International Journal of Science and Research (IJSR)
ISSN (Online): 2319-7064
Index Copernicus Value (2013): 6.14 | Impact Factor (2013): 4.438

Potential Toxicity of Egyptian Ashwagandha: Significance for their Therapeutic Bioactivity and Anticancer Properties

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Abstract: ***Chemotherapy is usually given early after diagnosis in several cancer subtypes to offer best results. However, chemotherapeutic drugs are often associated with some degree of toxicities, which are caused by reactive metabolites generated by the biotransformation of anticancer drugs in the liver. Phytochemicals are dietary phytoestrogens that may play a role in cancer prevention and treatment. Forty percent of Americans use complementary and alternative medicines (CAM) for disease prevention and therapy. Ashwagandha (Withania somnifera) contains flavonoids and active ingredients like alkaloids and steroidal lactones which are called 'Withanolides'. We hypothesize that the immune-modulatory and anti-inflammatory properties of Ashwagandha might contribute to its overall effectiveness as an anti-carcinogenic agent. Ashwagandha is one of the most versatile plants used in the traditional Indian medicine system (Ayurvedic).The goal of this study is evaluate the therapeutic bioactivity and cytotoxic effect of Egyptian Ashwagandha on hepatocellular carcinoma cell line (HepG2).The HepG2 cells treated by different doses of Ashwagandha root extract. The viability and cytotoxicity were measured by trypan blue and MTT assay. In conclusion: Ashwagandh can inhibit cell proliferation and induce cytotoxic effect on hepatocellular cancer cells.***

Keywords: Egyptian Ashwagandha , Hepatocellular Carcinoma Cell Line Antitumor activity

Abbreviation: HCC, Hepatocellular Carcinoma; HepG2, Human Hepatocellular Carcinoma Cell Line; W.S, Withania Somminera; IC_{50}, inhibition concentration dose; WA, Withaferin A; HeLa, Carcinoma of the Cervix; MCF-7, Breast Adenocarcinoma; EAW Ex ,Egyptian Ashwagandha Water Extraction.

1. Introduction

Hepatocellular carcinoma (HCC), which are the most health-threatening conditions drawing considerable attention from medical professionals and scientists[1]. Chemotherapeutic drugs are often associated with some degree of toxicities, which are caused by reactive metabolites generated by the biotransformation of anticancer drugs in the liver[2].Genotoxicity and cytotoxicity of anticancer drugs to normal cells is a major problem in cancer therapy and engender a risk of inducing secondary malignancies[3,4]. A dose of anticancer drug sufficient to kill tumor cells is often toxic to the normal tissue and lead to many side effects, which in turn, limits their treatment efficacy[5].However, HCC is well known to be a highly chemo resistant tumor, and the response is very poor [6]. To this end, alternative medicines are being actively sought from other sources with hopes to halt the disease's progression or even eliminate the tumors.[6,7]. Plant and plant products have utilized with varying success to cure and prevent diseases throughout history. Due to side effects of synthetic products, herbal products are gaining popularity in the world market[7]. Many active principles produced by animals, plants and microorganisms have been employed in the development of new drugs to treat diseases such as cancer[8].Medicinal plants are popular in indigenous system of medicine like Ayurveda, siddha, unani and homoeopathy and are used for its hepato-protective, antitumor, antihypertensive, analgesic, anti-inflammatory and antimicrobial properties[9].

Historically, the medicinal plant Ashwagandha *{Withania somnifera* (*W.s.*)} has been used for over centuries in Indian Ayurvedic Medicine to treat a wide spectrum of disorders[10]. Ashwagandha found throughout the drier parts of India, Baluchistan, Pakistan, Afghanistan, Sri Lanka, Congo, South Africa, Egypt, Morocco and Jordan[11], the plant has been used as an antioxidant, adaptogen, aphrodisiac, liver tonic, anti-inflammatory agent, astringent and new to treat ulcers, bacterial infection, venom toxins and senile dementia. The major biochemical constituents of Ashwagandha. are steroidal alkaloids and lactones, a class of constituents together known as withanolides (steroidal lactones with ergostane skeleton)[12].Further, it was able to induce dose dependent DNA fragmentation in treated cells[13].It promotes physical and mental health, rejuvenates the body in debilitated conditions, and increase longevity[14]. Ashwagandha is known to have anti-inflammatory[15], antitumor[16], antidiabetic[17], antioxidant[18], cardioprotective[19] and anti-stress effect[20]. The aim of this work is the study of antitumor activity and cytotoxic effect of Ashwagandha on hepatocellular carcinoma.

