Research Article **CODEN: AJPCFF** **ISSN: 2321 – 0915**

Asian Journal of Phytomedicine and Clinical Research

Journal home page: www.ajpcrjournal.com

https://doi.org/10.36673/AJPCR.2023.v11.i01.A01

IN VITRO EVALUATION OF ANTICANCER PROPERTIES OF NATURAL COMPOUND ANDROGRAPHOLIDE IN OVARIAN CANCER CELL LINES

Venkateshwari Ananthapur[*1], Shehnaz Sultana[1], Pratibha Nallari[1]

[1*]Department of Cell Biology, Institute of Genetics and Hospital for Genetic Diseases, Osmania University, Begumpet, Hyderabad, Telangana, India.

ABSTRACT

Andrographolide (AND) is a diterpenoid lactone molecule derived from Andrographis paniculate is emerging as a promising molecule for various anti-inflammatory, immunomodulatory hepatoprotective, antiviral and antitumoral effects. The present study aims to investigate anticancer properties of andrographolide in ovarian cancer cell lines (A2780, CP70 and SKOV 3 and OV90)to understand the underlying mechanisms. The results indicated chemotherapeutic activities with growth inhibitor properties by clonogenic and spheroid formation assay and upregulation of DNA damage repair proteins such as FANCD2, FANC J, RAD 51, γH2AX, P53, acetylated P53 and phosphorylated P53 by western blot analysis and induced single and double strand breaks. It also inhibits cell cycle progression by modulating the expression of cell cycle proteins. Thus, the study demonstrated the anticancer and cytotoxic effects of andrographolide in ovarian cancer cell lines and paves way for the development of alternate treatment strategies in ovarian cancer.

KEYWORDS

Andrographolide, Ovarian Cancer Cell lines, Cell cycle, Apoptosis and Cytotoxic.

Author for Correspondence:

Venkateshwari Ananthapur,
Department of Cell Biology,
Institute of Genetics and Hospital for Genetic Diseases, Osmania University,
Begumpet, Hyderabad, Telangana, India.

Email: venkateshwari@yahoo.com

INTRODUCTION

Ovarian cancer is assessed to be diagnosed in more than 225,000 women per year worldwide and remains a significant cause of gynecological cancer mortality (140,000 deaths/y). Unfortunately, the majority of women continue to present at advanced stages due to asymptomatic nature at early stage and the overall 5-year survival rate is around 40%[1]. The existing treatment for newly diagnosed ovarian cancer is a combination of optimal cytoreductive surgery and platinum-based chemotherapy. Advances in treatment strategies such as radical

surgery and chemotherapy have led to improved, albeit modest, clinical outcomes. Despite advances, there remains a significant risk of recurrence and resistance to therapy thus resulting in incurable ovarian cancer. However, the problems of drug resistance (intrinsic and acquired) and the toxic side-effects (*e.g.,* ototoxicity, nephrotoxicity and neurotoxicity) continue to be main factors limiting the efficacy of carboplatin and other platinum drugs. Hence, there is an urgent need for the search of efficient anticancer agents for the treatment of ovarian cancer. One of the possible ways to increase the efficacy of carboplatin in terms of overcoming drug resistance and reducing the side-effects is to combine it with a potent bioactive compound having different modes of antitumor action[2]. Platinum resistance is allied with anti-apoptotic factors and pathways such as (NF-κB) and (AKT), a number of phytochemicals have been shown to reduce expression of such proteins so that tumor-active phytochemicals might be perfect candidates for such combination.

Plant-derived natural products play an essential role in the area of cancer chemotherapy. Molecules such as vincristine, vinblastine, paclitaxel, camptothecin derivatives, epipodophyllotoxin and so forth, are precious contributions of nature to modern medicine. However, the pursuit to find out new therapeutic compounds for cancer treatment and management is a never-ending venture; and diverse plant species are persistently being studied for identification of prospective anticancer agents.

Andrographis paniculata nees, a well-known plant of Indian and Chinese traditional system of medicine, has drawn attention of researchers in recent times. Andrographolide, the key bioactive chemical component of the plant has shown credible anticancer potential in several investigations around the world. Andrographolide (AND) is a diterpenoid lactone molecule that possesses various biological activities, including anti-inflammatory[3], immunomodulatory[4], hepatoprotective[5], antiviral[6] and antitumoral effects[7]. Andrographolide treatment blocked the *in vitro* proliferation of a variety of tumor cell lines, such as neuroblastoma, melanoma, hepatoma, prostate cancer, and gastric cancer[8-11]. This compound exerts anticancer activity on tumor cells by several mechanisms, such as cell-cycle arrest[12], growth factor signaling modulation, cellular migration[13]and angiogenesis. Several *in vitro* studies have been demonstrated the capability of andrographolide compound of inducing cell-cycle arrest and apoptosis in a variety of cancer cells at different concentrations. It alsopossesses potent immunomodulatory and anti-angiogenic activities in tumor tissues[14]. In view of the above, the present study is aimed to determine the anti-proliferative and cytotoxic effect of andrographolide administered alone and in combination with carboplatin in various ovarian cancer cell lines.

