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Research Paper

Anticarcinogenic Potential of Ashwagandha: An *In vivo* Study

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A B S T R A C T

A popular ayurvedic herb Ashwagandha is commonly known as "Indian Winter cherry" has been reported for its anti-tumorigenic activity against various cancer cells. In these studies, we used alcoholic extract of root of this plant. We have selected four optimum doses of Ashwagandha (*Withania somnifera)* suspension 100, 150, 250, 300 and 350 mg/Kg. body weight for *in vivo* experiments. The methods adopted were total clastogeny and frequencies of aberrations in the bone marrow cells of albino mice *in vivo*. The data were collected at three durations of 16, 24, and 32 h for *in vivo* experiments. *Withania* extracts significantly reduce number of aberrant cells ranges from 31, 36 and 42 percent for 16, 24 and 32 h respectively, frequency of aberrations per cell was also reduced significantly at $p<0.05$ level *in vivo*.

***Key words*:** Genotoxicity, Clastogeny, Aberration, Aflatoxin B_1, *Withania* extracts, *In vivo*

A popular ayurvedic herb Ashwagandha is commonly known as "Indian Winter cherry". The root smells like horse ("*ashwa*") that is why it is called Ashwagandha. The species name *somnifera* means "sleep-inducing" in Latin, indicating its sedating properties. Some herbalists refer to ashwagandha as Indian ginseng, since it is used in ayurvedic medicine in a way similar to that *Panax ginseng* used in traditional Chinese medicine. This herb is commonly used in herbal formulation for its wide range of health benefits.

In *Ayurveda*, Ashwagandha is considered as a *rasayana* herb, which works on a nonspecific basis to increase health and longevity. *W. somnifera* has been in use for over 2500 years to treat all kind of diseases and human ailments. This herb is also considered as an adaptogen which is a nontoxic herb that works on a nonspecific basis to normalize physiological function. In Ayurveda, this plant is claimed to have potent aphrodisiac rejuvenative and life prolonging properties. The traditional use of 'Ashwagandha' was to increase energy, youthful vigour, endurance, strength, health, nurture the time elements of the body, increase vital fluids, muscle fat, blood, lymph, and semen and cell production. It also helps to counteract chronic fatigue, weakness, dehydration, bone weakness, loose teeth, thirst, impotency, premature aging emaciation, debility, and convalescence and muscle tension.

Administration of an extract from the powdered root of the plant *Withania somnifera* could stimulate immunological activity in Balb/C mice [1]. The influence of *W. somnifera* root powder on the levels of circulatory ammonia, urea, lipid peroxidation products for its hepatoprotective effect in ammonium chloride induced hyperammonemia [2]. The hypoglycemic, diuretic and hypocholesterolemic effects of roots of *W. somnifera* (Ashwagandha) on human subjects [3]. The roots are reported to contain alkaloids, amino acids, steroids, volatile oil, starch, reducing sugars, glycosides, hentriacontane, dulcitol, withaniol. Many biochemically heterogeneous alkaloids have been reported in the roots.

The antibiotic activity of the roots as well as leaves has recently been shown experimentally [4]. Ashwaganda showed the protective action against systemic Aspergillus infection. This protective activity was probably related to the activation of the macrophage function revealed by the observed increases in phagocytosis and intracellular killing of peritoneal macrophages induced by Ashwagandha treatment in mice [5].

Withaferin A, withanolide E exhibited significant antitumour activity in vitro against cells derived from human epidermoid carcinoma of nasopharynx (KB) and in vivo against Ehrlich ascites carcinoma, Sarcoma 180, Sarcoma Black (SBL), and E 0771 mammary adenocarcinoma in mice. The presence of an unsaturated lactone in the side-chain to which an allelic primary alcohol group is attached at C25 and the highly oxygenated rings at the other end of the molecule may well suggest specific chemical systems possessing carcinostatic properties [6-7]. Withaferin A has been shown to possess growth inhibitory and radio-sensitizing effects on experimental mouse tumours [8]. Administration of Withaferin A in mice inoculated with Ehrlich ascites carcinoma cells was found to inhibit tumour growth and increase tumour-free animal survival in a dose dependent manner [9-10]. The alcoholic extract of the dried roots of the plant as well as the active component Withaferin A isolated from the extract showed significant antitumour and radio-sensitizing effects in experimental tumours *in vivo*, without any noticeable systemic toxicity [11]. In the present work we have used extract of root of Ashwagandha as counter genotoxic agent against Aflatoxin induced genotoxicity.

