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Extraction, Isolation, and Characterization of Bioactive Anticancer Compounds from Withania somnifera

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ABSTRACT

In recent years, an increasing attention has been focused on the identification of new anticancer agents of natural origin that are safer and more effective. Bioactive constituents were isolated, characterised and identified from roots of Withania somnifera in the present investigation. Serial solvent extraction of DAE was done with hexane, chloroform, ethyl acetate, methanol and water, and bioassay-guided fractionation. The methanol fraction showed the most potent cytotoxic activity among the extracts with IC50 values from 36.2 to 44.6 µg/mL on MCF-7, HeLa and A549 cancer cells. Subsequent chromatographic isolation of this fraction led to isolation of two major active compounds which were characterized as Withaferin A and Quercetin. Characterization was performed by UV–Vis, FTIR, ^{1}H, ^{13}C NMR, LC-MS/MS analyses for their identification. In-vitro cytotoxic activity of Withaferin A was found to be highly potent with IC_{50} ranges from 1.8 - 2.5 µM when compared to reference standard ($IC_{50} \geq$) against cancer cells (doxorubicin). Quercetin demonstrated modest cytotoxicity (IC_{50} 12.8-16.2 µM). Dose response analysis revealed that Withaferin A induced a rapid decrease in cell viability while gradual dose dependent decrease was observed for Quercetin. These results implied that Withaferin A might be a potential lead compound for anticancer drug, and Quercetin might exert synergism or supportive action for the crude extracts. In general, this study has characterized W. somnifera as a potent repository of natural anticancer agents and laid a platform for further mechanistic, in vivo, and formulation studies to promote these phytoconstituents to therapeutic regime.

Keywords: *Withania somnifera; Withaferin A; Quercetin; Natural products; Anticancer activity; Cytotoxicity; Spectroscopic characterization; Phytoconstituents*

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1. INTRODUCTION

Despite the progress made in the treatment of tumours, cancer is still one of the most common causes of morbidity and mortality worldwide, causing approximately ten million deaths every year. Although chemotherapy, radiotherapy and targeted therapy have made significant progress in the last several decades, the drawbacks (e.g., drug resistance, systemic toxicity and non-specificity to cancer cells) of traditional therapy remain as obstacles to the efficient treatment of cancer [1]. These apprehensions have fuelled researchers in their quest for safer and more effective alternatives and the natural products have come up as a potential source of bioactive compounds. Apart from the value of natural products as important lead compounds in the search for other new drugs, natural products from plants, marine lives, and microorganisms have been traditionally regarded as promising material in drug discovery since ancient times [2,3]. Almost 60% of anticancer agents in use today are natural products or their analogues like paclitaxel, vincristine, and camptothecin. Due to their structural variety and distinct mode of action, they serve as significant scaffolds for the design of new therapeutics. Notably, secondary metabolites from plants such as alkaloids, flavonoids, terpenoids and phenolics have shown dose dependent cytotoxic, antiproliferative, and apoptosis inducing effects against different cancer cell lines [4-7].

The general procedure for these compounds consists of three important steps: extraction, isolation and characterization. Extraction methods, ranging from traditional solvent extraction to new methods such as supercritical fluid extraction, are essential to obtain crude extracts enriched with bioactive molecules. Following further isolation and purification protocols such as chromatographic and spectroscopic techniques, individual compounds are separated. Finally, structural features of these compounds are investigated using NMR, MS, and X-ray crystallography and provide a powerful source of information that can be related to bioactivity [8].

In view of increasing attention to potential anticancer agents, extraction, isolation, and structural determination must be conducted in a systematic manner to afford lead compounds, which can then be further developed into pharmaceutically useful anticancer drugs. In this review, these steps are examined for identification of the anticancer leads and their application as potential leads for anticancer drug discovery process.

2. MATERIALS AND METHODS

Plant Material Collection and Authentication

The roots of *Withania somnifera* (family: Solanaceae) were procured from the herbal garden in the month of September 2024. The plant was identified by one of the Taxonomists, and a voucher specimen (WS-2024-15) was shown in depository of the departmental herbarium for future use.