METHODS

Cell lines and antibody

The SKOV3 human ovarian cancer cell lines were cultured in eagle's medium, A2780 and CP70 cell lines were cultured in RPMI medium and complemented with 10% FBS, 100µg/ml streptomycin sulfate and 100U/ml penicillin. Cells were regularly tested for mycoplasma contamination by mycotest kit (*Invitrogen*) and cells within 10 passages were used in the experiments. Andrographolide and Carboplatin (Sigma, St. Louis, MO) stock solutions were prepared by dissolving in anhydrous DMSO and stored at -20°C. The stock solutions were further diluted to required concentration before adding to the cells. Antibodies to the following antigens used in this study include; FANC D2, FANC J, p53, acetylated p53, phosphorylated p53, RAD 51 and GAPDH (Santa Cruz Biotechnology, Inc) and γH2AX (Millipore). The secondary antibodies include anti-mouse IgG-FITC, anti-rabbit IgG-FITC (Molecular Probes).

Clonogenic survival assays

Cells were plated in triplicates in 6-well dishes and allowed to attach for 16 hours and treated with designated concentrations of the therapeutic agents and allowed them to form colonies by changing medium every three days. Later fixed in methanol

and stained with crystal violet and the colonies having more than 25 cells were counted using Gene Tools, Syngene Imaging system[15].

Cell cycle analysis by flow cytometry

Cell cycle profiles were analyzed by flow cytometry, after 12 hours and 24 hours of exposure with DMSO, andrographolide, carboplatin and combination of both, cells were harvested by fixing in ice cold 70% ethanol and stained with propidium iodide (PI)[15].

Sphere formation assays

Log phase ovarian cancer cells were harvested by trypsinization, counted and then seeded in ultra-low attachment 6 well dishes at 1000 to 10,000 cells/well. The cells were allowed to grow and form spheres[16]. Later the counted cells were dispersed as single cells, cultured in stem cell-specific serum free media (2mL) in an ultra-low attachment six well plates for 10-12 days. The media (1:1 DMEM/F-12) was added with 1% penicillin streptomycin, B27 and N2 supplements (Gibco) and growth factors [recombinant human epidermal growth factor (EGF) and fibroblast growth factor (FGF) *Invitrogen*] to support stem cell growth and the cells proliferate to form floating single cell cloned spheres. The cells were checked every day after seeding to confirm that spheres were forming as a result of multiplication from a single cell and not due to cell adherence. Every 72 h the fresh media containing growth supplements EGF (20ng/ml) and FGF (20ng/ml) was added. The spheres containing >50 cells were scored as large (true stem cell spheres), while spheres <50 but>15 cells were small spheres.

Western blotting

Cells were exposed to the specified agents and proteins after whole cell lysates were prepared subsequently washing the cells with ice cold PBS. Cells were lysed in ice-cold cytoskeletal (CSK) buffer freshly added with protease and phosphatase inhibitors (Roche). Samples were prepared in 4x SDS-PAGE sample buffer and heated to 100°C for 15 min after normalizing the protein concentrations. Before transferring to nitrocellulose membranes, denatured samples were resolved by SDS-PAGE. Membranes were incubated with indicated antibodies followed by respective HRP-conjugated secondary antibodies and blots were developed by chemiluminescence detection kits[17].

Apoptosis assay

Apoptosis assays were done by treating the cells with andrographolide, carboplatin and combination of both for a duration of 48 hours. Cells enduring apoptosis were measured after labeling with PE-annexin-V apoptosis detection kit (Dead Cell Apoptosis Kit with Annexin V Alexa Fluor® 488 and PI as per the manufacturer instruction (Life technologies Inc.) and analyzed by flow cytometry (BD Bioscience)[18].

Comet Assay

The comet assay was done as described by Singh *et al* (1988). Cells were seeded in tissue-culture plates and incubate for 24 h for cell attachment and treated with increasing concentration of doxorubicin hydrochloride and *E. hirta* for 24 h. After harvesting the cells by trypsinization, washed with PBS and resuspended in ice-cold PBS. About 7.5µL of the resuspended cells were mixed with 75µL of low melting point agarose at 37°C and the suspension spread over the well with the pipette tip. The slides were placed at 4°C in the dark till gelling occurred and then immersed in pre-chilled lysis buffer at 4°C. The buffer was aspirated and replaced with pre-chilled alkaline solution for 30 min at 4°C after 60 min incubation. After lysis and unwinding the slides were positioned in a horizontal electrophoresis tank filled with newly prepared alkaline electrophoresis buffer, then the slides were transferred to pre-chilled distilled water and immersed for 2 minutes and aspirated, repeated twice and replaced with cold 70% ethanol for 5 min. Afterward, the slides were allowed to air dry and 100µL/well of diluted Vista Green DNA dye was added to each slide for 15 min in the dark at room temperature for DNA staining. DNA migration was seen by means of fluorescence microscope at a magnification of 10X (Carl Zeiss Apo Tome, Germany). 100 randomly selected cells (50 cells from each of the two replicate slides) were analyzed for each concentration[19].