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Ashwagandha a known anti-inflammatory, anti-tumor, anti-stress, antioxidant, hemopoietic and rejuvenator has proven a variety of therapeutic effects with little or no associated toxicity [12].

MATERIALS AND METODS

The plant's long, brown, tuberous roots are used for medicinal purposes [13]. The whole plant is collected from the nearby area during the summer season and verified by the competent Botanist from Department of Botany, Shibli National College, Azamgarh.

100 g of *W. somnifera* dried roots were exhaustively extracted with various solvents (alcohol, hydro alcohol 50:50)). The extracts were dried and the percentage yields of extracts were determined. The powdered extract was re-dissolved in DDH_2O at a dose level of 1000. The experiments were performed with 8-10 week old Albino mice (25-35 g) following animal ethics of our institution, divided into 11 different group consisting of one mice in each group, 2 in control group (one normal control, one Aflatoxin induced) and 9 in test group *in vivo* with different concentration. All the mice were kept for overnight fasting. Aflatoxin B_1 and Withania extracts in different concentrations were administered by subcutaneous injection into the plantar side of the left hind paw. We have selected four optimum doses of *Withania somnifera* suspension of 100, 150, 250, 300 and 350 mg/Kg. body weight for *in vivo* experiments. Mice were exposed to different test chemicals by appropriate routes and were sacrificed at sequential intervals at 16, 24, and 32 h of treatment. Mice have been treated with each test substance once at the selected doses. Samples were collected at three times after treatment. The central sampling interval was 24 h. Since cell cycle kinetics can be influenced by the test substances, one earlier and one later sampling interval adequately spaced within the range of 6 to 48 h, had been applied, where the additional dose levels were tested in a subsequent experiment, samples have been taken at the predetermined most sensitive intervals.

Immediately after sacrifice, the bone marrow were obtained, and exposed to hypotonic solution and fixed. The cells were spread on slides and stained. Chromosome preparations were made from bone marrow cells following above mentioned procedure. The metaphase cells were scored for chromosomal aberrations. Prior to sacrifice, mice were further treated with colchicines, a spindle inhibitor to arrest the cells in metaphase. The slides were stained in 10% aqueous Giemsa solution and 100 bone marrow metaphase cells from each animal were scored under code [14]. The types of chromosomal aberrations considered were chromatid and chromosome gaps, breaks, and fragments, exchanges and pulverization (severely damaged cells). The reduction factor due to Ashwagandha (WS) treatment was calculated using the formula:

$$\text{Percent reduction} = \frac{\text{Aberrant cells in control} - \text{aberrant cells in } AFB_1 + WS}{\text{Aberrant cells in control} - \text{aberrant cells in negative control}}$$

RESULTS AND DISCUSSION

The results obtained after the treatment of *Withania somnifera* extract along with Aflatoxin B_1 (AFB1) at three different durations of 16, 24, and 32 h. were presented here. The data noticed due to this *Withania* extracts shows that the value after treatment with Aflatoxin B_1 alone at 16 h was 14.0 percent, but after the treatment with five different increasing concentrations of *Withania somnifera* extract along with Aflatoxin B_1, these values were reduced to 13.5, 12.7, 11.1, 10.1 and 9.6 respectively, whereas values for normal and DMSO control were 2.3 and 3.0 respectively. When the animals were treated with *Withania somnifera* alone, the value was 1.8%. In terms of percent reduction in the number of aberrant cells, the observed values were 3.57, 9.28, 20.71, 28.00 and 31.42 respectively for five different doses of *Withania somnifera* extract. The results show that *Withania somnifera* extract at each concentration except 1st and 2nd lower one can reduce the total aberrant cells significantly. This table also shows dose-dependent relationship between doses of *Withania somnifera* extract and the percent reduction of aberrant cells at 16 h (Table 1, Fig 1).

