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Alcoholic and aqueous leaf extracts of *Withania somnifera* induce cell death and arrest cell cycle in cancer cell line

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Abstract

Cancer is multifactorial disease and everyone looking for alternative therapeutic options to cure this. *Withania somnifera* is FDA approved nutraceutical and well reported as phytochemistry. In this study we investigated the efficacy of methanolic and aqueous leaf extract of this plant on cell proliferation, cell cycle and cell death in transformed HeLa cells. Well known model of cancer cell line were cultured in standard laboratory condition and treated with varied concentration of both methanolic and aqueous extracts of the plant leaves. Using MTT assay cell viability of these cells was found significantly affected. The inhibitory concentration was found to be 30-50 µg/ml for different fractions. We further tested IC50 concentration of the plant extracts on the HeLa cells and assessed the uptake of 7-AAD dye to check cell death. Dead cell were significantly increased in treated cell. It was observed that fraction-A, B, C induced significant cell death 35%, 37.5% and 21% respectively with compare to untreated control. Further, we assessed the effect of extracts on cell cycle using flow cytometry of propidium iodide stained cells. HeLa cells showed significant increase in G0 phase, decrease in G1 phase and arrested in S/G2/M phase with compare to control. Findings of our study demonstrate that *W. somnifera* extracts are rich in phytoactive component with therapeutic potential against cancerous cells. Both methanolic and aqueous extract are equally effective, and expected to possess similar component and exist similar mechanism which has to be confirmed in further studies.

Keywords: Cell cycle, apoptosis, cell viability, Hela cells, Propidium iodide

Introduction

People in India use some form of traditional medicine known as Ayurveda which practiced over five hundred decades. Ayurvedic medicine is a branch of Indian traditional medicine, which exists to the Indian subcontinent, and is a form of alternative medicine. As of now, the different parts of plant and herbs like; root, leaf, bark, fruits, seeds and flowers are used as ayurvedic medicine. Ashwagandha is such kind of medicinal plant has been utilized in traditional medicine. It belongs to the diverse Solanaceae family of flowering plants. *Withania* species show an exact wide distribution over drier climates of the world. Evidences from *in-vitro* and *in-vivo* studies suggest that ashwagandha possesses anti-tumorigenic properties. The bioactive compounds of ashwagandha are presents in the root and whole plants as alkaloids, flavonoids and steroidal lactones form^[1].

In non-pathogenic diseases, the most threaten category are cancer and diabetes throughout the world. Bray *et al.* done global estimation of cancer incidence and reported that the prevalence of cancer in 2008 is 28.8 millions^[2]. Cancer is a disease, involving abnormal cell proliferation with the capacity to invade different parts of the body. There are several factors to cause cancer. Numerous publications explained that one reason of cancer is certain occupations^[3]. Apart from genetic reasons, synthetic pharmaceutical agents are the cause of cancers. Studies on pregnant women who treated with diethylstilboestrol were shown that their female children had high rates of adenocarcinoma^[4]. To date the clear and exact mechanism for cancer progression is unclear. Though several established mechanisms reported for different cancer. In spite of major identification made in the field of drug discovery, researchers are trying to understand the cancer biology and the current therapeutics available. Long time therapy with *W. Somnifera* can inhibit cancer cell metastasis. Lower concentration of root extract was used to test against pro metastatic protein and found that it can prevent spread of breast cancer cells in rats^[5]. This medicinal plant also has anticancer property against prostate cancer. Use of this on the regular basis inactivate cdc2, regulate G2/M phase and causes cell death of prostate tumors cells^[6]. Bioactive components are basically responsible for anti-tumor activity.

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Research on the molecular mechanisms suggested that active components interact with cyclooxygenase-2 enzyme and inhibit prostaglandin synthesis which is responsible for systemic inflammation and rapid cell proliferation. However, it is also interacts with E2F, Bcl-2/caspase-3, p53 and cyclin D1 to control cell cycle progression and cellular homeostasis [1].