RESULTS AND DISCUSSION

Andrographolide exhibits chemotherapeutic activities against ovarian cancer cell lines

To assess the antineoplastic activities of andrographolide against human platinum sensitive and platinum resistant ovarian cancer cell lines, A2780, CP70 and SKOV 3 and OV90, cells were treated with different concentrations of andrographolide (2, 4, 6, 8 and 10μM) and 10μM carboplatin and their effects on tumor cell growth were measured by clonogenic survival assay. Clonogenic potential of the cell lines were significantly affected by andrographolide and carboplatin in a concentration dependent manner. The A2780, SKOV3 and CP 70 cell lines exhibited superior growth inhibitory properties than OV 90 cell line with an IC 50 value of 3μM for A2780, 4μM for CP70 and SKOV3 and 6μM for OV90 cell line. These inherent differences in sensitivities between the cell lines may be due to the genetic and epigenetic profile (Figure No.1).

Andrographolide treatment induces G2/M arrest in OC cell lines

To gain further insight into the mechanism of their antiproliferative activities, OC cell lines were treated with DMSO, 15μM andrographolide, 20μM carboplatin and combination of both andrographolide and carboplatin, and their effects were assessed for cell cycle progression at two time points i.e., 12 and 24 hr. post treatment. Exposure of OC cells to andrographolide and carboplatin attenuated cell progression through G2/M phase and S phase in 12 and 24 hr. treatment compared to DMSO treated cells respectively. A combination of andrographolide and carboplatin revealed an increased G 2/M phase indicating the accumulation of cells in this phase. A similar pattern of cell cycle distribution was observed in A2780 and Cp 70 cell lines. Such an observation indicates differential binding affinities to their targets. However, their ability to arrest at G2/M indicated that andrographolide may interfere with DNA replication directly or they may induce replication associated DNA damage which may in turn activate checkpoint responses to constrain the progression of cell cycle (Figure No.2).

Andrographolide induces DNA damage response in ovarian cancer cell lines

Western blot Analysis was carried out to check for the time kinetics of andrographolide on the expression of DNA damage repair proteins in OC cell lines by treating 15μM andrographolide at different time intervals (2hr, 4hr, 8hr and 12 hr.) and 20μM carboplatin for 12 hr. A gradual increase in the expression of DNA damage repair proteins such as FANCD2, RAD 51, p53, Acetylated p53 phospho p 53, γH2Ax was observed in a time dependent manner.

Western blot Analysis was carried out to check for the concentration gradient of andrographolide on the expression of DNA damage repair proteins in OC cell lines by treating different concentrations of andrographolide (1μM, 5, 10, 20, 40μM)) and 20μM carboplatin for 12 hr. and found gradual increase in the expression of FANC D2, pChk1, pChk2 and γH2AXin a concentration dependent manner from 1μM to 40μM.

Combination experiments were also carried out by treating the OC cells with 15μM andrographolide, 20μM carboplatin and a combination of both andrographolide and carboplatin for 12 hr and looked for the expression of DNA damage repair proteins. An increase in the expression of DDR proteins such as FANC J, FANCD2, RAD 51, γH2AX, P53, acetylated P53 and phosphorylated P53 indicating its role in the regulation of expression of DDR proteins (Figure No.3).

Andrographolide induced apoptosis in ovarian cancer cell lines

A2780 cell lines were exposed to andrographolide, carboplatin and combination of both andrographolide and carboplatin for 48 hours which resulted in increased apoptotic cells in the treated cells compared to control cells, thereby indicating the apoptotic activity of andrographolide in cancer cell lines (Figure No.4).

Andrographolide inhibits the Spheroid formation in ovarian cancer cell lines

Stem cell spheroid formation assay was also performed to check for the inhibitory efficiency of andrographolide in ovarian cancer cell lines such as A2780, CP70 and SKOV3. The study revealed the formation of equal number of small (< 50 cells in a sphere) and large spheres (≥ 50 cells in a sphere) in the control cells of A2780 and CP70 whereas the number of large spheres is less compared to small spheres in SKOV3 cell line. Such an observation indicates cell line specific formation of spheroids. Treatment of andrographolide in A2780 and its resistant cell line CP70 revealed reduction in number of large and small spheres in a concentration dependent manner from 2μM to 10μM. There were no large spheres in the cells treated with 10μM conc of andrographolide. Similar results were observed in SKOV3 cell line with the absence of large spheres at a conc of 8μM (Figure No.5).

Andrographolide induces both single and double strand breaks as evident by comet assay

Comet assay was evaluated in the cells treated with DMSO and andrographolide to check for the DNA damage. Comet tails were observed in the cells treated with 15μM concentration of andrographolide compared to the control cells indicating the cytotoxic nature of andrographolide (Figure No.6 A, B).