Table 1 Effect of *Withania somnifera* [WS] extract on the frequency of cells with chromosome aberrations induced by Aflation B_1 (AFB_1 x/kg.bw) at 16 h

Treatment	WS (Y/kg.bw)	Cells with pulverized chromosome	Types of chromatid aberrations				Aberrant cells		Percent reduction
			Gaps	Breaks	Fragments	Exchange	Number	(%)	
DH_2O	0	0	02	06	17	00	23	2.3	
DH_2O + DMSO	0	0	05	12	18	00	30	3.0	
AFB_1	0	19	88	24	112	02	140	14.0	
WS	WS_5	0	14	04	14	00	18	1.8	
AFB_1 + WS	WS_1	10	70	21	106	04	135	13.5	3.57
	WS_2	6	69	27	90	05	127	12.7	9.28
	WS_3	7	56	15	90	03	111	11.1	20.71
	WS_4	8	28	11	82	02	101	10.1	28.00
	WS_5	9	21	19	73	02	96	9.6	31.42

The total frequencies of the aberrant cells were 228 for Aflatoxin B_1 alone whereas 200, 196, 167, 129 and 173 for five different doses of *Withania somnifera* extract respectively. These reduced values were statistically very

significant in comparison to Aflatoxin B_1 alone. As the doses of *Withania somnifera* extract increases, the values of total frequency decreases, thus it shows dose-dependent inverse relationship. Cells with five or more than five aberrations were very few in numbers (Table 2).

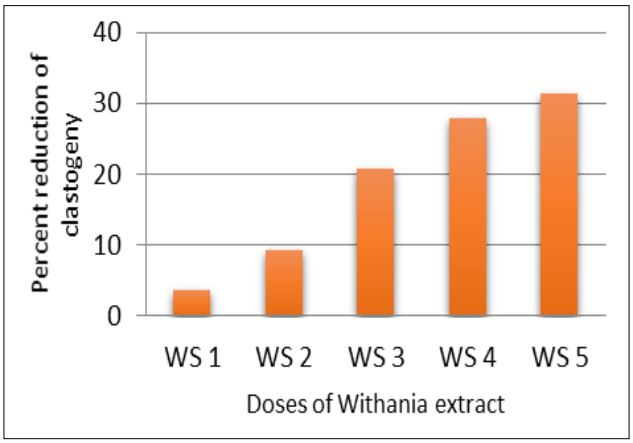

Fig 1 Showing *in vivo* antigenotoxic effect of *Withania somnifera* extract at 16h duration of treatment against AFB1 induced genotoxicity

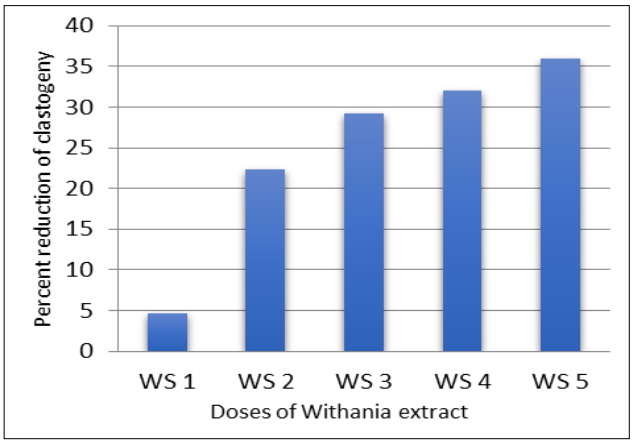

Fig 2 Showing *in vivo* antigenotoxic effect of *Withania somnifera* extract at 24h duration of treatment against AFB1 induced genotoxicity

Table 2 Effect of *Withania somnifera* [WS] extract on the total no. and types of frequency of cells with chromosome aberrations induced by Aflatoxin B_1 (AFB_1 x/kg .bw) at 16h