Therefore in current study we have investigated the anti cancer property of ashwagandha leaf extract by assessing the effects on cell proliferation, cell death and cell cycles of cancer cell line.

Materials and Methods

Preparation of extracts

Ashwagandha leaves were collected from northern part of India (Rewa, M.P.). These were cleaned with running tap water, sterilized and air dried under shed. Then grounded using laboratory grade grinder to fine powder. The dried powder (100-200 gm) subjected to different solvents like water and methanol and kept for 12 hours at 55-60°C under constant rotating condition in multimode extractor. After filtration with Whatman No.1 filter paper clear supernatant collected and dried under rotatory evaporator to get different solvent extracted powder and stored at -20°C until used for further analysis.

Phytochemicals analysis

Leaf extracts were subjected for phytochemical evaluation by adopting standard methods. After phytochemical analysis we used an advanced HPTLC machine (CAMAG, HPTLC) to separate and purify extracts in a proper solvent system for further experiments. Solvent mixture n-hexane, ethyl acetate and water (7:2:1) were used as mobile phase to separate the plant analytes. Sample application was performed on aluminium TLC plate pre coated with silica gel (5 x 10 cm) by an automatic applicator (Linomat-V). Plate was scanned (UV light: λ_{254} nm) in camag TLC Scanner 3 equipped win CATS Software.

Cell culture

HeLa cell line was purchased from National centre for cell science (NCCS: cell repository), pune, India, and maintained according to standard ATCC guidelines. Briefly, the purchased vial having 5×10^5 cryopreserved cells thaw in room temperature and added 10% FBS containing DMEM media and followed by two time washing (3000 rpm for 5 minutes each). After that, cells were plated into 35 mm petri dish (DMEM media: 10% FBS+0.01% penicillin-streptomycin) and allowed to grown at 37°C in CO₂ incubator till significant confluence (100%) not appeared.

Fractions treatment

After, 2-3 passaged, 0.5×10^5 cells were seeded into lysine coated 96 well microtitre plate for treatment. Different concentrations (0.1, 0.5, 1, 10, 30, 50, 70 and 100 $\mu\text{g/ml}$) of fractions were prepared in DMSO, filtered through 0.5 μm syringe filter, treated to cells and allowed to incubate for 24 hours at same conditions. Viability of vehicle as well as treated cells were assessed using MTT assay.

Cellular viability or proliferation assay (MTT-assay)

The MTT assay is a spectrophotometry based assay for assessing cellular metabolic status. Different cellular

oxidoreductase enzymes may define the cellular metabolic situation which directly correlated with proliferation. These enzymes are capable of reducing the tetrazolium dye MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to its insoluble formazan, which has a purple colour. The more colour conversion indicated more activity. In brief, after treatment for 24 hours, cells were washed with 1XPBS and then exposed to MTT 5mg/ml for at least 2 hours. At end the cell were washed twice with 1XPBS to remove remaining MTT. After that, DMSO was added to each well to fix the colour formation and measured absorbance at 572 nm using microplate reader (BIOTEK, USA). Each absorbance was calculated by subtracting background obtained at A630nm (reference absorbance) i.e. A572nm-A630nm. Result was expressed as percentage of viable cells by using below formula.

$$\text{Percentage of cell viability} = \left\{ \frac{\text{Sample (572-630)}}{\text{Control (572-630)}} \right\} \times 100$$

Calculation of IC50

Inhibitory concentration 50 indicates the concentration of any extraction which fifty percent of population dead over a time period. We calculated IC₅₀ value of each fraction from MTT data. Exact value was calculated by using Graph Pad Prism (Version-5). The percent control, or percent treatment data are represented by Y axis with their minimal and maximal values. The treatment concentrations were represented by X-axis then it automatically calculated IC₅₀ value which supports hills equation and slope. The following formula is.

