



## REVIEW ARTICLE

# A critical review of anticancer properties of *Withania somnifera* (L.) Dunal with respect to the biochemical mechanisms of its phytochemical constituents

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## ABSTRACT

Cancer is a leading cause of mortality worldwide, the conventional chemotherapeutic drugs have been known for their toxicity and numerous side effects. A new approach to treat cancer involves phytochemical drugs. In the present review, anti-cancer activity of a class of steroidal lactones called withanolides obtained from *Withania somnifera* (L.) Dunal is discussed. The commonly studied bioactive compounds namely withaferin-A, withanoside IV, withanoside VI and withanolide-A among others obtained from methanolic and chloroform extract of the leaves and various alcoholic, aqueous and chloroform extract of roots have shown inhibition to various human cancer cell lines including skin, breast, colon, prostate, liver, ovary, cervical and lung. Prominent mechanisms of action include induction of apoptosis by NOS upregulation, ROS production and NBS2 or COX-2 inhibition; cytotoxicity by humoral and cell mediated immune response, activation of p53 and pRB and inhibition of various viral oncoproteins; cell cycle arrest by Cdc2 facilitated mitotic catastrophe, cyclin-D1 down-regulation and inhibition of transcription factors. Cancers are also controlled by inhibition of angiogenesis and metastasis of the tumor cells. In addition to anti-tumorigenic properties, *W. somnifera* also holds properties that make it a potential adjuvant in integrated cancer therapeutics and in enhancing the effectiveness of ongoing radiation therapy.

## Introduction

One of the most common forms of medication involves phytotherapy; plants show a vast molecular diversity and produce numerous therapeutic phytochemicals. Over 60% of the people across the world rely on phytochemicals to cure various diseases. Moreover, currently, extensive studies are being conducted to develop and screen new drugs in phytotherapy (1). It is essential to evaluate and screen more such phytochemicals for pharmaceutical purposes (2). Most of these phytotherapeutic drugs are made out of phytochemicals mainly comprising lactones and alkaloids obtained from root, stem, leaves and other parts of a plant (3).

In fact, various herbal plants were reported to be historically used as prophylactic agents for treatment of cancer in India, Greece, China and Egypt (4). Indian winter cherry is also known as Indian ginseng due to resemblance in its usage with Chinese *Panax ginseng*. It is more commonly referred to as Ashwagandha (meaning “horse-smell”) because the smell of its roots resembles that of a horse (5). Its scientific name *Withania somnifera* (L.) Dunal originates from Latin, and it means “sleep-inducing” or sedative (3, 6).

The taxonomic position of *W. somnifera* is as follows:

Kingdom	– Plantae (Plants)
Subkingdom	– Tracheobionta (Vascular plants)

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Super division	– Spermatophyta (Seed plants)
Division	– Angiosperms
Class	– Dicotyledoneae
Order	– Solanales / Tubiflorae
Family	– Solanaceae
Genus	– <i>Withania</i>
Species	– <i>W. somnifera</i>

*W. somnifera* is described as a small, woody, erect and evergreen shrub of about 2 feet tall belonging to the Nightshade family. The roots of *W. somnifera* are whitish brown in color and stout fleshy in appearance. Leaves are simple, with ovate margin and glabrous surface; they are relatively smaller and opposite when closer to the flower. The flowers borne by *W. somnifera* are small, inconspicuous and greenish to lubrid yellow in color; the axillary flower shows umbellate cyme inflorescence. Its ripened fruits are globose, orange red berries enclosed within



Fig. 1. Morphology of *Withania somnifera* (L.) Dunal during fruiting stage.

persistent calyx (Fig. 1) and shows properties to coagulate milk. The seeds appear yellow and reniform.

The origin of *W. somnifera* can be traced back in the Asian countries like India, Pakistan, Afghanistan and other dry regions like South Africa. It grows in Africa, Mediterranean region and India. In India, it's found to grow in dry regions of Manasa, Neemuch, Jhawad, Hehsils of Mandsaur, districts of Madhya Pradesh, Punjab, Sindh and Rajasthan (6).

### **Pharmacological activity of *W. somnifera***

Various steroidal lactones obtained from root, stem and leaves of *W. somnifera* show therapeutic properties that affect the immune system, circulatory system, CNS etc (7). It is a well-known sedative, hypnotic/narcotic and has the ability to boost energy. Studies have shown that *W. somnifera* extracts have positive effects on overall health, especially during pregnancy and increase non-specific resistance against various physical, chemical and biological agents (8). It has been used to treat arthritis, venom toxins, muscle strain, fatigue, skin infections, rheumatoid joint inflammation, aches and rheumatism (9).

It has been prominently used as anti-ulcerogenic, anti-diabetic, anti-Parkinson's, anti-fungal, anti-malarial, anti-hypertension, anti-inflammatory, anti-microbial, anti-stress, anti-ageing, anti-fatigue, analgesic, immunomodulatory, hepatoprotective, free radical scavenging, detoxifying, neurogenerative, chondroprotective and cardio-protective agent. It also acts as an anti-depressant, anti-coagulant and anti-oxidant and is a well-known rejuvenator (9).

### **Phytochemistry and bioactive compounds obtained from *W. somnifera***

Major active compounds among the *W. somnifera* phytochemicals are over 20 alkaloids including anaferin, cuscohygrine, anahygrin and steroidal lactones such as withanolides, withaferin, withanamides, withanosides (Fig. 2), withanolide glycosides, liganamides and steroidal saponins (10-13). Withanolides are a family of structurally complex steroidal lactones which act as potential drugs and aid in various biological and physiological activities (14). They are characterized by an ergostane skeleton whose C-22 and C-26 are oxidized to form  $\delta$  lactones with 2-ene-1-one in ring A of the steroid (15). They are given the name 22-Hydroxy ergostane-26-oic acid 26,22-d-lactone. Steroidal alkaloids and steroidal lactones (withanolides) are major constituents of alcohol extract of *W. somnifera* (16). Withanolide biosynthesis shows high levels of tissue specificity. The highest abundance of withaferin-A is found to be in leaf tissue followed by the bark, stem and roots. Conversely, withanolide-A is abundantly biosynthesized in roots followed by other tissues. Therefore in general both root and leaf tissues are considered to study the biosynthesis of withanolides (16).