Discussion

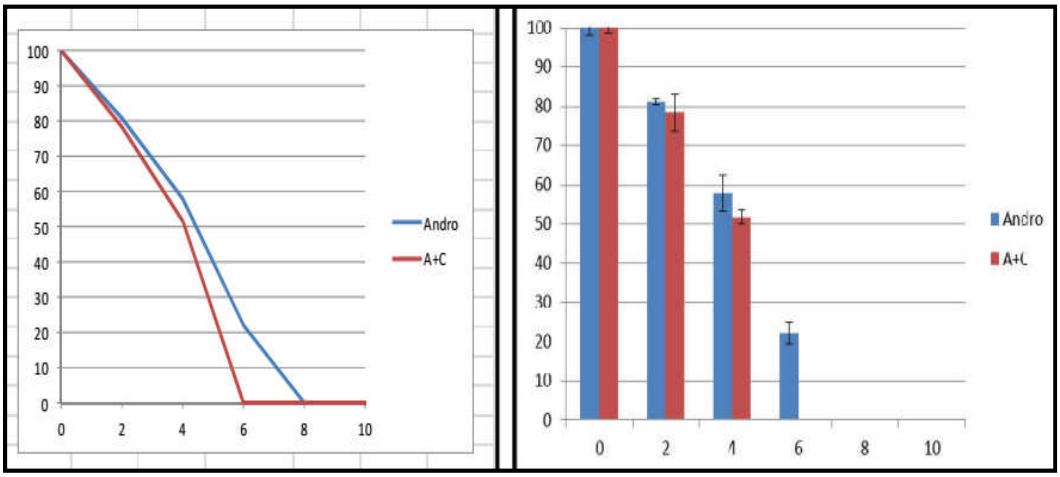
Natural anticancer drugs derivative from plants are considered to be very effective with limited side effects[20].Andrographolide inhibits MV4-11 cell proliferation through inhibition of fatty acid synthesis, iron uptake, protein synthesis and FLT3 signaling pathway in a dose- and time-dependent manner[21]. In the present study clonogenic potential of the cell lines were significantly affected by andrographolide and carboplatin in a concentration dependent manner. Andrographolide induces cell cycle arrest at G0/G1 stage in cancer cells[22]. In human acute myeloid leukemic HL-60 cells, increase in G0/G1 phase cells and substantial decrease in cells at S and G2/M phase was observed after andrographolide treatment (12μg/ml) for 36 h[23]. Through modulating the expression of cell-cycle related proteins andrographolide inhibits cell cycle progression. The initiation of cell-cycle inhibitory proteins p16, p21, p27 associated with decreased expression of cyclin A, cyclin D, CDK4 and CDK2, required for G1 to Stransition results in the initiation of cell-cycle arrest at G0/G1[10,24]. Inhibition of human colorectal carcinoma Lovocells were reached by andrographolide treatment (10-30μM)[24].

Jieliang *et al*, (2007) studied the cytotoxicity of andrographolide towards the HepG2 human hepatoma cells and found that the growth of HepG2 cells was exaggerated in the presence of andrographolide. Andrographolide also induced cell cycle arrest at G2/M phase and a late apoptosis of the cells through the collapse of mitochondrial membrane potential (MMP) and an intracellular increase of hydrogen peroxide (H2O2) but adecrease of superoxide radicals (O2−) and reduced glutathione[25].In the present study, exposure of ovarian cancer cells to andrographolide and carboplatin attenuated cell progression through G2/M phase and S phase in 12 and 24 hr treatment, which indicates that andrographolide may interfere with DNA replication directly or they may induce replication associated DNA damage which may in turn activate checkpoint responses to inhibit the progression of cell cycle. The exact molecular target of andrographolide that blocks the G1 stage is yet to be known. Ingrid *et al* (2019) studied the efficacy of andrographolide in prostate cancer by means of both *invitro* and *invivo* models, were androgen-independent (PC3) and androgen dependent (22RV1) cell lines treated with andrographolide. In *invitro*, andrographolide decreased prostate cancer cell migration, invasion, and increased cell apoptosis. The prostates of severe combined immunodeficient (SCID) mice were injected with 22RV1 and the mice were treated three times per week with andrographolide 10mg/kg and assessed for the tumor growth in orthotopic xenograft model. Tumor volume, MMP11 expression and blood vessels formation was

decreased *in vivo* through andrographolide. In tumors treated with andrographolide, gene expression analysis identified altered major molecular and cellular functions such as cellular compromise, cell cycle and "DNA recombination, replication and repair". In DNA repair genes, increased expression of genes involved in DNA double strand break repair were observed. Altogether which shows that andrographolide inhibits prostate cancer by promoting DNA damage[26].In the present study we have checked the time kinetics and concentration gradient of andrographolide in ovarian cancer cell lines and observed a gradual increase in the expression of DNA damage repair proteins FANCD2, pChk1, pChk2, γH2Ax in a time and concentration dependent manner. Tadanobu *et al*, (2021) demonstrated the enhanced anti-cancer activity between andrographis paniculata and oligomeric proanthocyanidins (OPCs) in colorectal cancer in terms of their ability to inhibit cancer cell growth, suppress colony formation and induce apoptosis. The results were further corroborated in subcutaneous xenograft model and in patient derived primary epithelial 3D organoids.

Involvement of metabolic pathways and ferroptosis-associated genes, including HMOX1, GCLC and GCLM, which might be responsible for the increased anti-tumorigenic activity by andrographis paniculata and oligomeric proanthocyanidins were identified by transcriptomic profiling[27].

Andrographolide can inhibit cholangiocarcinoma (CCA) cell migration by suppression of claudin-1 through the activation of p-38 MAPK signaling pathway[28]. The present study evaluated the inhibitory efficiency of andrographolide in ovarian cancer cell lines such as A2780, CP70 and SKOV3, and the results indicated cell line specific formation of spheroids. Andrographolide shows cytotoxicity towards the cancer cells through the NFkB signal transduction pathway without persuading pyroptosis and blocks breast and ovarian cancer invasion by hindering MMP-7 expression through TIMP1 up-regulation and has the potential to be developed as a drug targeting ovarian and breast cancers[29].In conclusion, the present study highlights the role of andrographolide as cytotoxic and anti-tumorigenic nature of the compound in the causation of DNA damage in ovarian cancer cell lines.