$$Y = \min + \left(\frac{(\max - \min)}{1 + 10^{((X - \log \text{IC}_{50}) \times \text{Hill slope})}} \right)$$

7-AAD uptake assay

7-AAD is fluorescent, live cell impermeable DNA binding dye. To calculate percentage of live and dead cells upon treatment, we harvested treated cells from media by scraping and/or trypsin digestion and subjected to staining. Briefly, single cells were centrifuged with binding buffer (1X PBS+2% BSA) at 3000 rpm for 5 minutes. This washing step repeated three times. Final pellet was resuspended in 200 μl of binding buffer which contained 5 μl of 7-AAD (5 $\mu\text{M/ml}$). Sample was kept at room temperature for 10-15 minutes and then acquired by flow cytometer. The data was analyzed in accuri C6 software on the basis of fluorescence changes which detected in FL-3 band pass filter and expressed as percent positive cells. Thus, 7-AAD positive cells were considered as dead cells.

Cell cycle analysis

To analyse the different cell cycle phases (G1, S and G2/M) of HeLa cells, we performed the propidium iodide (PI: DNA binding dye) staining. Briefly, single cells (0.5×10^6) were fixed into 70% alcohol (3:1; alcohol: cells) for 12 hours at 4 °C. After fixation, cells were centrifuged at 300g for 5 minutes and removed alcohol. The pellet was washed three times with 1X PBS at 4 °C. Final pellet was resuspended into 200 μl of 1X PBS which contained 50 $\mu\text{g/ml}$ of RNase A (to degrade RNA) and 30 $\mu\text{g/ml}$ of propidium iodide and incubated at 37 °C for 30 minutes. Then, cells were acquired by flow cytometer to use FL-2 (red channel: PI) and results expressed as percent cells presents in different phases.

Statistics

In our experiment mean and variation were calculated for treated and untreated controls. Mean + SEM (standard error mean) were represent in bar graphs. One way analysis of variance was carried out to compare among three experimental groups and confirmed by Bonferroni t test. All studies were repeated for at least three times. Statistical analysis was done in Microsoft excel and Graph Pad Prism (version 5). A p value ≤ 0.05 was considered as significant.

Results

Phytochemical investigation confirmed that methanolic extract contained alkaloids, steroids, tannins and terpenoid along with phenol and flavonoids. Similarly Water extract contained all except steroid and terpenoid. Yellow precipitate in extracts confirmed the presence of alkaloids; similarly, formation of greenish color, brownish color and brown ring in indicated the presence of steroid, tannin and terpenoids. HPTLC investigation of aqueous and methanolic extracts confirmed the one or more flavonoids and phenolics present in multiple samples respectively. Methanolic extract separated in two fractions which was mentioned as fraction A and C and aqueous extract separated as fraction B.

Anticancerous activity of Fraction-A

Anti-cancerous potentials of fraction-A in imparting suppressive action to HeLa cells was evaluated. Among various treated doses of the fraction from 0.1 to 100 $\mu\text{g/ml}$, it was observed that treatment to fraction-A induced cellular death by reducing cell viability to less than 40% in HeLa cell line at concentration of 30 $\mu\text{g/ml}$ (Figure:1A, $p < 0.05$). The calculated IC50 value of fraction A was 30 $\mu\text{g/ml}$. Further, to proof the cell death induced by fraction-A, we performed flow cytometry based 7-AAD uptake assay. It was observed that fraction-A induced significant cell death (35%, $p < 0.05$) with compare to untreated control (4.5%) (Figure: 1, B and C). Thus, the results indicate that fraction-A has anticancer potential.