2. Materials and Methods

Plant: *Withania Somnifera* (Egyptian *Ashwaghandha*), roots were harvested from Rafah, El-Arish, North Sinai, Egypt in September 2008.

Figure 1: leaves and root of *Egyptian Ashwagandha*

1) Tested Compounds

Dry powder of Egyptian Ashwaghandha Water Extraction (EAW Ex) roots was prepared by suspending 10 g of dry powder in 100 ml of distilled water and stirring it overnight at 45±5°C, followed by filtration under sterile conditions. The filtrate thus obtained was treated as 100% EAW Ex. It was stored at –20°C in 1 ml aliquots until further use.

2) Cell Culture

Hep-G2, a human hepatocellular carcinoma cell line was obtained frozen in liquid nitrogen (-180oC) from the American Type Culture Collection. The tumor cell line was maintained in the National Cancer Institute, Cairo, Egypt, by serial sub-culturing. Cells were cultured in RPMI-1640 medium (RPMI-1640, Sigma–Aldrich, USA). The medium was supplemented with antibiotic-free 10% fetal bovine serum (FBS, Sigma, USA), 100 U/ml penicillin and 2-mg/ml streptomycin. The cells were sub cultivated after trypsinization (Trypsin-EDTA, Cambrex Bioscience Verviers, Belgium) once or twice per week and re-suspended in complete medium in a 1:5 split ratio. Cell line was maintained as monolayer in T75 cell culture flasks with filter screw caps (TPP, Trasadingen, Switzerland) at 37 °C in a humidified 5% CO2 incubator.

3) Cell treatment

Cells were treated with EAW Ex at concentration range from 0.65 to 1.00 %. The percentage of viable cells was determined by trypan blue exclusion. Hep-G2 cells were used when 90% confluence was reached in T25 flasks, adherent cell lines were harvested with 0.025% trypsin. Then cells were exposed to different concentrations of EAW Ex. The cellular viability was determined using light microscope employing 0.4% trypan blue dye exclusion technique at 12 h and 24 h time intervals.

4) MTT cytotoxic assay

Anti-proliferative activity against liver tumor cell line was estimated by the 3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay, which is based on the cleavage of the tetrazolium salt by mitochondrial dehydrogenases in viable cells21. Cells were treated for 24 h with various concentrations of EAW Ex before submitted to the MTT assay. The relative cell viability was expressed as the mean percentage of viable cells comparing to control 0 treated cells and the half maximal growth inhibitory concentration (IC50) was calculated by the trend line equation.

5) Flow cytometric cell cycle analysis

Hep-G2 cells (5x105cells/well) were plated in 6 well micro plates. Then collected after treatment with IC50 concentration of EAW Ex for 6h, washed two times with PBS, re-suspended in 300µl of PBS, and fixed with 4 ml of ice-cold 70% ethanol. When ready to stain with propidium iodide (PI), cells were centrifuged; the ethanol was removed and washed once in PBS. The cell pellets were then re-suspended in one ml of PI/Triton X-100 staining solution (0.1% Triton X-100 in PBS, 0.2 mg/ml RNase A, and 10 mg/ml PI) and incubated for 30 min at room temperature. The stained cells were analyzed using a MoFlo flow cytometer (Dako Cytomation, Glostrup, Denmark) 22.

6) Trypan Blue Exclusion

The dose response curve of viable cells was determined by Trypan blue exclusion. The method was carried out according to that of Sheldon and Preskorn, (1996)23 . (in brief); Cells were cultured in 24-well plate and incubated for 24hr. Drug was added and incubated for another 24hr. Medium was collected and cells were released by trypsinization in falcon tubes. Supernatant was removed and pellets were re-suspended. Cells were counted to determine viability using Trypan blue dye.

7) Counting of the Viable Cells:

Cell suspension was prepared at a high concentration (~10^6 cells / ml) by trypsinization. A volume of 50 µl of 0.05% Trypan blue solution was added to 50 µl of the single cell suspension and left 1-2 min. The cells were examined under the inverted microscope using the hemo cytometer. Non stained (viable) cells were counted and the following equation was used to calculate the cell count/ml of cell suspension.

Viable cells / ml = Sum of viable cells X No. of counted squares (5) X 10^4 X dilution factor.

The cells were then diluted to give the concentration of single cell suspension required for each experiment.