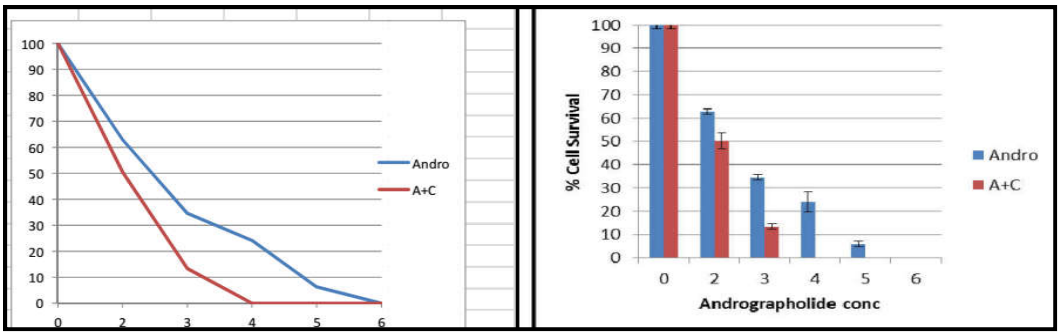
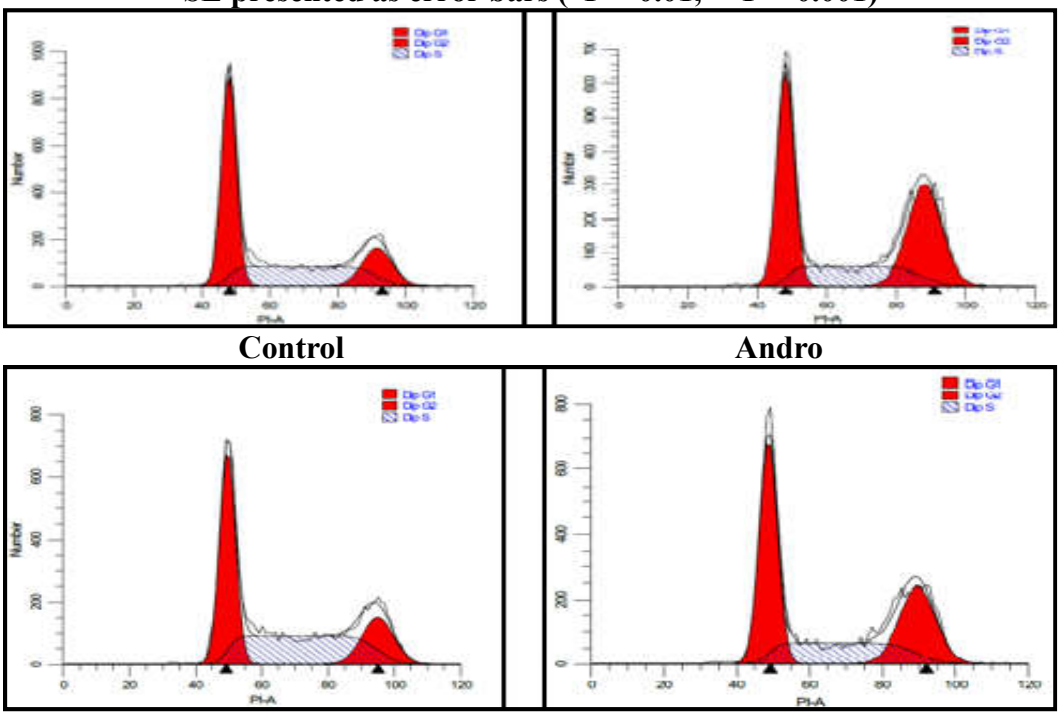


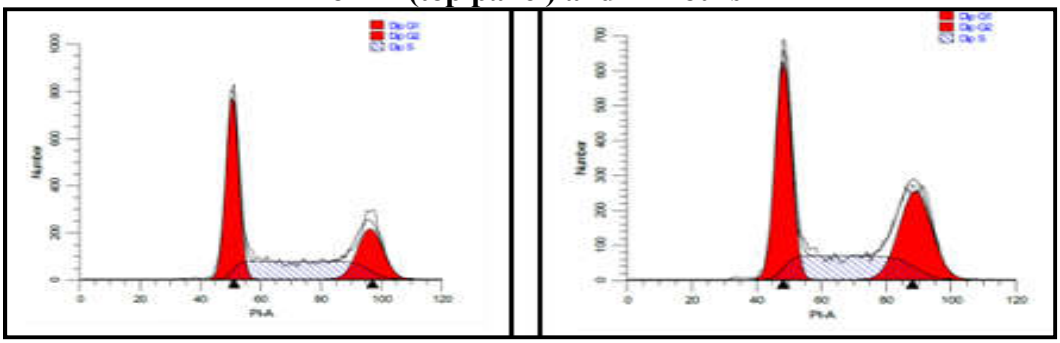
Figure No.1: Clonogenic assay-andrographolide and carboplatin exhibit cytotoxic effects to ovarian cancer cells, Clonogenic survival assays show andrographolide and carboplatin inhibits survival of A2780 (A), CP70 (B) cells in a dose dependent manner. Data presented are an average of triplicates and ± SE presented as error bars (*P < 0.01, **P < 0.001)

