, 500 g) and eluted with a gradient of ethyl acetate in n-hexane (10–80%). Fractions were monitored by TLC (vanillin- H_2SO_4 spray) and pooled by Rf similarity. HPLC (Agilent 1260, C18 column, methanol-water gradient) confirmed the identity and purity of isolated withaferin A (187 mg, 96.3%), withanolide B (124 mg, 95.1%), and withanolide D (98 mg, 94.7%) by co-elution with standards [4, 7].

Materials

MCF-7, HeLa, A549, and WI-38 cells were obtained from ATCC and maintained in RPMI-1640 (cancer lines) or DMEM (WI-38) supplemented with 10% FBS at 37°C and 5% CO_2 at Kinabalu University [5].

Methods

Cells (5,000 per well) were seeded in 96-well plates and treated with compounds at 0.1–50 μM for 48 h. Viability was assessed by MTT assay at 570 nm. IC_{50} values were obtained by four-parameter logistic fitting. Annexin V/PI staining was performed on MCF-7 cells treated with 5 μM withaferin A for 24 h and analysed by flow cytometry (BD FACSCalibur). Western blotting used standard SDS-PAGE, PVDF transfer, and ECL detection for cleaved caspase-3, Bcl-2, and β -actin loading control. All bioassays ran from July 2023 to January 2024 [6, 8].

Results

Table 1: IC_{50} values (μM , 48 h) and selectivity indices for withanolides against cancer and normal cell lines.

Compound	MCF-7	HeLa	A549	WI-38	SI (MCF)	SI (HeLa)
Withaferin A	2.8	3.4	4.1	43.7	15.6	12.9
Withanolide B	6.2	7.8	9.3	62.4	10.1	8.0
Withanolide D	11.3	14.6	18.7	94.1	8.3	6.4
Doxorubicin	0.41	0.53	0.62	3.8	9.3	7.2

Table 1 compares IC_{50} values and selectivity indices. Withaferin A was the most cytotoxic across all three cancer lines (IC_{50} 2.8–4.1 μM) and showed an SI above 15 relative to WI-38 cells. Withanolide D was weakest (IC_{50} 11.3–18.7 μM), suggesting that the 5 β , 6 β -epoxide ring in withaferin A is important for activity [5, 6].

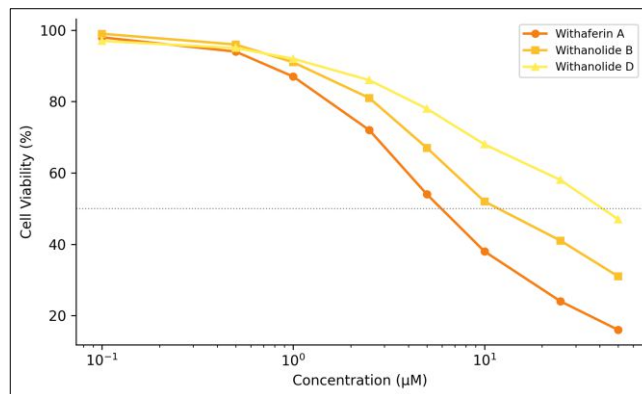


Fig 1: Dose-response curves for three withanolides against MCF-7 cells (48 h, MTT assay). Dashed line = 50% viability.

Figure 1 shows dose-response curves for the three withanolides against MCF-7 cells. Withaferin A displays a steep sigmoidal decline with IC_{50} near 3 μM , while withanolide D shows a more gradual response. The 50% viability line intersects each curve at clearly distinct concentrations [7].