Anticancer activity of Fraction-B

In this experiment, we checked the anticancer property of

fraction B to compare with fraction A. MTT result shown that fraction B started to reduce the cell survival at concentration 50 $\mu\text{g/ml}$ (Figure:2A, 50% viability, $p < 0.05$) with compare to untreated control. Thus, the IC50 value of fraction B was evaluated at concentration 50 $\mu\text{g/ml}$. Similarly, a significant cell death (37.5%, $p < 0.05$) was observed at that particular concentration with compare to untreated control (Figure: 2, B and C). 7-AAD uptake assay and MTT assay both are indicating significant anti cancer potential of fraction B. However, fraction B having the less anticancer potential with compare to fraction A.

Anticancer activity of Fraction-C

In this experiment, it was observed that fraction C also started to reduce the cell survival at concentration 50 $\mu\text{g/ml}$ (Figure: 3A, 50% viability, $p < 0.05$) with compared to untreated control. Thus, the IC50 value of fraction C was evaluated at concentration 50 $\mu\text{g/ml}$. Similarly, 7-AAD results shown that significant cell death (21%, $p < 0.05$) was observed at that particular concentration with compare to untreated control (Figure: 3, B and C). 7-AAD uptake assay and MTT assay both are indicating significant anti cancer potential of fraction C.

Effects of different fraction on cell cycle distribution in HeLa cells

Decreasing cell proliferation and increasing cell death were noted in cells treated with extract. Further, various fraction induced stress and their impact on cell cycle distribution were checked and observed after 24 hours of treatment. Propidium stained cells clearly shows a significant increase in G0 phase, decrease in G1 phase and arrested in S/G2/M phase with compare to control. (Figure: 4, $**p < 0.05$). Data for different fractions are as follows: For Control group (G0=5.5 \pm 0.7, G1=44.7 \pm 2.5, S=25 \pm 5.5 and G2/M=17.6 \pm 2.5% population), for fraction A group (G0=19.5 \pm 2.7, G1=11.8 \pm 1.5, S=41 \pm 5.5 and G2/M=28.1 \pm 5.5% population), for fraction C (G0=17.5 \pm 2, G1=13.5 \pm 1.5, S=45 \pm 5.5 and G2/M=24.1 \pm 5.5% population) and for fraction B group (G0=21 \pm 1.3, G1=13 \pm 1.5, S=42 \pm 2.5 and G2/M=28 \pm 3.5% population).

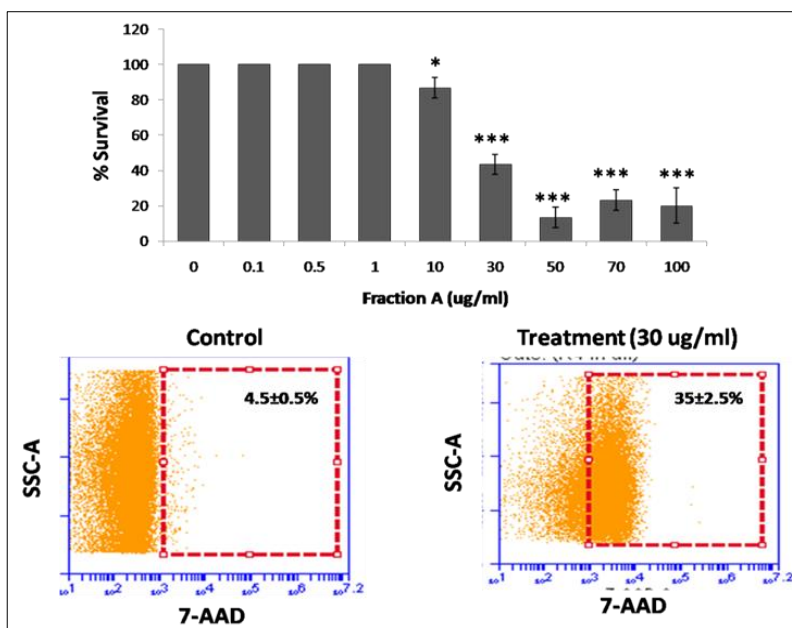


Fig 1: A: Cellular viability as determined by MTT assay (Fraction A). Fraction-A (30 $\mu\text{g/ml}$) induced cellular viability reduced upto 40%. B and C panel shown that % 7-AAD positive cells which were treated with fraction-A. Data is expressed as mean \pm SEM, *, *** $p < 0.05$.