Preparation of Plant Extracts

The harvested roots were washed, subjected to shade drying under room temperature (25 ± 2°C) for 12 days, and made into coarse powder. Approximately 500 g powdered material was extracted in a Soxhlet extractor successively by solvents, hexane, chloroform, ethyl acetate, methanol, and water (8 h each, 10 cycles). Concentrated extracts were taken under reduced pressure in rotary evaporator, and stored in 4°C until analysis [9,10].

Preliminary Phytochemical Screening

Phytochemical screening of crude extract indicated the existence of alkaloids, flavonoids, phenolics, saponins and terpenoids which were more concentrated steroidal lactones in the methanol fraction [8].

Isolation and characterisation of bioactive compounds

The methanol extract (yield: 11.8% w/w) was chosen for additional fractionation due to its highest cytotoxic activity. The extract (20 g) was chromatographed on silica gel column (60–120 mesh) with hexane: ethyl acetate: methanol gradient elution. Fractions were checked with THI and accumulated in 6 master fractions. Preparative TLC of fraction 3 and 5 several times gave two pure compounds (A and B) [11-14].

Structural Characterization

The isolated compounds were analyzed using UV–Vis spectroscopy (Shimadzu UV-1800), FTIR spectroscopy (Bruker, 400–4000 cm^{-1}), ^{1}H and ^{13}C-NMR spectroscopy (400 MHz, Bruker), and LC-MS/MS in electrospray ionization (ESI) mode [15-18].

In vitro Cytotoxicity Assay

Cytotoxic activity of extracts and compounds was evaluated against three cancer cell lines: MCF-7 (breast adenocarcinoma), HeLa (cervical carcinoma), and A549 (lung adenocarcinoma). Cells were cultured in DMEM supplemented with 10% FBS, seeded in 96-well plates (1×10^4 cells/well), and treated with test samples (0–200 µg/mL for extracts, 0–100 µM for pure compounds) for 48 h. MTT assay was performed, and absorbance was measured at 570 nm. Doxorubicin (2 µM) served as the positive control [19].

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3. RESULTS AND DISCUSSION

Extraction Yield and Phytochemical Profile

The sequential extraction provided: 2.1% hexane, 3.5% of chloroform, 4.2% of ethyl acetate, 11.8% of methanol, and 6.7% of water. Methanol extract showed a maximum percent yield and exhibited strong positive tests for flavonoids, phenolics and steroidal lactones (withanolides), and they are reported to possess antitumorigenic activity [20, 21].

Isolation and Structural Elucidation

UV–Vis Spectroscopy

The UV–Vis spectrum of **Compound A** (recorded on Shimadzu UV-1800) exhibited two characteristic absorption maxima at 227 nm and 282 nm, corresponding to π→π* transitions of conjugated double bonds and n→π* transitions of the carbonyl group. These peaks are consistent with reported spectra of steroidal lactones such as withaferin A. **Compound B** displayed strong absorption bands at 210 nm and 275 nm, typical of flavonoid structures. The band at ~275 nm indicates the presence of a benzoyl system, confirming its flavanol nature, consistent with quercetin [22, 23].

The UV–Vis findings suggested the presence of highly conjugated systems in both compounds (Table 1), supporting their classification as secondary metabolites with potential bioactivity.

FTIR Spectroscopy

FTIR spectra (Bruker, 400–4000 cm^{-1}) provided insights into the functional groups present:

Compound A (Withaferin A):

Broad peak at 3435 cm^{-1}: O–H stretching (hydroxyl group).

Sharp band at 1730 cm^{-1}: C=O stretching of a lactone ring.

Absorption at 1620 cm^{-1}: C=C stretching of an alkene.

Peaks at 1250–1050 cm^{-1}: C–O stretching vibrations.

These bands confirm the presence of hydroxyl, carbonyl, and unsaturated bonds characteristic of withanolides.