8) Morphological Studies

Cells were cultured in 6-well plates and incubated for 24hr then extract was added and incubated for 24hr. After that cells were examined under inverted microscope and morphological changes were recorded. Cells were photographed using digital camera.

3. Statistical Analysis

The experimental data were expressed as mean ± standard deviation (SD). A p value less than 0.05 was considered significant. Microsoft Excel and computer program package (SPSS version 15) was used for all statistical testing and management of the database.

4. Results

Dose response curve:Hep-G2 cells were treated with graded concentrations (0.6 – 1%) of EAWEx roots. The viability was monitored. The data indicated that treatment of cells with resulted in significant inhibition of viability of the cells

at 24- 48 hr as compared with control as shown in data that have been summarized in Fig. 2,3.

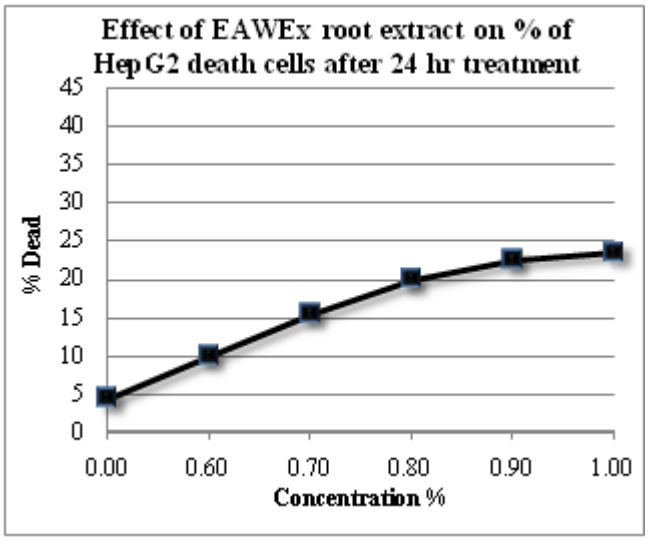

Figure 2: Effect of EAWEx root extract on% of HepG2 death cells after24 hr treatment.

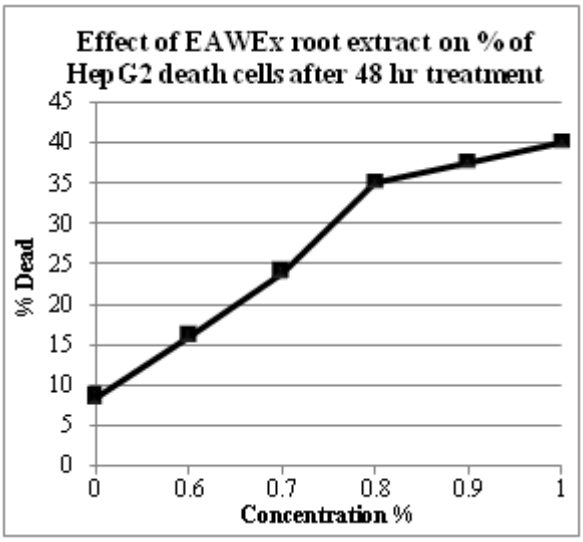

Figure 3: Effect of **EAWEx** root extract on % of HepG2 death cells after 48 hr treatment.

Table 1: After 24 hr incubation HepG2 seems to be more resistant and observed very resistant after 48 hr to EAWEx roots.

Concentration	*Observation*
Control	Cells very healthy, over-growth (over-sheeted), few floated dead cells found.
0.6%	Cells are slightly affected, there is no over-sheet
0.7%	Effect is very little. Cells begin to be unhealthy
0.8%	More dead cells observed.
0.9%	Slightly similar to previous concentration.
1%	Cells highly affected than other concentrations.

1) MTT Assay

To determine IC_{50} (inhibition concentration 50) value of EAWEx roots on HepG2, cytotoxicity (MTT) assay has been performed. Results showed that there is an anti-proliferative activity of the tested extract on the growth of HepG2 cell line after incubation for 24 hr. The effect of EAWEx roots on HepG2 showed that inhibited the cell growth in a dose dependent manner, with IC_{50} value of 0.9 %, () as shown in data that have been summarized in Fig. 4, cytotoxicity was measured and expressed as the survival fraction compared with untreated cells.