There are over 40 known withanolides of which 12 major withanolides obtained from *W. somnifera* are withaferin A, sitoindosidic, 25-hydroxy-27-desoxywithaferin-A, 2-3 dihydrowithaferin, Physagulin-D, PhysagulinD(1->6)- $\beta$ -D-glucopyranosyl-(1->4)- $\beta$ -D-glucopyranoside, 27-O- $\beta$ -D-glucopyranoylphysagulin D, withanoside IV, 27-O- $\beta$ -D-glucopyranosylviscosalactone B, 4,16-dihydroxy-5 $\beta$ ,6 $\beta$ -epoxyphysagulin D, viscosalactone B, and diacetylwithaferin A. Out of these the most studied are withaferin A, withanolide A and withanone (17).

Other plants reported to produce withanolides are mostly from Solanaceae and rarely from

Taccaceae, Fabaceae and Lamiaceae. Within Solanaceae, species from *Withania* genus are the

cell under minimal adversities. Most of the chemotherapeutic agents target various unique

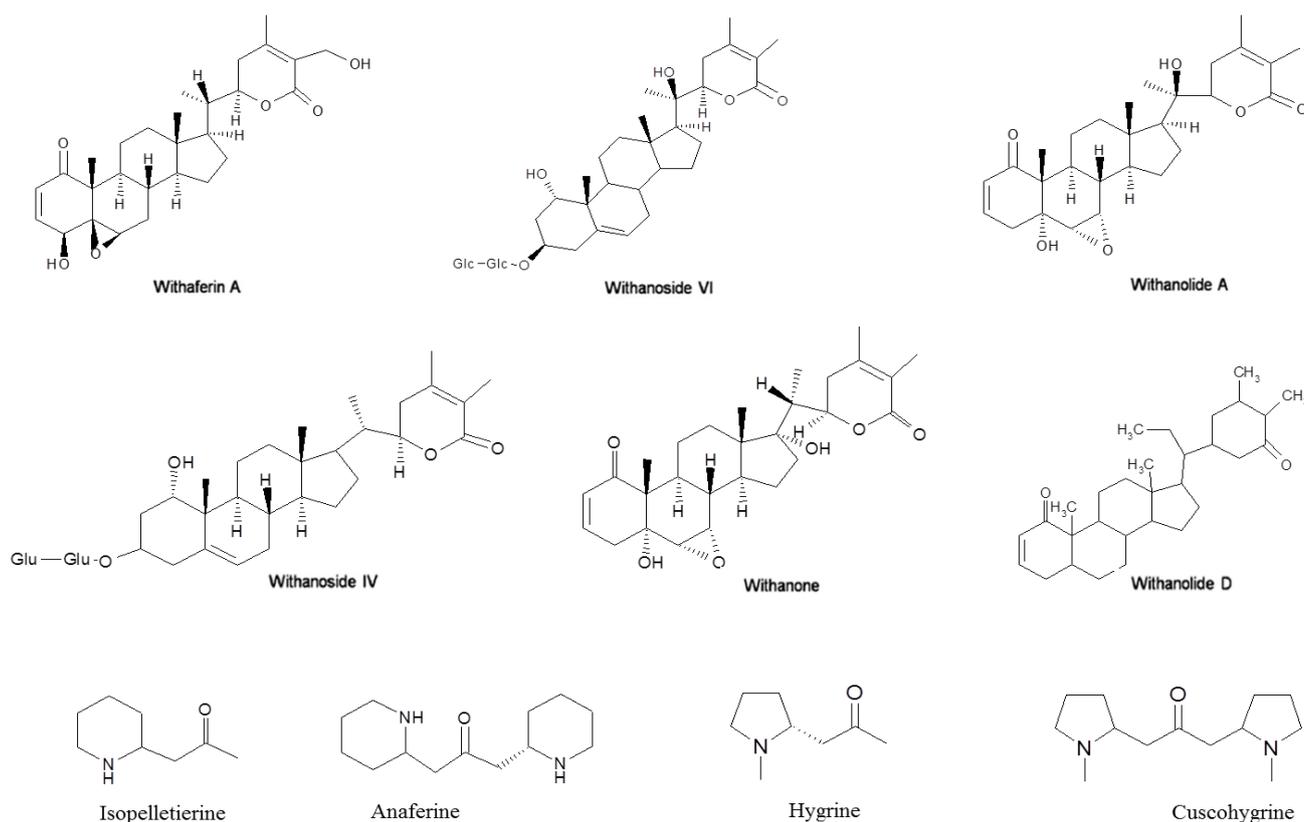


Fig. 2. Some important phytochemical constituents of *Withania somnifera* (L.) Dunal.

major producers, but 14 other reported withanolide producing genera are *Acnistus*, *Datura*, *Deprea*, *Dunalis*, *Iochroma*, *Jaborosa*, *Lycium*, *Nicandra*, *Physalis*, *Salpichroa*, *Tubocapsicum*, *Discopodium*, *Treconates* and *Witheringia* (18-21).