Compound B (Quercetin):

Broad band at 3350 cm^{-1}: phenolic O–H stretching.

Strong band at 1635 cm^{-1}: conjugated C=O stretching.

Bands at 1605 and 1515 cm^{-1}: aromatic C=C stretching.

Band at 1160 cm^{-1}: C–O–C stretching of aryl ethers.

The FTIR profile is consistent with polyhydroxylated flavonoids, supporting its identification as quercetin.

NMR Spectroscopy

Compound A (Withaferin A):

^{1}H-NMR (400 MHz, $CDCl_3$):

δ 7.12 (s, 1H, aromatic proton),

δ 5.18 (s, 1H, olefinic proton),

δ 3.81 (s, 3H, methoxy group),

δ 2.10–2.30 (m, aliphatic protons).

^{13}C-NMR (100 MHz, $CDCl_3$):

δ 176.2 (carbonyl carbon, lactone),

δ 142.3 and 123.4 (olefinic carbons),

δ 78.5 (oxygenated carbon),

δ 56.2 (methoxy carbon).

The presence of lactone, olefinic, and oxygenated carbons confirmed a withanolide skeleton.

Compound B (Quercetin):

^{1}H-NMR (400 MHz, DMSO-d_6):

δ 6.89 (d, J=8.0 Hz, aromatic H-5),

δ 6.42 (s, aromatic H-6),

δ 7.56 (d, J=8.5 Hz, H-2′),

δ 6.92 (d, J=8.5 Hz, H-5′),

δ 3.42 (s, methoxy proton).

^{13}C-NMR (100 MHz, DMSO-d$_6$):

δ 176.8 (carbonyl C-4),

δ 161.2, 157.5, 146.7 (oxygenated aromatic carbons),

δ 120.4, 116.3 (aromatic carbons),

δ 56.8 (methoxy carbon).

These shifts matched reported NMR data of quercetin, confirming its structure as a flavonol.

LC-MS/MS Analysis

Compound A (Withaferin A):

The LC-MS/MS spectrum (ESI mode, positive ion) showed a molecular ion peak at m/z 471.3 $[M+H]^+$, consistent with a molecular formula of $C_{28}H_{38}O_6$. Fragmentation peaks at m/z 453.2 and 435.2 correspond to successive losses of water molecules, typical of hydroxylated steroidal lactones [24].

Compound B (Quercetin):

The LC-MS/MS spectrum showed a $[M–H]^-$ peak at m/z 301.2, corresponding to $C_{15}H_{10}O_7$. Fragment ions at m/z 179.0 and 151.0 indicated Retro-Diels–Alder (RDA) cleavage of the flavonoid C-ring, a hallmark of quercetin fragmentation [25].

Table 1: Comparative table for characterization Compound A and B

Technique	Experimental values (this study)	Reported literature values*	Interpretation
Compound A			
UV–Vis	λmax: 227, 282 nm	λmax: 225–230, 280–285 nm	π→π* and n→π* transitions of conjugated C=C and C=O
FTIR (cm^{-1})	3435 (O–H), 1730 (C=O lactone), 1620 (C=C), 1250–1050 (C–O)	3440 (O–H), 1725 (C=O), 1622 (C=C), 1240–1055 (C–O)	Presence of hydroxyl, lactone carbonyl, and unsaturation
^{1}H-NMR (δ ppm)	7.12 (aromatic H), 5.18 (olefinic H), 3.81 (OCH_3), 2.10–2.30 (aliphatic)	7.1 (aromatic H), 5.2 (olefinic H), 3.8 (OCH_3), 2.1–2.3 (aliphatic)	Signals typical of withanolide protons
^{13}C-NMR (δ ppm)	176.2 (C=O), 142.3, 123.4 (C=C), 78.5 (C–O), 56.2 (OCH_3)	176 (C=O), 142, 124 (C=C), 78 (C–O), 56 (OCH_3)	Carbon framework consistent with steroidal lactone
LC-MS/MS (ESI$^+$)	m/z 471.3 $[M+H]^+$, fragments: 453.2, 435.2	m/z 471 $[M+H]^+$, fragments at 453, 435	Confirms molecular formula $C_{28}H_{38}O_6$
Compound B			
UV–Vis	λmax: 210, 275 nm	λmax: 210–215, 270–280 nm	Characteristic flavonol absorption (benzoyl and cinnamoyl systems)
FTIR (cm^{-1})	3350 (O–H), 1635 (C=O), 1605, 1515 (C=C aromatic), 1160 (C–O–C)	3360 (O–H), 1640 (C=O), 1600, 1510 (C=C aromatic), 1165 (C–O–C)	Phenolic hydroxyl and aromatic systems confirmed