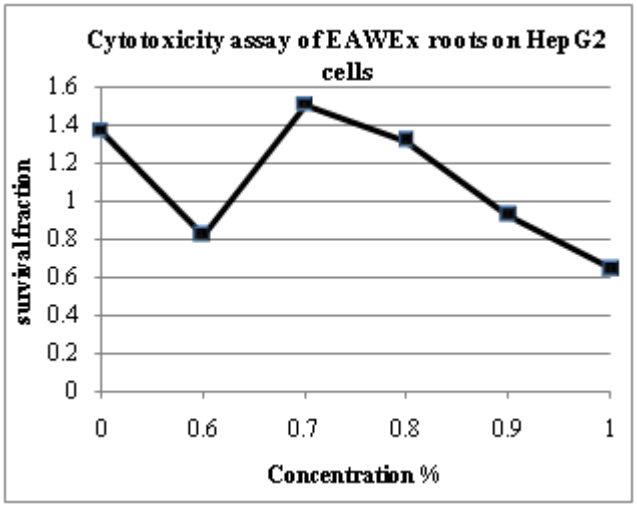

Figure 4: Cytotoxicity assay of EAW Ex roots on HepG2 cells

2) Cell Cycle Analysis

The effect of EAW Ex roots extract on the cell cycle distribution of $HepG_2$ cells is shown in figure (4,5). The control untreated cells show the regular distribution of G_0-G_1, G_2-M and S phases were 64.27, 5.79, and29.93 respectively. While cells treated with EAW Ex roots extract, show significant increase in cell number at G_2-M phase.

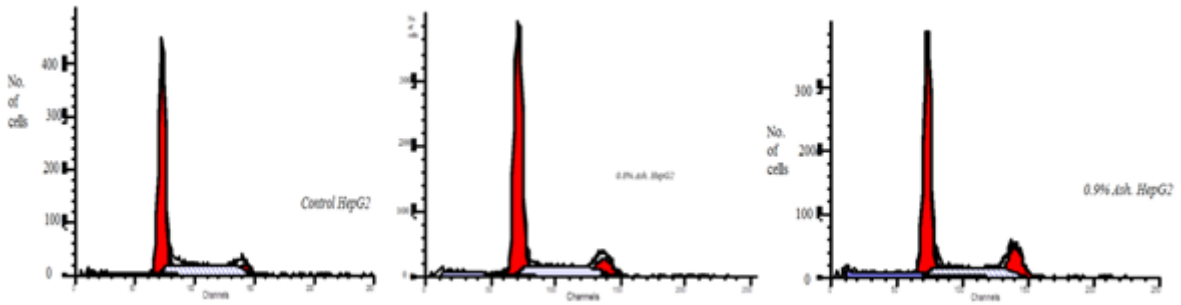

Figure 4: Influence of EAWEx roots on cell growth inhibition

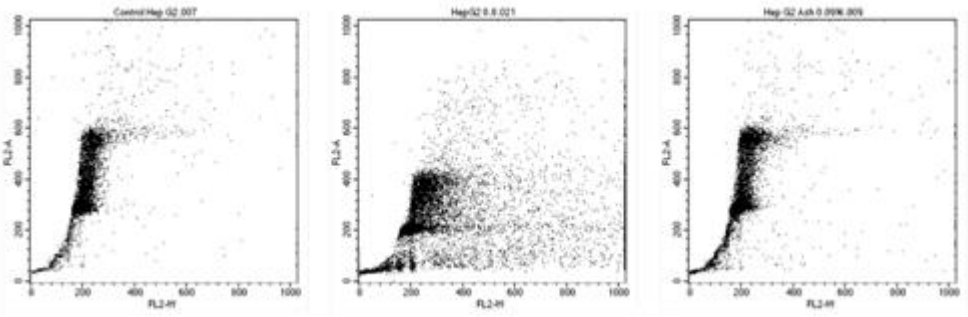

Figure 5: Flow cytometric analysis of EAWEx roots induced apoptosis in HepG2 cells.

3) Cell death assessment by DNA fragmentation

Treatment of HepG2 cells with different concentration of EAW Ex roots extract (0.5, 1.0, 5.0 and 10%) for 48hrs induced significant DNA ladder formation, suggesting apoptotic cell death.