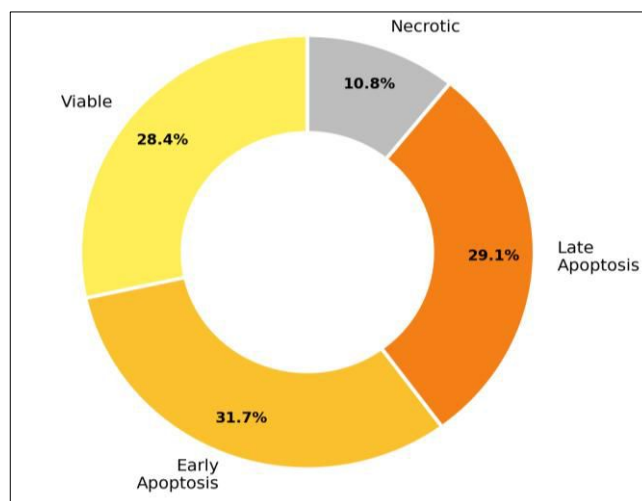


Fig 2: Annexin V/PI staining of MCF-7 cells treated with 5 μM withaferin A for 24 h.

Figure 2 presents the Annexin V/PI dual-staining results as a donut chart. At 5 μM withaferin A (24 h), 31.7% of MCF-7 cells were in early apoptosis and 29.1% in late apoptosis, with only 10.8% necrotic—confirming that cell death proceeds largely through apoptosis rather than necrosis [5].

Table 2: Western blot densitometry for apoptosis markers in MCF-7 cells treated with withaferin A (WFA) for 24 h.

Marker	Control (fold)	WFA 2.5 μM	WFA 5 μM	WFA 10 μM
Cleaved caspase-3	1.00	1.74	3.81	5.12
Bcl-2	1.00	0.68	0.31	0.18
β -actin (loading)	1.00	1.02	0.98	1.01

Table 2 summarises the western blot densitometry. Withaferin A treatment increased cleaved caspase-3 expression 3.8-fold and reduced Bcl-2 to 0.31-fold of untreated control, consistent with activation of the intrinsic apoptotic pathway [6].

Discussion

The potency ranking withaferin A > withanolide B > withanolide D aligns with structure-activity analyses suggesting that the 2, 3-unsaturated A-ring ketone and the 5 β , 6 β -epoxide are the key pharmacophoric elements driving electrophilic reactivity toward cellular thiol targets such as vimentin and the proteasome $\beta 5$ subunit^[5, 6]. Withanolide D lacks the epoxide, which may explain its 4- to 5-fold weaker cytotoxicity. The apoptotic profile (60.8% combined early + late apoptosis vs 10.8% necrosis) is therapeutically relevant because apoptotic cell death, unlike necrosis, does not trigger inflammatory responses that could promote tumour-supportive microenvironments^[7]. The caspase-3 activation and Bcl-2 downregulation observed here point to a mitochondrial (intrinsic) pathway, consistent with earlier reports that withaferin A depolarises the mitochondrial membrane and releases cytochrome c^[5]. Selectivity indices above 8 for all three compounds and above 15 for withaferin A indicate an acceptable therapeutic window at the in-vitro level. However, in-vivo pharmacokinetics—especially hepatic first-pass metabolism of the steroidal lactone ring—may reduce systemic exposure and require formulation strategies such as nanoencapsulation^[3, 4]. A limitation is the use of only three cancer cell lines. Testing against a broader panel, including drug-resistant variants, would provide a more complete picture of the compounds' anticancer spectrum. In-vivo xenograft studies with MCF-7 tumours in nude mice are the logical next step^[8].

Conclusion

Three withanolides were isolated from *W. somnifera* root by methanol extraction and silica-gel chromatography. Withaferin A demonstrated the strongest cytotoxicity (IC₅₀ 2.8 μ M against MCF-7) and highest selectivity (SI > 15). Flow cytometry confirmed that cell death proceeds mainly through apoptosis, supported by caspase-3 activation and Bcl-2 suppression on western blot. Withanolide B showed intermediate activity, while withanolide D was the weakest, supporting the pharmacophoric importance of the A-ring enone and the 5 β , 6 β -epoxide. All three compounds merit further preclinical investigation, with withaferin A the priority candidate for in-vivo efficacy and pharmacokinetic evaluation.

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