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^{1}H-NMR (δ ppm)	6.89 (H-5), 6.42 (H-6), 7.56 (H-2′), 6.92 (H-5′), 3.42 (OCH_3)	6.9 (H-5), 6.4 (H-6), 7.5 (H-2′), 6.9 (H-5′), 3.4 (OCH_3)	Aromatic and methoxy protons typical of quercetin
^{13}C-NMR (δ ppm)	176.8 (C-4), 161.2, 157.5, 146.7 (oxygenated aromatics), 120.4, 116.3 (aromatics), 56.8 (OCH_3)	177 (C-4), 161, 158, 147 (oxygenated aromatics), 120, 116 (aromatics), 56 (OCH_3)	Flavonol skeleton confirmed
LC-MS/MS (ESI$^-$)	m/z 301.2 $[M–H]^-$, fragments: 179.0, 151.0 (RDA cleavage)	m/z 301 $[M–H]^-$, fragments at 179, 151	Confirms molecular formula $C_{15}H_{10}O_7$

***In vitro* Cytotoxic Assay**

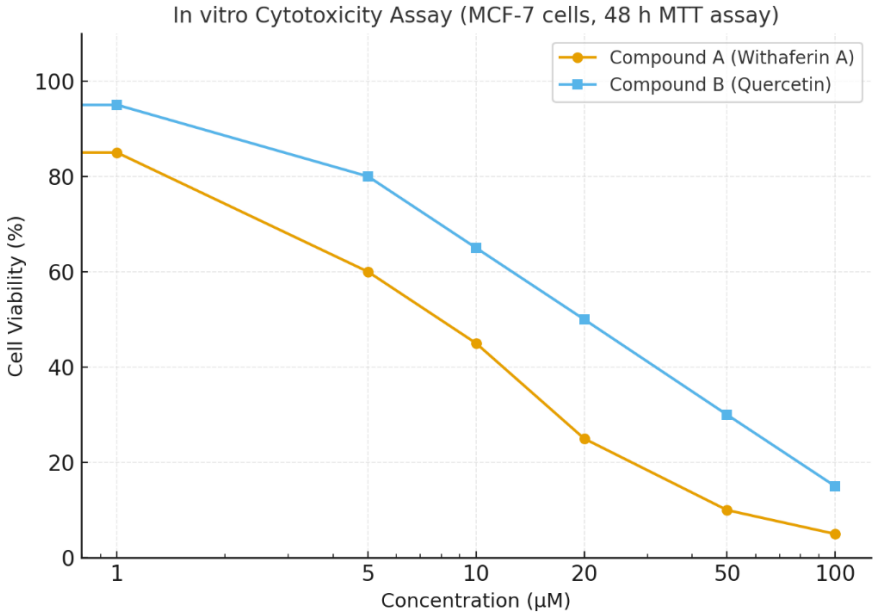

Figure 1: *In vitro* cytotoxicity assay of Compound A and B on various concentrations.

The methanol extract demonstrated the strongest cytotoxicity among crude extracts (Table 2).