Treatment	WS (Y/kg.bw)	Cells with aberrations							Total number of aberrations
		0	1	2	3	4	5	6-9	
DH_2O	0	977	21	02	00	0	0	0	25
DH_2O + DMSO	0	970	25	05	00	0	0	0	35
AFB_1	0	860	88	29	16	3	3	1	228
WS	WS_5	972	24	04	00	0	0	0	32
AFB_1 + WS	WS_1	865	97	22	08	4	3	2	200
	WS_2	873	92	21	06	4	2	2	196
	WS_3	889	79	17	10	3	1	1	167
	WS_4	899	80	15	05	1	0	0	129
	WS_5	904	80	12	02	1	1	0	173

Note: The animals were sacrificed 16 h after AFB_1 treatment. 1000 cells from 10 animals were analyzed for each point

Table 3 Effect of *Withania somnifera* [WS] extract on the frequency of cells with chromosome aberrations induced by Aflatoxin B_1 (AFB_1 x/kg.bw) at 24 h

Treatment	WS (Y/kg.bw)	Cells with pulverized chromosome	Types of chromatid aberrations				Aberrant cells		Percent reduction
			Gaps	Breaks	Fragments	Exchange	Number	(%)	
DH_2O	0	0	02	10	12	00	22	2.2	
DH_2O + DMSO	0	0	02	03	22	00	25	2.5	
AFB_1	0	26	104	40	60	15	130	13.0	
WS	WS_5	0	2	08	16	00	24	2.4	
AFB_1 + WS	WS_1	10	52	26	94	02	124	12.4	4.61
	WS_2	8	28	10	83	02	101	10.1	22.30
	WS_3	8	21	19	71	01	92	9.2	29.23
	WS_4	6	15	08	78	01	88	8.8	32.00
	WS_5	8	14	18	65	00	83	8.3	36.00

The similar trend was obtained after 24 h of treatments with some increasing values. The obtained value of percent

aberrant cells was 13.00% for Aflatoxin B_1 and 12.4, 10.1, 9.2, 8.8 and 8.3 percent for five different concentrations of *Withania somnifera* extract along with Aflatoxin B_1 respectively. The percent reductions in the aberrant cells were 4.61, 22.30, 29.23, 32.00 and 36.00 shows very significant effect of different concentrations of *Withania somnifera* extract. It also shows inverse dose dependent relationship between the doses of *Withania somnifera* extract and percentage reduction of the aberrant cells (Table 3, Fig 2). The frequencies of total number of aberrations in the aberrant cells were observed in the (Table 4). It also shows similar trend as in the (Table 3) with increasing frequencies.

Table 4 Effect of *Withania somnifera* [WS] extract on the frequency of cells with chromosome aberrations induced by Aflatoxin B_1 (AFB_1 x/kg.bw) at 24 h

Treatment	WS (Y/kg.bw)	Cells with aberrations							Total number of aberrations
		0	1	2	3	4	5	6-9	
DH_2O	0	979	18	03	00	00	00	00	24
DH_2O + DMSO	0	974	23	03	00	00	00	00	29
AFB_1	0	860	88	29	16	03	03	01	228
WS	WS_5	975	22	03	00	00	00	00	28
AFB_1 + WS	WS_1	867	98	21	08	04	02	02	204
	WS_2	876	94	19	05	02	03	01	177
	WS_3	889	79	17	10	03	01	01	167
	WS_4	904	82	10	02	01	01	00	117
	WS_5	919	46	19	08	05	03	00	143

Note: The animals were sacrificed 24 h after AFB_1 treatment. 1000 cells from 10 animals were analyzed for each point

Table 5 Effect of *Withania somnifera* [WS] extract on the frequency of cells with chromosome aberrations induced by Aflatoxin B_1 (AFB_1 x/kg.bw) at 32 h