### Cancer and its treatment

Cancer is a group of heterogeneous hyper proliferative disorder that causes alterations in cellular signaling pathways leading to tumorigenesis due to uninhibited proliferation and deregulation of cell cycle and its apoptosis mechanisms (22). Various hallmarks of cancer are transformation, apoptotic dysregulation, proliferation, invasion, metastasis, angiogenesis, replicative immortality and tumor promoting inflammations (23). Self-sufficiency in growth signaling, metastasis, alteration of cellular bioenergetics and invasion of immune detaching tissue, leading to alteration in apoptosis which causes apoptosis (24), progression of this tumor is due to gene instability and mutations (25). Across the world, cancer is a major factor contributing to increased death rates. Cancers that show maximum death rates are colorectal, stomach, breast, lung and prostate cancer (3). Only 5% of all cancers have hereditary causes; while the rest are due to either internal or external environmental factors (10).

Based on the common hallmarks of cancer, most treatment techniques selectively interfere with replication of tumor and regulate homeostasis of the

cancer hallmarks in their course of treatment (26). Common cellular pathways that are targeted include p53 signaling, GM-CSF signaling, death receptor signaling, apoptosis signaling pathways respectively and the G2-M DNA damage regulation pathway (27). Common therapeutic approaches to treat cancer are surgical removal of tumor, radiation therapy, chemotherapy and other specific targeted therapies. Biotransformation of chemotherapeutic drugs in the liver generates toxic and highly reactive metabolites (28). While targeted therapies are most efficient to treat benign tumors with least noted side effects, they are difficult to treat metastatic cancers. Various plants are known to be direct sources to major chemotherapeutic drugs against cancer worldwide. *Catharanthus roseus* is a source for vinblastine and vincristine, *Podophyllum spp.* are source for podophyllotoxin and its derivatives etoposide and teniposide, *Taxus brevifolia* is a source for paclitaxel (29). While many plants are an indirect source to major chemotherapeutic drugs, vinorelbine and doctaxel are semi synthetic derivative of *Taxus baccata*; irinotecan and topotecan are semi synthetic derivatives of *Camptotheca acuminata* (30-32).

### Major active compounds in anti-cancer activity

Studies show that withanolides obtained from roots and leaves of *W. somnifera* are cytotoxic to tumor cells (33, 34) and possess immunomodulatory and neuroprotective properties (1, 3, 35-41). In fact, most withanolides inhibit various hallmarks of cancer and

are anti-proliferative, anti-metastatic, anti-angiogenic, anti-invasive and pro-apoptotic (42). Most of these phytochemicals are classified under chemotype III (43, 44). Withanolides are also biosynthesized in other *Withania* spp. such as *W. aduensis*, *W. riebeckii* and *W. coagulans* (42); as well as root and leaf extracts of non *Withania* spp. such as *Tubocapsicum anomalum*. However, only 2 species that show economic and medicinal significance in terms of amount of withanolide synthesized, its composition, ease of extraction and desired bioactivity are *W. somnifera* and *W. coagulans* (41).

A study identified inhibitory concentration in  $\mu\text{g/ml}$  of a mixture of withanolide-A for 50% reduction of lung, CNS, breast and colon cancer cells as  $0.24 \pm 0.01$  to  $11.6 \pm 1.9$  respectively for withaferin A and its derivatives, and  $0.32 \pm 0.05$  to  $0.47 \pm 0.15$  for visosalactone B and  $7.9 \pm 2.9$  to  $17.3 \pm 3.9$  for 27-O-glucoside derivative. No inhibition was seen in Physagulin D (45).

The major known anti tumorigenic active compound is a withanolide called withaferin-A (46). Studies have demonstrated various mechanisms of anti-tumor activity by withaferin-A involving activation of p53 and ROS signaling (7, 34, 47), increasing intracellular antioxidant levels (48), induction of apoptosis (49-52), inhibition of activation of NF-KappaB (53), inhibition of notch signaling (54, 55), cytoskeletal architecture alteration, angiogenesis inhibition (56), down regulation of cyclin B1, cyclin A, cdk2, expression of p-cdc2 and increase in cellular levels of p-chk1 and p-chk2, all leading to cell cycle arrest. Other mechanisms include down regulation of oncoproteins, p53 induction and accumulation, increase in cellular levels of P-21, decrease in cellular levels of STAT 3, increase in levels of p53 mediated apoptotic markers such as Bcl 2, BAX, caspase 3, cleaved PARP and PAR 4, down-regulation of AKT and EMT signaling, disruption of certain cytoskeletal elements such as actin, vimentin and intermediate filaments (57-60), inhibition of cell proliferation and alteration of cell differentiation (61), cell cycle arrest (rarely at S phase and mostly at G2/M phase), DNA degradation which is a hall mark of apoptosis, inhibition of angiogenesis and metastasis (62, 63). Inhibition of external onco-proteins synthesis is also seen in case of oncoproteins E6 and E7 of human papilloma virus predominantly causing cervical cancer (64).

### **Mechanism of action against cancer**

A recent study demonstrates the detailed mechanism of action of withaferin-A in prostate cancer cell lines of PC-3 and DU-145. Withaferin-A arrests the cell cycle of these tumorigenic cell lines at G2/M phase. The mechanism for this arrest involves upregulation of phosphorylated wee 1, phosphorylated histone H3, P21 and aurora B, with a simultaneous down regulation of cyclin A2, B1 and E2, and phosphorylated cdc 2 Tyr (15). As a result, cellular levels of phosphorylated chk 1 (Ser 345) and chk 2 (Thr 68) decrease. This leads to activation of cdc 2, which in turn facilitates mitotic catastrophe in abnormally duplicated cells by arresting its cycle at M phase, ultimately causing death of the cell.