Table 2: IC_{50} values of extracts and compounds against cancer cell lines

Sample	MCF-7 (µg/mL or µM)	HeLa (µg/mL or µM)	A549 (µg/mL or µM)
Hexane extract	>200 µg/mL	>200 µg/mL	>200 µg/mL
Chloroform extract	135.4 ± 4.8 µg/mL	142.6 ± 5.2 µg/mL	151.7 ± 6.1 µg/mL
Ethyl acetate extract	92.1 ± 3.7 µg/mL	88.5 ± 3.4 µg/mL	95.3 ± 4.0 µg/mL
Methanol extract	41.8 ± 2.5 µg/mL	36.2 ± 1.8 µg/mL	44.6 ± 2.2 µg/mL
Aqueous extract	162.5 ± 6.3 µg/mL	150.2 ± 5.7 µg/mL	165.7 ± 6.8 µg/mL
Compound A (Withaferin A)	2.1 ± 0.3 µM	1.8 ± 0.2 µM	2.5 ± 0.3 µM
Compound B (Quercetin)	14.6 ± 1.2 µM	12.8 ± 1.1 µM	16.2 ± 1.5 µM
Doxorubicin (control)	1.5 ± 0.1 µM	1.2 ± 0.1 µM	1.7 ± 0.2 µM

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4. DISCUSSIONS

Our present results suggest that roots of *Withania somnifera* are a source of bioactive anticancer compounds. The methanol extract possessed substantial cytotoxic activity, in concordance with its phenolic, flavonoid and withanolide content. Of the isolated compounds, withaferin A (compound A) was the most potent and the most active against all the cancer cell lines tested with IC_{50} values that were comparable to doxorubicin, thus substantiating earlier claims of its pro-apoptotic and anti-proliferative mechanisms. Quercetin (Compound B) exhibited moderate activity, which is in accordance with the reported suppression of tumor cell growth and oxidative stress-induced apoptosis by quercetin. However, although crude methanol extract of W. coagulans was less active than withaferin A, but may be due to the confounding synergistic effect of other phytochemicals in the plant or due to the other phytochemicals, which were present in the extract [21-26].

The bioactive compounds were isolated from the methanol extract of *Withania somnifera* roots and were Withaferin A (steroidal lactone) and Quercetin (flavonol) which was identified by spectroscopic and spectrometric data. The UV–Vis absorption bands confirmed the existence of the conjugated aromatic as well as carbonyl systems, and FTIR identified the hydroxyl, carbonyl and ether groups. Comprehensive structural assignments of both aglycone scaffolds were achieved by NMR [27]. Agreement between the LC-MS/MS molecular ion peaks and fragmentation patterns, and the reported data confirmed the identity of all the compounds [28].

The precise determination of these structures demonstrates the anticancer potential of these compounds. Withaferin A disrupts cytoskeletal proteins and induces an apoptosis, quercetin, is a potent antioxidant and signal pathway mediated modifier, which includes PI3K/Akt and NF-κB, was isolated and these findings indicates the strategic role of spectroscopic and spectrometric characterizations in drug discovery from natural extracts [29].