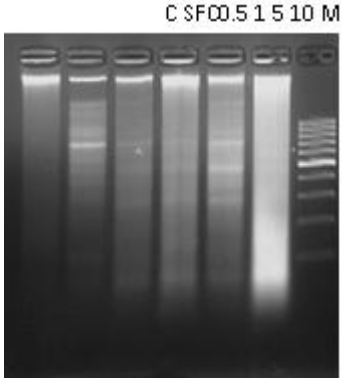

Figure 6: Cell death assessment by DNA fragmentation showing effect of EAW Ex roots extract on DNA of $HepG_2$ cells.
C: Control; SFC: Serum free control; M: DNA marker

4) Morphological Studies

Morphological studies were carried out to evaluate the effect of EAW Ex roots extract on cell morphology in relation to concentration and time of incubation. HepG2 cells were cultured in the presence of different extract concentration for 24 and 48 hrs, and morphological changes were examined under microscope. As shown in Fig.7, incubation for 24 hrs mainly showed no significant effect on cells. But at higher concentration (1%) extract treatment caused cytotoxicity and cell death. Cells rounded up in about 24 hr sand seemed to undergo apoptotic cell death. However, at lower concentration cells appeared to be growth arrested and showed morphology that appeared similar to control cells. Treatment for 48 hrs showed significant morphological changes; they showed condensed, shrank and aggregated shapes.

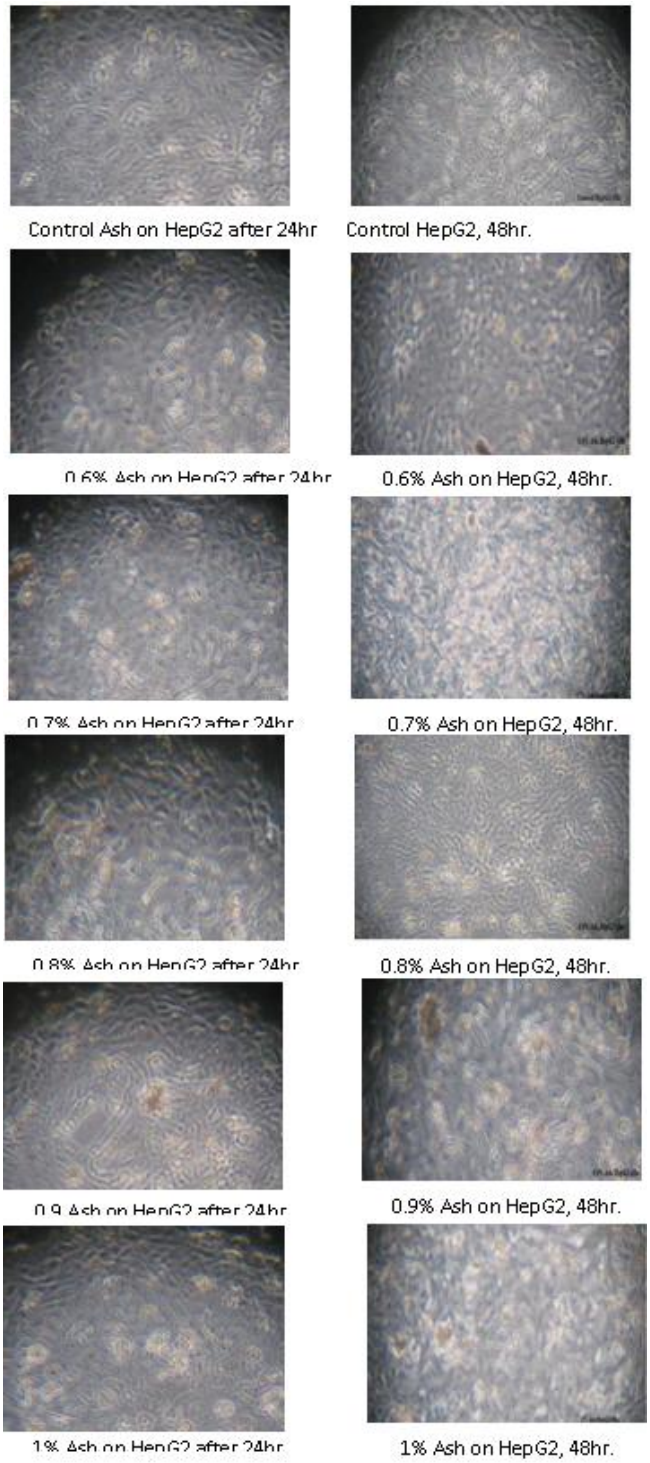

Figure 7: After 24 and 48 hr incubation of HepG2 with EAWEx roots it seems to be very resistant and indicate apoptotic cells

5. Discussion

A number of studies suggest that agents derived from plants including dietary fruits and vegetables are helpful in either inhibiting or reversing the development of cancer[24-27]. The development of effective therapeutic agents for liver cancer is necessary to improve current chemotherapy. The present study confirmed that the active constituents in Ashwagandha roots, the crude water extraction of roots inhibited the growth of $HepG_2$ cancer cell lines comparable to control. Although Ashwagandha has been shown to inhibit various cancers such as cancers of the breast[28, 29], colon[30], ovarian[31], and prostate[32], To our knowledge, this is the first study that done to observe the effect of Ashwagandha roots on HepG2 cell line.