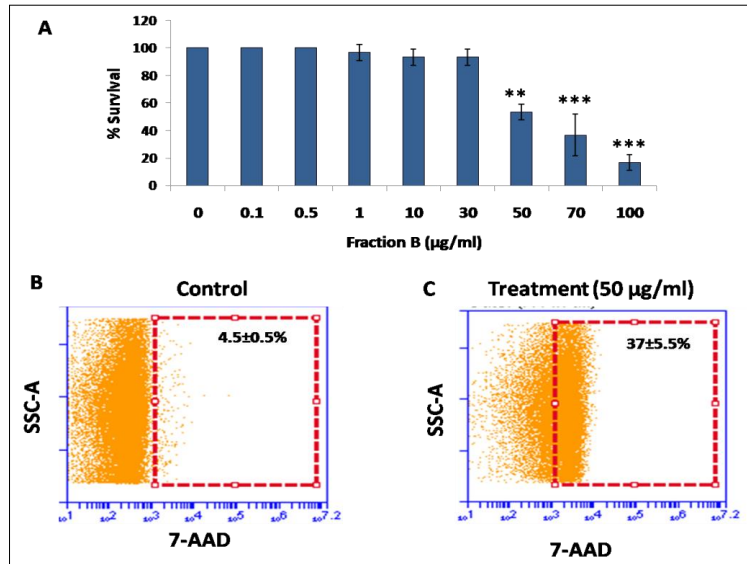


Fig 2: A: Cellular viability as determined by MTT assay (Fraction B). Fraction-B (50µg/ml) induced cellular viability reduced upto 50%. B and C panel shown that % 7-AAD positive cells which were treated with fraction-B. Data is expressed as mean ± SEM, *, ****p*< 0.05.

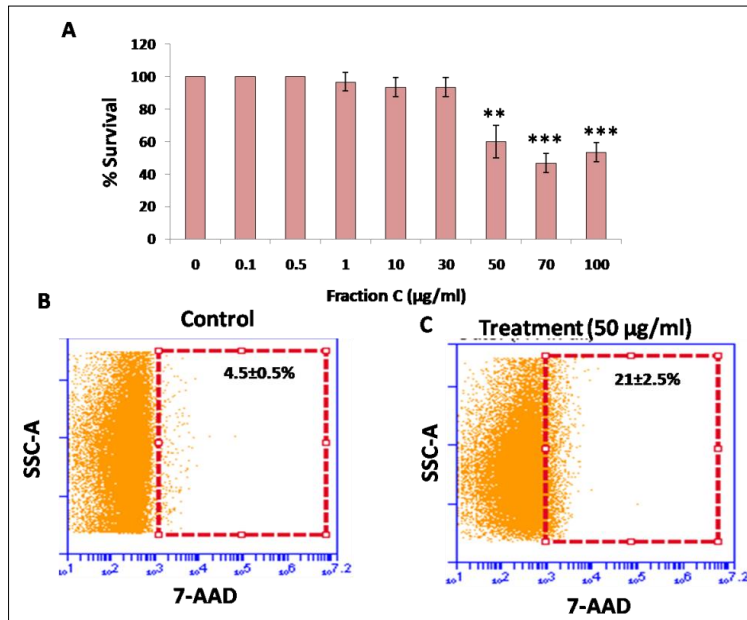
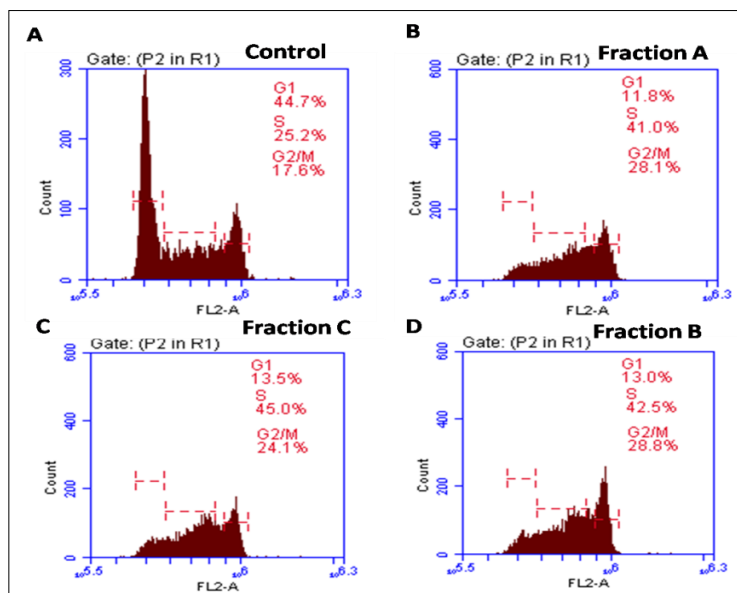