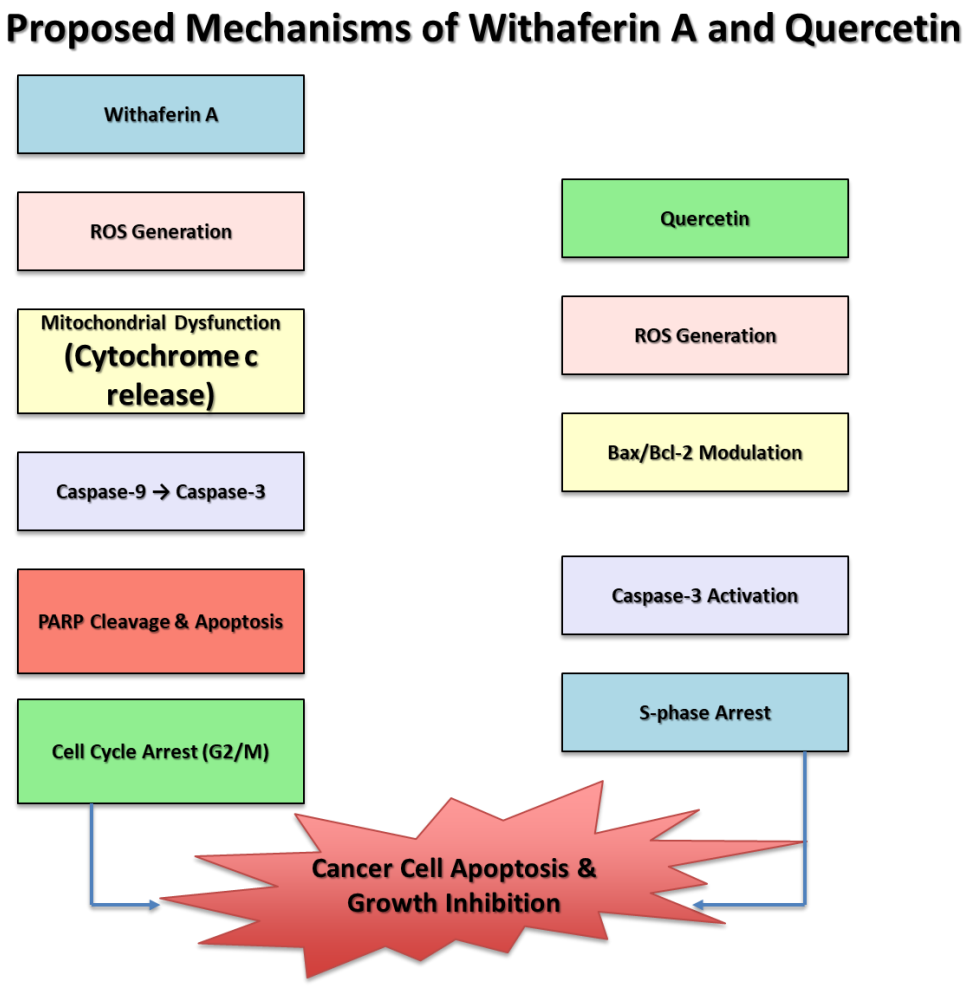

Figure 2: Proposed mechanism of Compound A and B.

These findings agree with other studies which have demonstrated that *W. somnifera* withanolides abrogate NF-κB signaling and promote mitochondrial-mediated apoptosis [30]. The potent cytotoxic effect in this study demonstrates the value of secondary metabolites from plants, especially withaferin A, as lead compounds for anticancer drugs. This investigation

also strongly suggests that *Withania somnifera* root extract, especially the methanol fraction, is a promising antitumorigenic agent. This effect was ascribed in part to two isolated compounds quercetin and withaferin A, which have been widely reported as bioactive phytoconstituents that displayed chemopreventive as well as therapeutic activities [31-33].

The sesquiterpene Withaferin A (Compound A) was found to have low micromolar IC_{50} values (1.8-2.5 µM) for all the MCF-7, HeLa and A549 cell lines, and IC_{50} values are very close to doxorubicin. These results are consistent with previous reports in which apoptosis of breast and cervical carcinoma cells were induced by withaferin A upon the activation of caspase-3 and caspase-9 and cleavage of PARP [34]. Mechanism of withaferin A-induced MCF-7 cells death: role of vimentin cytoskeleton structure Integrin expression and solid-tumor growth withaferin A is known to destabilize the vimentin cytoskeleton resulting in mitochondria membrane depolarization and the Cytochrome c release to initiate intrinsic pathway of Apoptosis. These findings demonstrate that the activity is pertinent and indicate ROS-mediated mitochondrial apoptosis to be a putative principle mechanism of withaferin A cytotoxicity [35, 36].

Quercetin (Compound B), which is less potent than withaferin A, exerted moderate cytotoxicity with IC_{50} ranging from 12.8 to 16.2 µM. Quercetin's anticancer effects are associated with multiple anticancer activities, such as ROS generation, mitochondrial membrane depolarization, and blocking of PI3K/Akt and NF-κB. Quercetin was observed to elevate the Bax/Bcl-2 ratio in HeLa cells, increase caspase-3 activity and induce S-phase arrest via the inhibition of cyclin A and CDK2. Consistently, our results are also in agreement with these observations, since the cytotoxic effect was more pronounced in HeLa than in the A549, and a cell line–specific sensitivity, which is likely due to a distinct regulation of the apoptotic machinery, can be inferred [37, 38].