General studies with various *W. somnifera* extracts have reported their ability to inhibit enzymes essential for the cell's TCA cycle such as malate dehydrogenase and isocitrate dehydrogenase in test animals with induced colon cancer (65). Myeloid cells are known to regulate tumor progression. Myeloid derived suppressor cells (MDSC) and tumor associated macrophages (TAM) are two of the most common tumor regulating myeloid cells. The growth of tumor is promoted by these MDSCs as they exhibit inherent immunosuppressive abilities. MDSCs suppress tumor specific T lymphocytes by releasing T cell suppressive reactive oxygen species (ROS), thereby causing immunosuppression against cancer (66). They also convert macrophages into a tumor enhancing phenotype (67-70). A study demonstrates the *in vitro* ability of withaferin-A to reduce the secretion of interleukin (IL) 6, TNP and  $\alpha$ -cytokines, all of which otherwise increase the concentrations of MDSC. As a result, withaferin-A successfully reduces/inhibits the formation of these reactive oxygen species by STAT 3 dependent mechanism. Hence, a tumor rejecting phenotype is achieved by reduction of myeloid cell immune suppression by withaferin-A. In fact, a common approach to immunotherapy of cancer involves the antitumor immune response activation. In most cancer patients, withaferin-A blocks MDSC and inhibits metastasis of tumor, (71, 72); it also enhances the activation of tumor reactive cytotoxic T lymphocytes (73).

A study on human leukemia cell line showed that mitochondrial dependent and mitochondrial independent apoptosis was induced by withaferin-A, thus leading to dysfunction in the cell and subsequent restriction in growth of the tumor (74). A crude extract of *W. somnifera* has the ability to induce the hall mark of apoptosis in a cancer cell line by causing its DNA fragmentation (75). Another known mechanism of cancer proliferation is the activity of telomerase; non-cancerous cells' life span is regulated by telomere shortening. Tumor cells overcome this mortality by activation of telomerase which adds a sequence of TTAGGG at telomeric ends thereby allowing the cells to proliferate indefinitely (76). Even cancerous cells that don't contain telomere have shown alternative methods to increase the telomere length. Glioblastoma multiforme, gastric carcinoma, neuroblastoma, liposarcoma, epithelioid sarcoma, chondrosarcoma, astrocytoma, malignant fibers and histiocytoma are some of the common tumors that have high prevalence of these alternate telomere lengthening mechanisms (26, 77, 78). In the absence of telomere lengthening, replicative mortality is achieved by DNA damage signals which lead to apoptosis. Hence, telomerase inhibition is a potential cancer therapy.

Withaferin-A inhibits telomerase activity and the subsequent upregulation of DNA damage response due to telomere dysfunction. Studies have also shown that the activity of withaferin-A against alternative mechanisms of telomere lengthening is even more efficient. Withaferin-A causes transcriptional suppression of an MRN complex protein NBS-1 mediated by myc-mad. NBS-1 is an essential component of alternative telomere lengthening (79).

Conversion of the cellular lipid arachidonic acid to prostaglandins which causes inflammatory response is carried out by enzymes cyclooxygenase (COX) 1 and 2. An inflamed or cancerous cell has high expression of COX 2 (80-82). An effective chemopreventive approach for cancer is to selectively inhibit COX 2, which in turn inhibits COX 1 activity. This selective inhibition of COX 2 is more effective than the conventional non-selective-non-steroidal-anti-inflammatory drugs (83). Studies have shown that *W. somnifera* leaf extract possesses excellent ability to selectively inhibit COX 2 (84).

The active compound triethylene glycol present in the aqueous extract of *W. somnifera* leaves can activate tumor suppressor genes like p53 and pRB. In a normal cell, there is an increased degree of phosphorylation of pRB gene which increases the cellular concentration of cyclin B1; the latter in turn decreases the concentration of cyclin D1. This facilitates normal progression of the cell cycle. In a cancerous cell, pRB is hypophosphorylated which decreases the cellular concentration of cyclin B1, thereby increasing the concentration of cyclin D1; this arrests the cell cycle and cell growth. Triethylene glycol also downregulates MMP 3 and MMP 9, which are metastasis regulators in cancerous cells, while normal cells remain unaffected (9).

#### **Anti-tumorogenic activity of various extracts**

##### **Leaf extract**

Studies on mice assay have shown that the methanolic leaf extract of *W. somnifera* containing withanolide-A, withanoside IV, withaferin-A and withanoside VI is non-toxic and anti-tumorogenic predominantly on neuroblastoma. The cancer inhibition factor of the leaf extract affects apoptosis signaling, death receptor signaling, G2-M phase DNA damage regulation pathway, GM-CFS signaling and most commonly p53 signaling. The methanolic and chloroform leaf extract containing withaferin-A causes nuclear translocation of p53. It also activates p53 pathway and disrupts cell cycle in tumor cells. Some studies have even claimed that p53 induced cell cycle disruption can only be carried out by the leaf extract and not the root extract of *W. somnifera* (7, 85). A study on withaferin-A of the chloroform leaf extract reported that it arrests cell cycle of human osteogenic sarcoma cell lines at G2, G1 and S phases with varying cell fractions. Along with this cancer inhibition factor, the hydroalcoholic leaf extract also contains polyphenolic compounds that are known to control various cancer cell lines of the breast, ovary and lung cancers including MCF-7, A549 and PA-1 (86). The aqueous extract of *W. somnifera* leaves activate p53, which in turn increase the concentration of cyclin B1 (9). Another mechanism of anti-cancer activity of the leaf extract is its ability to selectively inhibit COX 2 enzyme activity in tumor cells.

##### **Root extract**

*W. somnifera* chloroform root extracts have shown to contain 1-oxo-5- $\beta$ ,6- $\beta$ -epoxy witha-2-enolide and 5,6-de-Epoxy-5-en-7-one-17-hydroxy withaferin A. Studies have shown that this active compound down

regulates tumor in skin carcinoma cell line due to UV exposure in rats; other reports have shown activity against liver, colon, prostate and breast cancer as well (87). The alcoholic root extract has also shown the ability to inhibit various transcriptional factors such as NF-Kappa B and AP 1 (88). It can also induce mitochondria-mediated cytochrome C release to activate caspases for apoptosis of cancer cells (89). Studies have also shown the ability of ethanolic, chloroform and aqueous root extract to upregulate Th1 dominant polarization which causes humoral and cell mediated immune response (39), down regulate P34cdc2 expression (90) and bring about nitric oxide synthase induced protein expression, NOS also produces nitric oxide which in turn causes DNA damage and mutation by depurination and AT-GC transition (91).