Treatment	WS (Y/kg.bw)	Cells with pulverized chromosome	Types of chromatid aberrations				Aberrant cells		Percent reduction
			Gaps	Breaks	Fragments	Exchange	Number	(%)	
DH_2O	0	0	03	05	16	00	21	2.1	
DH_2O + DMSO	0	0	03	02	24	00	26	2.6	
AFB_1	0	21	88	24	112	02	140	14.0	
WS	WS_5	0	03	05	20	00	25	2.5	
AFB_1 + WS	WS_1	12	71	21	105	04	133	13.3	5.00
	WS_2	9	53	26	94	02	124	12.4	11.42
	WS_3	8	62	16	65	00	81	8.1	42.00
	WS_4	6	21	19	75	01	96	9.6	31.00
	WS_5	8	62	16	65	00	81	8.1	42.00

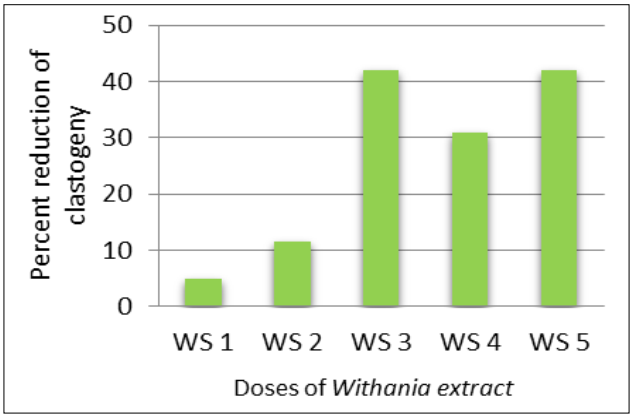

Fig 3 Showing *in vivo* antigenotoxic effect of *Withania somnifera* extract at 32h duration of treatment against AFB1 induced genotoxicity

After 32 h of experiments the values obtained were 14.0 for Aflatoxin B_1 alone and 13.3, 12.4, 8.1, 9.6, 8.1 for each concentrations of *Withania somnifera* extract plus Aflatoxin B_1. In terms of percent reductions in the number of aberrant cells, *Withania somnifera* extract shows very effective in reducing the aberrant cells. The values for five different concentrations of *Withania somnifera* extract were 5.00, 11.42, 42.00, 31.00 and 42.00 percent respectively (Table 5, Fig 3).

The total frequencies of aberrations in the aberrant cells were also follows similar trend. The values recorded were 234 for Aflatoxin B_1 and 176, 129, 113,109 and 97 for *Withania somnifera* plus Aflatoxin B_1. Thus, frequencies were also reduced significantly and show dose dependent relationship between the frequency and doses of *Withania somnifera* (Table 6).

Table 6 Effect of *Withania somnifera* [WS] extract on the frequency of cells with chromosome aberrations induced by Aflatoxin B_1 (AFB_1 x/kg.bw) at 32h

Treatment	WS (Y/kg.bw)	Cells with aberrations							Total number of aberrations
		0	1	2	3	4	5	6-9	
DH_2O	0	978	20	02	00	00	00	00	24
DH_2O + DMSO	0	975	22	03	00	00	00	00	28
AFB_1	0	890	92	25	08	05	04	04	234
WS	WS_5	976	22	02	00	00	00	00	26
AFB_1 + WS	WS_1	876	95	18	05	02	03	01	176
	WS_2	899	80	15	05	01	00	00	129
	WS_3	908	77	11	02	02	00	00	113
	WS_4	912	75	11	02	00	00	00	109
	WS_5	917	71	10	02	00	00	00	97

Note: The animals were sacrificed 32 h after AFB_1 treatment. 1000 cells from 10 animals were analyzed for each point

Withania somnifera, despite being under-appreciated in the area of oncology experiments in test tubes and in animal models demonstrated that *Withania somnifera* plays an anticancer role by inducing apoptosis and cell cycle arrest, enhancing the immune system, and inhibition of angiogenesis and metastasis. Interestingly, as *Withania somnifera* exhibits both anti-oxidant and pro-oxidant activities, it has been reported to sensitize tumors to radiation while presenting itself a radio/chemo-protector for normal cells. These observations are in accordance to [15-17].