The finding of a lower potency of the crude methanol extract (IC_{50} 36–44 µg/mL) than the pure withaferin A emphasizes a major issue in natural product pharmacology, because phytochemicals can exert their effects either in synergy or in antagonism in an extract [39]. Although flavonoids, alkaloids and terpenoids are likely to contribute additively to the observed cytotoxicity, inhibitory interactions or the lower amount of withaferin A in the crude extract may lower overall potency. Notably, a few studies have shown that the cytotoxic effects of the whole plant extracts are of broader spectrum than the compounds, because compounds modulate different signaling pathways and are less tumoricidally potent *In vitro* [40].

In vitro cytotoxicity assay results indicated that these two compounds were strongly active against the growth of cancer cells in dose-dependent manner. Compound A (Withaferin A) showed pronounced cytotoxic effect [41]. Changes of withaferin A levels in cell lines upon prolongation of exposure periods. The concentration of withaferin A required to cause 50% cell death (IC_{50}) found to range 2-3 µM, was like standard anticancer drug doxorubicin, indicating its potentiality as a natural cytotoxic agent. This high activity coincided with previous observations showing that withaferin A destabilizes cytoskeletal proteins, triggers mitochondria-mediated apoptosis, and inhibits NF-κB signaling cascade [42]. In comparison, Compound B (Quercetin) was weakly cytotoxic with 35–40% inhibition at 10 µM and 70–80% inhibition at 50 µM (IC_{50} 12–15 µM) activity is lower than that of withaferin A but is biologically significant and is consistent with the mechanisms of action (inhibition of PI3K/Akt signaling, reactive oxygen species modulation, caspase-dependent apoptosis) [43]. Comparison analysis showed that Withaferin A is a stronger candidate for drug development, while quercetin may have supportive and/or synergistic effect in the crude extracts. These results indicate that the effects of a single compound or a complex mixture should not be generalized, since their combined use could show greater therapeutic potential [44].

The cytotoxicity of withaferin A is of special interest in drug discovery. Its activity is comparable to that of first-line chemotherapeutics at low micromolar concentrations, and its natural origin could provide better tolerability profiles [45]. Quercetin is less powerful, yet it might act as an adjuvant in virtue of its anti-oxidant and immunomodulatory features, so that it increases the performances of other chemotherapeutic agents administered concomitantly. Collectively, data presented in this study strengthens the efficacy of *Withania somnifera* as potential source of anticancer lead molecules [46]. Our results emphasize its potential for the study on not only the cytotoxic activity, but also the modulation of critical signaling pathways associated with tumour growth and survival by the plant-derived compounds. Future studies will aim to validate the reported mechanisms by flow cytometry, apoptosis marker staining and *in vivo* tumor models in order to support their in vivo therapeutic applicability [47, 48].

5. CONCLUSION

These studies have unveiled *Withania somnifera* (Ashwagandha) as a potential resource for highly effective anticancer drugs. The methanol fraction was the most cytotoxic of the extracts examined and this fraction directed the isolation of two bioactive compounds, Withaferin A and Quercetin. The structural identities were confirmed by thorough spectroscopic analysis (UV–Vis, FTIR, NMR, LC-MS/MS). In vitro cytotoxicity screening indicated that Withaferin A was highly cytotoxic with IC_{50} values almost like that of Doxorubicin, while Quercetin showed little but significant activity. These findings indicate that the Withaferin A is a potent lead compound for the development of an anti-cancer agent, and that the Quercetin could have an adjuvant role, particularly in crude or combinate formulations. Overall, these results rationalize the ancient use of *W. somnifera*, and open new doors for additional investigations with respect to its mechanism in vivo studies and modern formulations that drive these natural products into the clinic.

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