##### **Fruit**

The fruits of *W. somnifera* contain L-asparaginase which is known to inhibit the growth of lymphoblastic leukemia (92). A study involving a crude aqueous extract of the fruit assisted by potassium chloride and ammonium sulphate from which L-asparaginase was isolated by various chromatographic columns and electrophoretic techniques was found to be cytotoxic to human lymphoblastic leukemia cell culture at various inhibitory concentrations (93).

#### **Anti-tumorogenic activity to specific cancer cell lines**

##### **Hepatocellular carcinoma**

Hepatocellular carcinoma is the sixth most common form of cancer and the second largest cause of cancer related mortality in East Asia and Sub-Saharan Africa (93), and hence its control and treatment requires great attention (94, 95). Over 80% of hepatocellular carcinoma cases are seen to occur in East Asia and sub-Saharan Africa (96). Hepatitis C virus causes cirrhosis which is a main cause of hepatocellular carcinoma. The major risk factors of human hepatocellular carcinoma include its late symptom presentation, aggressive proliferation and poor sensitivity to conventional medication, along with multiple side effects (97, 98). Common treatment strategies of hepatocellular carcinoma include radiofrequency ablation, liver transplantation, transarterial chemoembolization, radioembolization, curative resection and systemic targeted agents (99); but toxicity, pain, fatigue, anemia, emotional distress are some of the reported side effects of these treatment plans. The above mentioned treatments are also expensive (100). Hence, various herbal plants are used as complementary medicine to reduce the tumor without pain (101). Studies have shown that the use of methanolic and aqueous leaf extract of *W. somnifera* inhibits hepatocellular carcinoma by induction of apoptosis and anti-oxidant activities; it also employs other mechanisms for inhibition of cell proliferation and alteration in cell differentiation (61). The mechanism to restrict tumor proliferation in hepatocellular carcinoma involves withaferin-A mediated upregulation of Bim, t-Bid and caspase 8 (102), all of which cause inhibition of TNF  $\alpha$ ; this in turn causes down regulation of cytokines and other

proteins involved in systematic inflammation, and hence the inhibition of tumor (56). The aqueous leaf extract also acts against Hep G2 cell line of hepatocellular carcinoma by shrinking and decreasing the viability of the tumor (103).

### Skin cancer

Melanoma is the most aggressive form of cancer causing over 75% deaths due to cancer across the world. Common chemotherapeutic drugs consumed against melanoma are not target specific and have shown various side effects. Many studies on *W. somnifera* alcoholic (mostly ethanolic and methanolic) root extract have proven it as a target specific treatment for melanoma with no side effects. However, most of these studies have used melanoma induced mouse as a target and very few are conducted on human melanoma cell lines. It is also seen that the efficacy of this root extract against melanoma cells depends on dosage and treatment/incubation time (8). Dimethyl benzanthracene present in *W. somnifera* is known to enhance the activity of antioxidant enzymes like glutathione peroxidase and catalase, which in turn reduce skin carcinogenesis (48).

### Breast cancer

Invasive breast cancer is one of the highest causes of mortality due to cancer. There have been over 232,340 cases of invasive breast cancer and 64,640 cases of non-invasive breast cancer of all breast cancer cases in USA women in 2013. It has also been predicted that African American women are more likely to be affected by invasive breast cancer than other American women. Tumor metastasis in lymph nodes, liver and lungs is the main cause of high mortality in invasive breast cancer (104). 10 to 20% of all breast cancer cases are triple negative and invasive; it is resistant to most conventional chemotherapeutic drugs and radiation therapy. It is also difficult to treat due to its high metastasis and aggressive proliferation. *W. somnifera* extracts can treat this form of breast cancer effectively (105, 106). A study conducted on nude mouse xenografts to treat induced breast cancer of MDA-MB-231 cell line, showed that *W. somnifera* was effective in the treatment. It is also known to induce metastasis in MCF 7 and MDA 231 cell lines of human breast cancer by inhibiting Notch signaling cascades. Withaferin-A also reduced metastasis in triple negative invasive breast cancer (26).

### Prostate cancer

Various studies and surveys have claimed that prostate cancer is the third most common cause of cancer related deaths worldwide and second most common cause of cancer death in United States (107). A common therapeutic approach to reduce the proliferation of prostate cancer is to downregulate androgen synthesis. Hence hormone ablation therapy is the primary treatment for prostate cancer (108). Many cases of castration resistant prostate cancers are now emerging to which primary treatment is ineffective (109). *W. somnifera* extracts causes downregulation of proinflammatory cytokines, which in turn facilitates the upregulation of P38MAPK,

Caspase 6, Cyclin D1 and p13k, all of which inhibit the growth of tumor in prostate cancer. Another mechanism specifically to prostate cancers of PC-3 and DU-145 cell lines is the ability of withaferin-A to regulate G2/M phase of the cell cycle and stop the tumorous cell growth (64).

### Lung cancer

Lung cancer is one of the leading causes of mortality due to cancer worldwide with about 1 million deaths per year (110). Conventional chemotherapeutic drugs are often prescribed in any of these 3 combinations, vinblastine and cisplatin along with mitomycin; cyclophosphamide, doxorubicin and methotrexate, procarbazine (111); or vincristine with cisplatin and etoposide (112) or paclitaxel. Studies have shown that withanolides suppress NF-KB and its regulated gene products, which ultimately lead to anticancer activity towards lung cancer cell line NCL-H460. Withaferin-A, a chlorinated steroidal lactone 27-acetoxy-4 $\beta$ , 6 $\alpha$ -dihydroxy-5 $\beta$ -chloro-1-oxowitha-2,24-dienolide and a diepoxywithanolide-5 $\beta$ , 6 $\beta$ , 14 $\alpha$ , 15 $\alpha$ , diepoxy 4 $\beta$ , 27-dihydroxy-1-oxowith-2,24-dieolide, extracted from the aerial parts of *W. somnifera* have shown growth inhibition towards lung cancer. Withaferin-A is the most cytotoxic one of these three withanolides (113).