The present study showed that Ashwagandha roots induced inhibition of cell viability in liver cancer cell lines. The effects of Ashwagandha roots in inhibiting liver cancer cell colony formation was demonstrated in HepG2 cancer cell lines. We would like to know whether the inhibition of liver cancer cell growth and viability is due to decreased cell proliferation rate, induction of cellular apoptosis, or both. We found that Ashwagandha roots caused both G2/M phase cell cycle arrest and cellular apoptosis in HepG2 cells, indicating that inhibition of liver cancer cells is attributed to both induction of apoptosis and cell cycle arrest in these cell lines.

Recent preclinical studies have revealed that Ashwagandha targets multiple molecules for mediating cell death in a variety of cancer cells[32]. Simultaneous blocking of cancer cell growth and survival pathways while activating apoptosis is a powerful approach to effectively suppress cancer. Recent studies indicate the involvement of apoptosis in Ashwagandha dependent inhibition potential for this safe and non-toxic agent[32]. A number of prior studies demonstrating involvement of apoptosis signaling in Ashwagandha dependent suppression of human breast and prostate cancer cell growth as well as the soft tissue sarcomas[33–35].

Su et al. observed that WA was more effective in inhibiting the growth of colon and breast cancer cell lines than Adriamycin[36]. Therefore, it can be postulated that theconsumption of Ashwagandha leaves as a dietary supplement may prevent or decrease the growth of tumors in cancer patients as well as the formation of new tumors. Since some of the withanolides are reported to induce phase-II enzymes, Ashwagandha leaves may be a potential source for the development of cancer therapeutics[36].

The results of the present study demonstrate that Ashwagandha roots exhibited selective cytotoxicity against Hep G2 of human cancer cell lines in vitro compared with normal cells and effectively inhibited tumor growth. The preparation of EAW Ex was to have a herbal product exhibiting its immune-stimulatory properties besides its anticancer activity. Cellular immune-response is weakened in cancer patients or tumor bearing animals[37].

Khan et al., showed the potential role of the chemically standardized leaf extract of Ashwagandha and its identified component in activating immune system. Thus it may be concluded that Ashwagandha is also a potent immune-stimulatory agent easily available in abundance and may be found useful in enhancing the immune response in immune compromised diseases like tuberculosis, leishmaniases, leprosy AIDS and cancer[40].

Sharma et al., reported that, the fishes fed with Ashwagandha root showed enhanced Phagocytic activity, total Immunoglobulin level and lysozyme activity compared with the control group. The survivability was higher in experimental diets than the control group[42].

The cytotoxic effect of Ashwagandha on HepG2 cell line was measured by MTT test with graded concentrations (0.6 – 1%) the result showed significant inhibition of viability of the cells at 24- 48 hr as compared with control. Further evaluation by flow cytometry revealed that, the effectively inhibited the proliferation of Hep G2 cancer cell line by generally blocking cell cycle at all stages.

Serena el al, reported that, the active ingredient in the chloroform extract fraction of Ashwagandha leaves exert its cytotoxic effect by inducing apoptosis[13]. DNA fragmentation is a late event in apoptosis as observed by Collins et al[43]. Further, it was able to induce dose dependent DNA fragmentation in treated cells; this demonstrates the possibility of using Ashwagandha in the development of anti-cancer drugs[13]. We also found a dose dependent increase in DNA fragmentation by performing Diphenylamine test.

Zhang et al, showed that Ashwagandha induced inhibition of cell viability in various ovarian cancer cell lines. The effects of WA in inhibiting ovarian cancer cell colony formation the inhibition of ovarian cancer cell growth and viability is due to decreased cell proliferation rate, induction of cellular apoptosis, or both. They found that Ashwagandha caused both G2/M phase cell cycle arrest and cellular apoptosis[30]. In conclusion Ashwagandha considered has anti-proliferative activity and cytotoxic effect on HepG2 cell line and can be used as a promising treatment of liver cancer .

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