It was found that when crude alcoholic extract from WS roots was injected in mice, complete regression of injected sarcoma occurred within 100 days [18]. WS has been proposed to regulate the cell cycle and apoptosis pathways in several ways depending on the cell type. First, a methanolic leaf extract of WS was demonstrated to restore normal p53 function in tumor cells bearing mutated copies [19]. Second, in cases when tumors were p53-null, i.e., HL-60 leukemia cells, the WS caused apoptosis by down-regulation of bcl-2, cytochrome C release from the mitochondria and caspase-3 activation [20]. Third, the methanolic root extracts, at doses of 65–265 µg/ml, down-regulated expression of p34 cdc2, a cell-cycle regulatory protein and could lead to the growth arrest at the G2/M phase [21]. Fourth, WS sensitized human cancer cells to anti-cancer drugs suggesting that the WS may be employed in combination with anticancer drugs to yield an effective combinatorial anti-cancer formulation [22].

A study by [23] confirmed the antitumor and radio-sensitizing properties of Ashwagandha. *W. somnifera* significantly altered the level of leucocytes, lymphocytes, neutrophils, immune complexes and immunoglobulins (Ig) A, G and M in experimental colon cancer in mice induced by azoxymethane [24]. The results of this study revealed that azoxymethane induced colon cancer and immune dysfunction was controlled by *W. somnifera* [25]. The anticancer activity of Withaferin-A (WA), which exhibits potential for further development for targeted chemotherapy or chemoprevention strategies in the context of colon cancer [26]. *In vitro* studies have shown that root extracts of WS exhibited cytotoxic properties against lung, colon, central nervous system, and breast cancer cell lines [27]. WS has been shown to posses tumor preventing activity against urethane induced lung-adenomas in adult male albino mice by inducing a state of nonspecific increased in resistance (Adaptogen) and immunostimulant properties [28]. Recent studies showed that ashwagandha extract inhibited the growth of human breast, lung, and colon cancer cell lines in the laboratory. This inhibition was comparable to that achieved with the common cancer chemotherapy drug doxorubicin. In fact, researchers reported that withaferin A, a specific compound extracted from ashwagandha, was more effective than doxorubicin in inhibiting breast and colon cancer cell growth [29-30]. Withaferin A major chemical constituents of WS, primarily induces oxidative stress in human leukemia HL-60 cells and in several other cancer cell lines and the results of these studies demonstrate that withaferin A induced early ROS generation and mitochondrial dysfunction in cancer cells trigger events responsible for mitochondrial -dependent and -independent apoptosis pathways [31].

CONCLUSION

Scientific studies conducted in mice revealed that the roots of WS have capability to inhibit fore-stomach and skin carcinogenesis in mice [32]. Administration of an extract of WS reduced two stage skin carcinogenesis induced by DMBA (dimethyl benzanthracene) and croton oil [33]. Leaf extract of WS has been shown to produce antiproliferative activity on MCF-7 (breast) human tumor cell lines [34]. The chemical constituent Withaferin A enhanced radiation-induced apoptosis in human renal cancer cells (Caki) cells through ROS generation, down-regulation of Bcl-2 and Akt dephosphorylation [35]. In another study, treatment of Caki cells with withaferin A induced a number of signature ER stress markers, including phosphorylation of eukaryotic initiation factor-2α (eIF-2 α), ER stress-specific XBP1 splicing, and up-regulation of glucose-regulated protein (GRP)-78. In addition, withaferin A caused up-regulation of CAAT-enhancer-binding protein homologous protein (CHOP), suggesting the induction of ER stress. Pretreatment with N-acetyl cysteine (NAC) significantly inhibited withaferin A-mediated ER stress proteins and cell death, suggesting that reactive oxygen species (ROS) mediate withaferin A-induced ER stress. Furthermore, CHOP siRNA or inhibition of caspase-4 activity attenuated withaferin A-induced apoptosis. Taken together, the present study provides strong evidence supporting an important role of the ER stress response in mediating withaferin A-induced apoptosis [36]. We have also found that *Withania* extracts significantly reduced the genotoxic effect of Aflatoxin in our experiments.

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