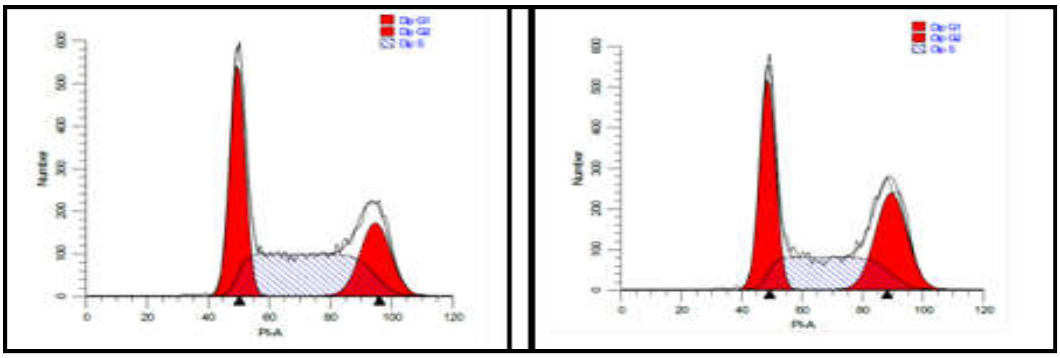


Control **Andro**

Carboplatin **Andro+ Carbo**

Figure No.2: Cell cycle analysis in CP70 at 24 hr. Andrographolide induces G2/M arrest CP 70 cells which were exposed to 15µM andrographolide, 20µM carboplatin and combination of Andro and Carbo for 12 (top panel) and 24 hours