Fig 3: A: Cellular viability as determined by MTT assay. Fraction-C (50µg/ml) induced cellular viability reduced upto 50%. B and C panel shown that % 7-AAD positive cells which were treated with fraction-C. Data is expressed as mean ± SEM, *, ****p*< 0.05.



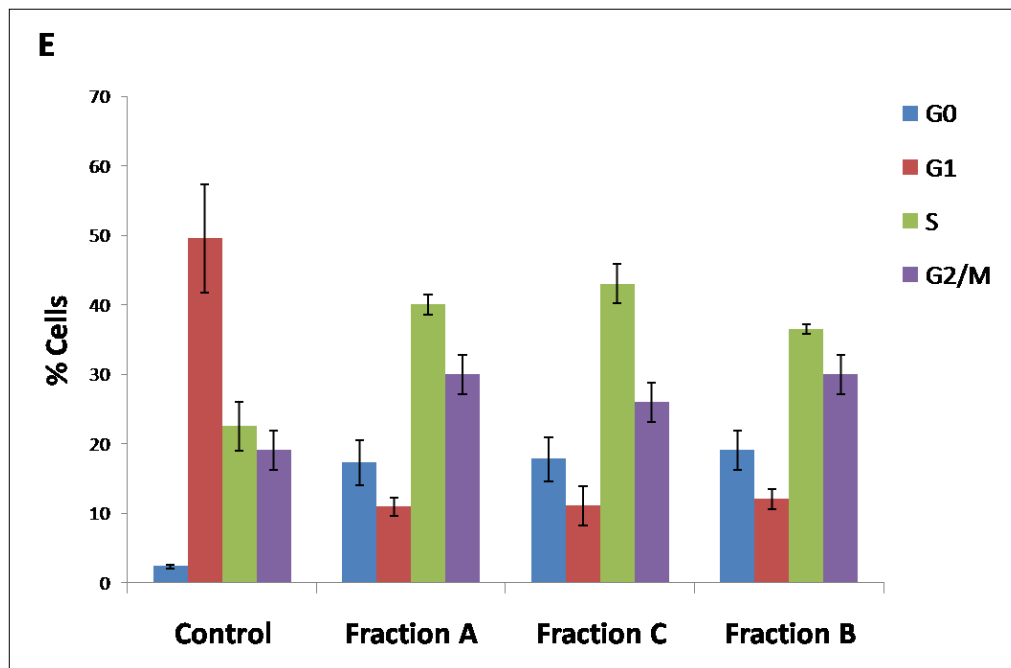


Fig 4: Cell cycle analysis of HeLa treated with different fractions. A: control group B: fraction A group, C: fraction C group and D: fraction B group and E: Bar graph represented the cell cycle dynamics. Data is expressed as mean \pm SEM, * p < 0.05. FL-2-A indicated Propidium uptake area.