### Colon and Colorectal cancer

Colorectal cancer is common worldwide (114), and the fourth leading cause of deaths due to cancer across the world (115). A common treatment approach is chemotherapy, but this mode of treatment is not effective due to drug resistance in tumor cells, as well as high toxicity of these chemotherapeutic drugs towards gastrointestinal tract, skin and bone marrow. STAT 3 is crucial for proliferation of colorectal cancer (116). A study conducted on xenograft of nude mouse for colon and colorectal cancer cell line HCT116 showed that the activation of STAT 3 signaling causes progression of cell cycle, metastasis and angiogenesis, thereby increasing proliferation in the cell culture (117). STAT 3 inhibitions physiologically or chemotherapeutically can cause prevention of colon cancer and promote its apoptosis (118-120). Hence, a therapeutic alternative is to directly inactivate STAT 3 (121). Withaferin-A is seen to block interleukin 6 (IL 6), which induces STAT 3 activation, and thus cease the progression of colon and colorectal cancer tumor proliferation. Other mechanisms by which *W. somnifera* inhibits colon and colorectal cancer proliferation include the generation of cytotoxic reactive oxygen species, inhibition of proteasome, p53 stabilization, endoplasmic reticulum stress induction, activation of p38 MAPk, Akt phosphorylation inhibition, inhibition of notch signaling cascades (122-124).

### Cervical cancer

Various studies have shown that a major cause of cervical cancer is human papilloma virus (HPV) infestation leading to over 70% of total cervical cancer cases worldwide. HPV 16, 18, 31 and 33 are mainly the causative HPV for cervical cancer (125). Tumor suppressor proteins, p53 and pRb which are essential for cell cycle regulation and protection of genome integrity (126) are inactivated by E6 and E7

viral oncoproteins (127). p53 and p103-Rb are marked by E6 and E7 oncoproteins, but the resultant mutation is reversible (127). Hence, inhibition of these viral oncoproteins can reactivate p53 and lead to apoptosis of cervical cancer cells (128-130). The cell cycle arrest is carried out by transcriptional activation of p21 cip1/waf1 which is a cyclin dependent kinase (131); this further activates Bax, a proapoptotic gene product (129, 130). A study conducted on CaSKi cell line of human cervical cancer has found withaferin-A to possess the ability to downregulate oncoproteins E6 and E7 expression by HPV. This induces accumulation of p53 in tumor cells and upregulation of p21 cip1/waf1, which in turn interact with proliferating cell nuclear antigen (PCNA), leading to B1, p34 cdc2 and PCNA levels-modulated-G2/M-cell-cycle-arrest. Another known mechanism of cervical cancer inhibition by withaferin-A includes phosphorylation of Tyr-705 and Ser-727 and STAT 3, all of which lead to altered expression of BCL 2, Bax, Caspase 2 and cleaved PARP, which are p53 mediated apoptosis markers.

### Ovarian cancer

Epithelial ovarian cancer is the fifth most leading cause of cancer deaths among American women (132, 133). Common therapeutic measures in ovarian cancer patients include cyto-reductive surgeries followed by chemotherapy using carboplatin by platinum taxane, combined with paclitaxel. Although in 70 to 80% of the cases, this treatment approach is initially effective, in over 70% of the cases, the recurrent cancer developed due to cellular platinum resistance leads to tumor relapse (134). Studies have shown that this chemo-resistance is due to cancer stem cells (135) which are one of the major causes for chemo resistance, tumor progression and relapse of the cancer, post initial treatment (136, 137). The most common chemotherapeutic drug cisplatin also introduces various side effects including nausea, vomiting, neurotoxicity, severe toxicity,

DNA adducts that lead to tumor suppression. A study on A2780 cell line of human orthotopic ovarian cancer tested on mice has shown a reduction of tumor upto 70-80 % and total inhibition of metastasis on treatment with withaferin-A. Withaferin-A was also found to target cancer stem cell for enhanced anti tumorigenic activity with or without the combination of cisplatin. It can reduce the dosage requirement and time dependence of cisplatin by generating reactive oxygen species that lead to DNA damage, thereby reducing the tumor proliferation (141, 142). Hence, a combined chemotherapeutic treatment with cisplatin as well as withaferin-A can enhance DNA damage.

Other mechanisms of action of withaferin-A against ovarian cancer include inactivation of Akt and NF-kB leading to apoptosis, induction of Par 4, activation of caspase 3 and caspase 9 which leads to DNA damage, inhibition of HSP 90, inhibition of FOXO 3a and Bim, inhibition of Notch 1 and downregulation of HPV E6 and E7 oncoprotein expression.

### *W. somnifera* as an adjuvant during radiation and chemotherapy

*W. somnifera* has shown the ability to proliferate or normalize stem cells. A study on 75% methanolic *W. somnifera* extract revealed its ability to increase bone marrow cellularity, reduce  $\gamma$  radiation induced leucopenia, increase leukocyte count and normalize normo-chromatic erythrocytes and poly-chromatic erythrocytes ratio. *W. somnifera* can control the proliferation of tumor and hence enhance the effectiveness of radiation therapy and decrease its side effects (85).