Andrographolide induces DNA damage response in ovarian cancer cell lines

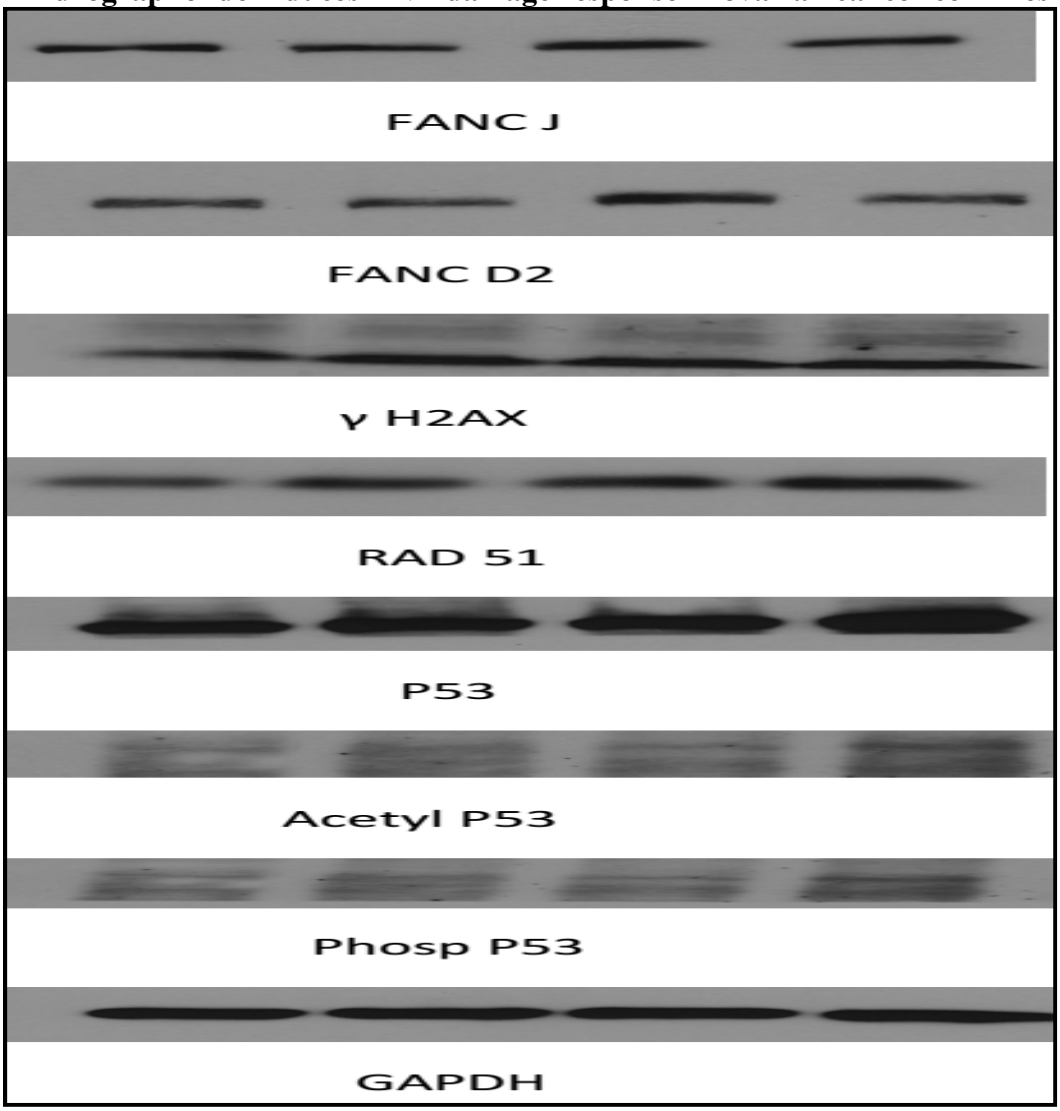


Figure No.3: Andrographolide exposure induces replication associated DNA damage and activates cell cycle checkpoints in A2780cells. Exponentially growing A2780cells (A) were exposed to 15µM andrographolide, 20µM carboplatin and combination of Andro and Carbo for 12 hr and cell lysates were prepared after indicated times. The normalized proteins were resolved on SDS-PAGE and blotted for different DDR proteins

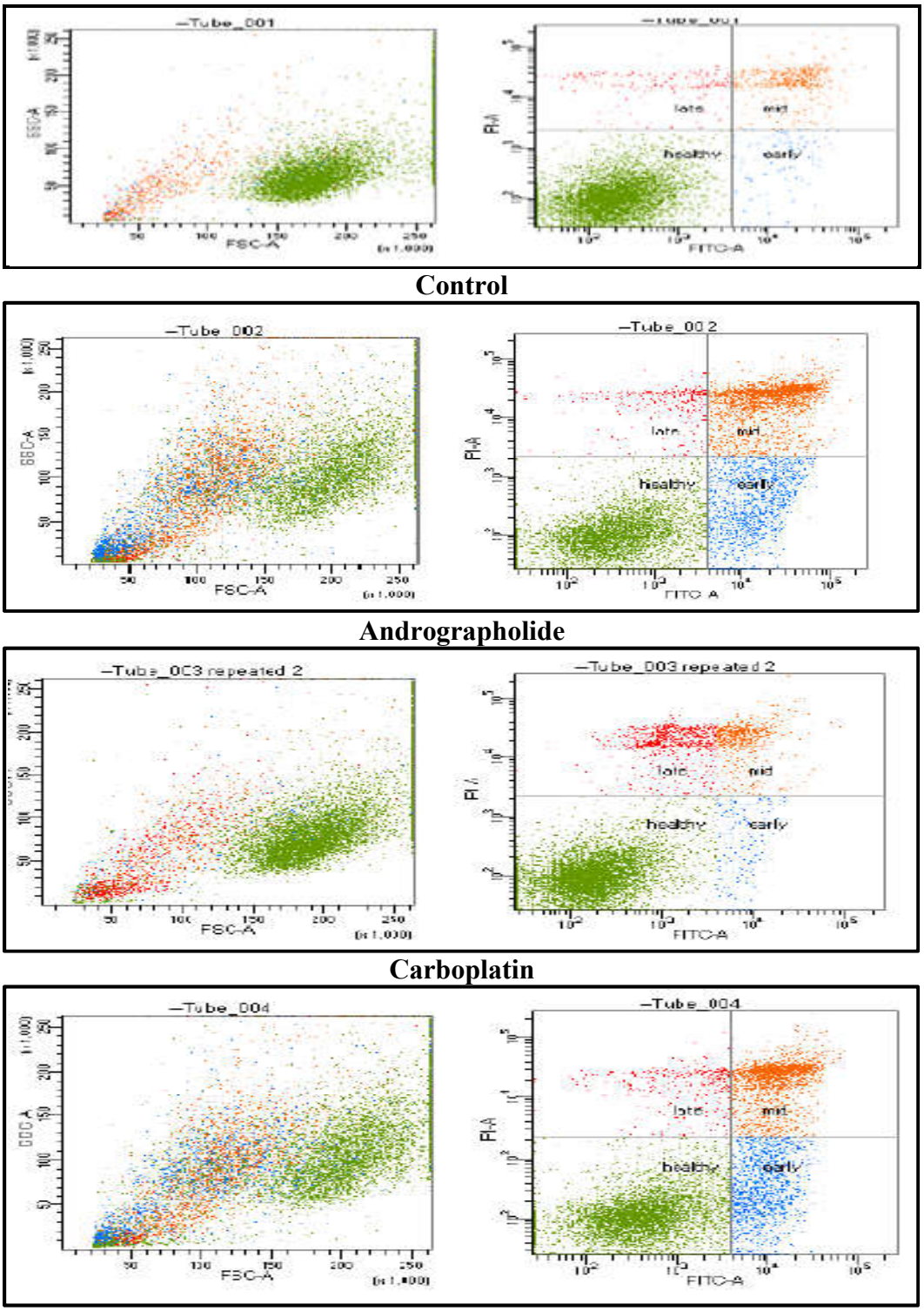
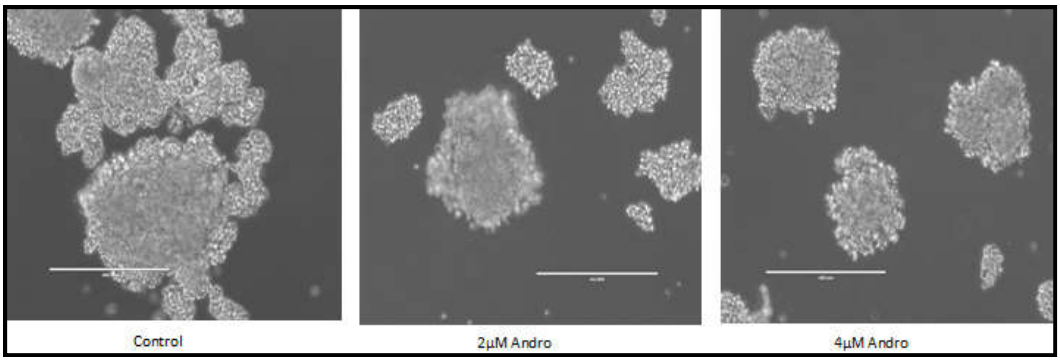