Discussion

In the current study we have investigated the therapeutic potential of *Withania somnifera*. Specifically, we have focused on anti tumorigenic or anti cancerous activity of leaf extract. Results obtained indicated that extracts have several different categories of phytochemicals including phenolic, flavonoids, alkaloids, steroid, tannins and terpenoids. Thus, keeping this in perspective, the anti-cancerous potentials of different fractions in imparting suppressive action to HeLa cells was evaluated in a dose-dependent manner where the concentration of the fraction ranged from 0.1 to 100 μ g/ml. Several procedures are reported to check the negative impact of any drugs on cellular survival. Such assays are cell proliferation assays (MTT), apoptosis markers (7-AAD and PI assays) etc. Basically cancer cells are transformed cells and they have the rapid proliferation property and grown on any diverse condition. Thus, it is most important to find a compound which inhibits the proliferation of cancerous cells. In order to investigate such property in the plant extracts we performed metabolic survival assay and 7-AAD uptake assay. In previous Studies it was reported that Withaferin A and Withanolide D are withanolide; a group of flavonoids and steroidal molecules shown anti-tumor activity and radiosensitizing to cancer cells [7]. Further, 1-oxo-5 β , 6 β -epoxy-witha-2-enolide is another component of *W. somnifera* noted to decrease the skin carcinoma induced by UV radiations [8].

As the calculation of IC₅₀ value for an unknown molecule is very important steps for any kind of drug development and clinical studies. In our studies, we evaluated the IC₅₀ value of different fractions. We observed that treatment with fraction-A induced significant reduction of HeLa cells viability and increase death at concentration 30 μ g/ml and thus its estimated IC₅₀ is 30 μ g/ml. Further, to proof the cell death induced by fraction-A, we performed flow cytometry based 7-AAD uptake assay and noted that fraction-A induced significant cell death with compare to untreated control. It supports that fraction A had anticancer potential. Fraction-A

having different flavonoids like components thus it is expected that these flavonoids could be responsible for cell death. Withaferin A (flavonoids/steroids) treatment in mice inoculated with Ehrlich ascites carcinoma cells was noted to suppress tumour growth and accelerate tumour-free animal survival in a dose dependent manner and also decrease the growth of breast, lung, central nervous system and colon cancer in *in-vitro* [9]. In this study we also checked the anticancer effect of fraction B and C. MTT assay result shown that fraction B and C started to reduce the cell survival at concentration 50 μ g/ml with compare to untreated control or the IC₅₀ value of fraction B and C is 50 μ g/ml. 7-AAD uptake assay and MTT assay both are indicating significant anti-cancer potential of fraction B and C. However, fraction B having the less anticancer potential with compare to fraction A. Fractions B and C were shown IC₅₀ value more than fraction A but have the good anti cancer potency.

Next findings of this study indicated that various fraction induce stress given impact on cell cycle distribution which was observed after 24 hours of treatment of Ashwagandha. Treatment significantly increased apoptotic count (G0 phase) and an arrest condition in S/G2/M phase reveals the DNA damage capability of fractions. It is reported that after cellular DNA damage apoptotic cascade is initiate immediately. Thus ashwagandha leaf fractions have significant anti cancer property by modulating cellular homeostasis. However detail mechanism still need to be confirmed in further studies.

Conclusion

Phytochemicals is now considered as nutraceuticals to be an inexpensive, readily applicable, acceptable and accessible approach to cancer control and management. Based on the results, it can be concluded that Ashwagandha aqueous and methanolic leaf extracts were shown significant anticancerous property in a concentration dependent manner against HeLa cells. Decreased level of metabolic cell survival and arrest in cell cycle upon fraction treatment were observed.

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