### Clinical studies on anti-cancer properties of *W. somnifera*

There are very limited pre-clinical and clinical trials conducted on herbal extracts for their claimed medicinal properties, a major reason for this is the

Table 1. Effective dosages and cancer cell line of various withanolides used in recent clinical studies

Active compound	Dosage	Route of administration and other variables of the study/ host organism model	Cancer type/cell line	References
Various Withanolides	5 $\mu$ M-Withaferin A; 20 $\mu$ M-4 $\beta$ -hydroxywithanolide; 20 $\mu$ M-anomanolide A	Administered for 48 hours	MDA-MB-231, MCF-7 (Breast cancer cell lines)	(154)
	0.24 $\pm$ 0.01 to 11.6 $\pm$ 1.9 Ag/mL-Withaferin A; 0.32 $\pm$ 0.05 to 0.47 $\pm$ 0.15 Ag/mL- Viscosalactone B	-	MCF-7 (Breast cancer), NCI-H460 (Lung cancer), HCT-116 (Colon cancer), SF-268 (CNS cancer cell line)	(45)
Withaferin A	7 $\mu$ M, 14 $\mu$ M and 28 $\mu$ M	-	SCC-4 (Oral cancer cell line)	(155)
	0.7%, 5.8%, 12.4% and 22.66%	Cispatin resistant cancer cell line used	SCC-4 (Oral cancer cell line)	(155)
	4mg/kg body weight	Female nude mice	Estrogen dependent MCF-7, Estrogen independent MDA-MB-231 (Breast cancer cell line)	(51)
	6mg/kg body weight	-	MiaPaCa2, BxPc3 (Pancreatic cancer cell lines)	(156)

nephrotoxicity, hepatotoxicity and myelo-suppression (138-140). The mechanism of action of cisplatin involves its binding to the tumor cell DNA forming

loopholes in guidelines that either exempts herbal medicines from clinical trials or lacks strict bylaws (143). For the same reason, clinical studies are also

very limited with respect to the anticancer aspects of *W. somnifera* and its phytochemical constituents (Table 1).

One of the earliest preclinical trials for anticancer property of withaferin-A was conducted on golden Syrian hamsters with oral cancer induced by DMBA. Withaferin-A through oral intake of 20 mg per kg of the host body mass for duration of 14 weeks fully cured the cancer (144). A follow up study was conducted revealing a circadian pattern of withaferin-A activity against cancer. It was found that withaferin-A showed better anticancer property on the host from 8 am to 12 pm with 100% protection from tumor formation, whereas when the host was treated at 12 am, it only served 50% protection from tumor formation. The diurnal nature of antioxidant enzymes activity and lipid peroxidation in the host organism. Studies also showed that treatment with DMBA and withaferin-A is more effective in reduction of protein expression of p53 and Bcl2 than treatment with DMBA alone (145).

Another study was conducted on mammary carcinogenesis developing in mmtvneu mice, which was reduced by 33% with a treatment of 750 mg of *W. somnifera* root extract per kg for a period of 10 months (146). An extended study used the same model but the dosage was changed to 100 µg per mouse of withaferin-A alone, 3 times a week for 28 weeks and similar results were obtained with lower metastasis of the lung and lower macroscopic tumor weights (147). It was concluded that withaferin-A inhibits self-renewal of breast cancer stem cells by altering its intermediary metabolism by reducing proteins involved in TCA and glycolysis (148). The tumor stem cell machinery is also obstructed by lowering ALDH1 activity. Further studies on mammary cancer xenograft reveals that withaferin-A has the ability to downregulate the cell proliferation marker ki-67, surviving, XIAP and upregulate TUNEL positive apoptotic cell and increase the protein expression of pERK, pRSK, CHOP and DRS (149).

A clinical study on DBA/2 female mice with skin carcinogenesis initiated by 2 weeks of DMBA application along with exposure to tumor promoter 12-o-tetradecanoyl phorbol-13-acetate was given a 20 µg dose of withaferin-A once a day, 5 times a week for 14 weeks. Withaferin-A showed 100% efficacy of tumor prevention by blocking the upregulation of acetyl CoA carboxylase induced by the carcinogen. In male nude mice it was observed that 4-8 µg of withaferin-A per kg body weight daily for 28 days inhibited the tumor growth factor PC-3 and inhibited the activity of proteosomal chymotrypsin (123). Some studies injected withaferin-A into the host by patches as well, a patch delivering 4 mg withaferin-A per kg inhibited 60% of A549 lung cancer cases (150).

Clinical studies on human test subjects on this regard are very limited. One of the recent studies conducted in International Institute of Herbal Medicine (IIHM), Lucknow, on prostate cancer, dermatofibrosarcoma, breast cancer, fibroids of uterus and squamous cell carcinoma of the penis in last stages of the cancer without conventional drug treatment when modern treatment was refused by

the subjects, some of those were fully cured with *W. somnifera* extract and radiological intervention alone (151). While side effects drastically reduced with the use of this extract as an adjuvant to traditional treatment in most of the other subjects (152).

### Conclusion and Future perspectives

The present review indicates that methanolic and aqueous extracts of root and stem of *W. somnifera* shows anti-cancer activity by either induction of apoptosis, cell cycle arrest or cytotoxicity of the cancer cells. It also has the ability to control progression of cancer by inhibition metastasis and angiogenesis and can be used as an adjuvant during radiation and chemotherapy. The prominent mechanisms for its activity specifically against hepatocellular carcinoma, skin cancer, breast cancer, prostate cancer, lung cancer, colon and colorectal cancer, cervical cancer and ovarian cancer were elaborated. Some of these mechanisms are Nitric oxide synthase (NOS) upregulation and production of reactive oxygen species (ROS) leading to mutations and DNA damage or mitochondrial dysfunction and telomerase inhibition all of which cause apoptosis. Inhibition of viral oncoproteins and other immune responses and activation of p53 and pRb leading to cytotoxicity, inhibition of angiogenesis and metastasis by MDSC and STAT 3 inhibition and VEGF down regulation and cell cycle arrest caused by inhibition of transcription factor NF-kappaB. Fig. 3 gives a diagrammatic representation of all the above mentioned mechanisms of anti-cancer activity.

Although biochemical mechanisms are proposed, most of the studies are only held on animal test subjects. Anti-tumorigenic activity of *W. somnifera* is yet to be identified for various newly emerging cancer cell lines. Most of the research studies referred in this study have not proposed a definite biochemical mechanism for their findings yet. Anti-cancer activities against some cell lines have only been proposed based on molecular docking without consideration of any experiments involving test subjects. Even the pre-clinical and clinical studies conducted so far are mostly on animal test subjects. Moreover, a large number of them use withaferin-A as the therapeutic agent but not *W. somnifera* extract. It is important to understand that the plant extract is a mixture of numerous phytochemical active compounds in varying concentrations and testing the viability of withaferin-A alone doesn't give much clarity on the effect of the whole extract which is mostly consumed in reality and as a result, test result with withaferin-A must be extrapolated for the extract. Another major drawback of the previously conducted human clinical trials includes consideration of a small sample space which mostly consists of older population.

Hence in the future, studies must be conducted on human test subjects for the cell lines identified to be affected by treatment with *W. somnifera* extract. Definite mechanisms must also be proposed for cancer cell lines regulated by *W. somnifera* yet lacking a biochemical mechanism. Effect of *W. somnifera* treatment must be analyzed as a candidate

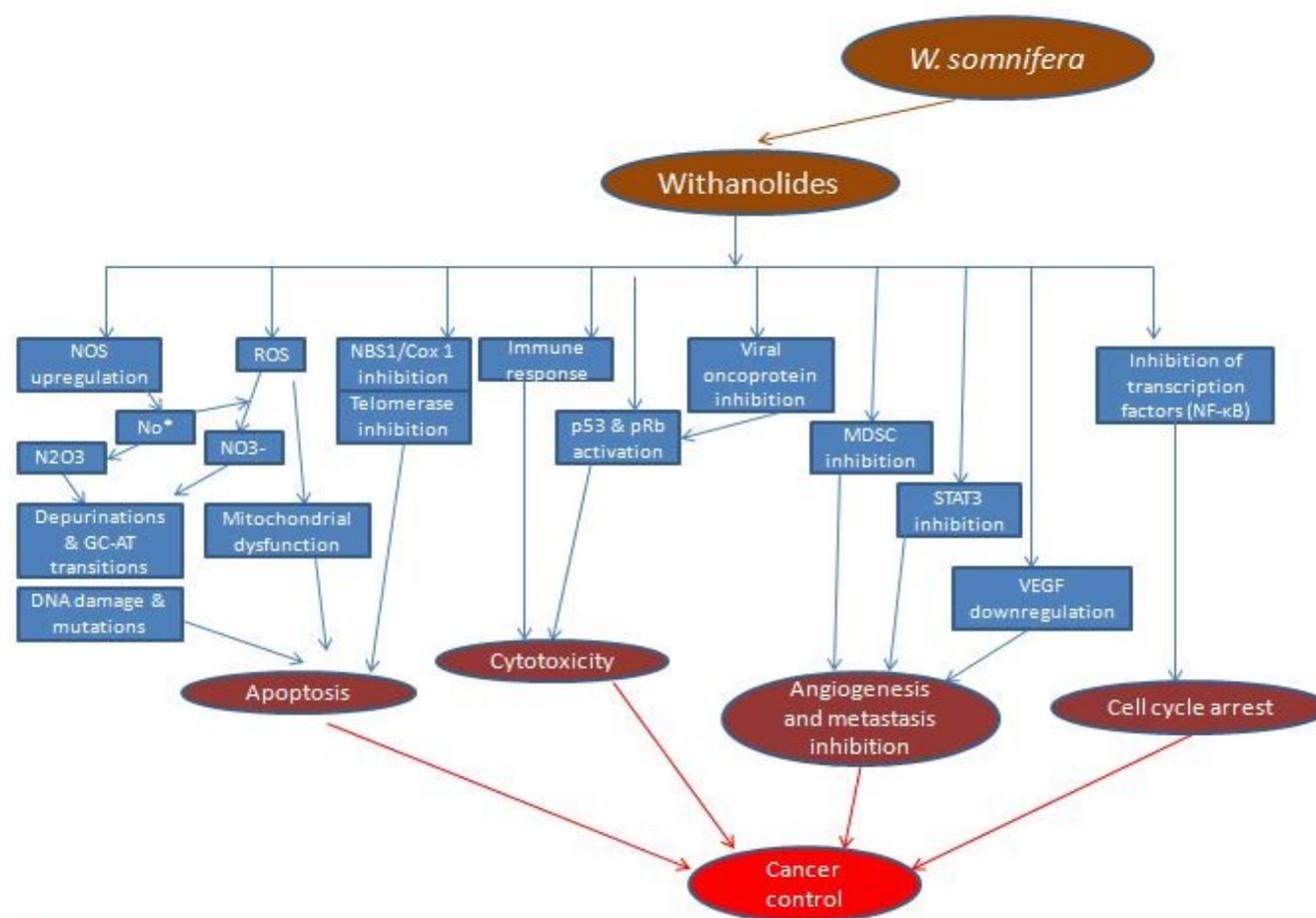


Fig. 3. Mechanism of action of withanolides in controlling cancer from *Withania somnifera*.

phytotherapeutic drug and a complete substitute of the conventional chemotherapeutic drugs against various cancer cell lines.

Studies have also shown various adverse effects on human consumption of *W. somnifera* including fever, rash, diarrhea, edema and abnormal LFTs (153). Further inspection on this aspect is crucial, research must also be undertaken to reduce these adverse effects.

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### Authors' contributions

RSU, PMN and PN did extensive literature survey and collected the articles. RSU wrote the review. VU and PMN helped in providing regular assistance during the process and PN was involved in finalization of the manuscript and all authors read and approved the final manuscript.

### Conflict of interests

All the authors declare that there is no conflict of interest.

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