Figure No.4: Andrographolide-induces apoptosis in ovarian cancer cells. A2780 (top panel) cells were exposed to andrographolide, carboplatin and combination of andrographolide and carboplatin for 48 hours and cells were co-stained with PI and Annexin V antibody and analyzed by flow cytome



(A2780)

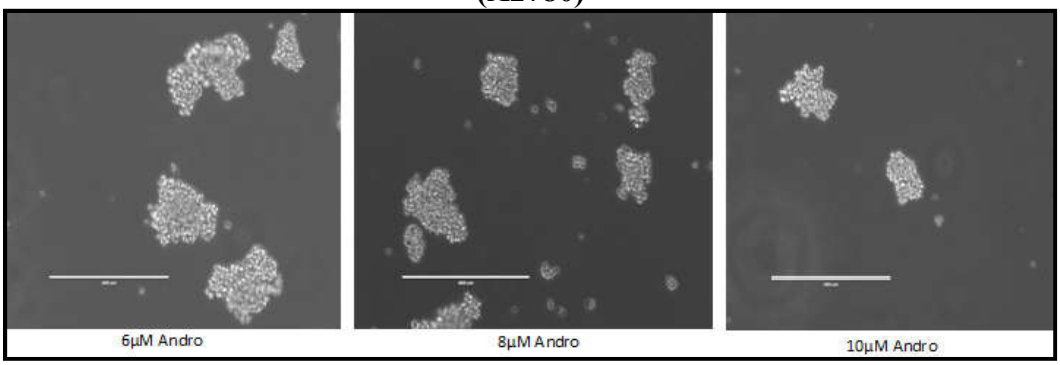
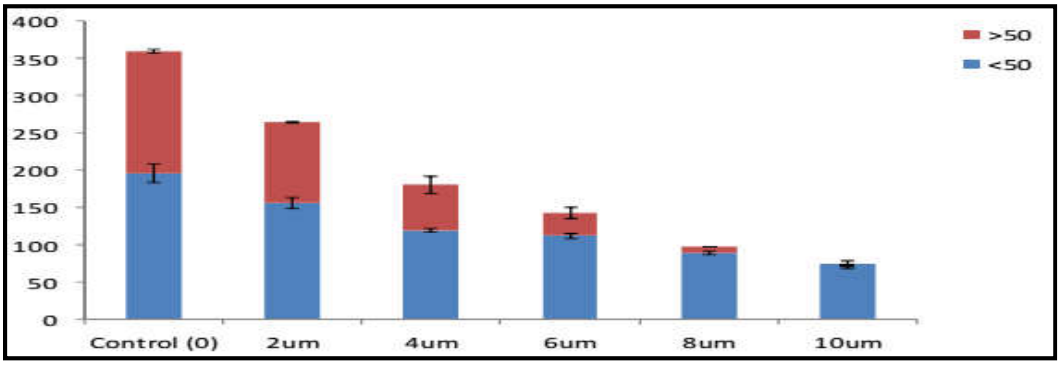
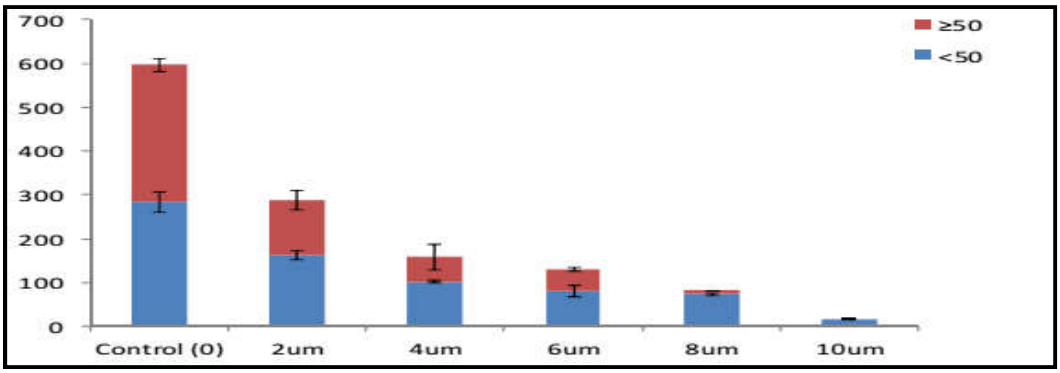


Figure No.5: Spheroid formation; A. Isogenic platinum-sensitive (A2780) platinum-resistant (A2780/CP70) ovarian cancer cells were tested for capacity for anchorage-independent growth

A2780



CP70



SKOV3

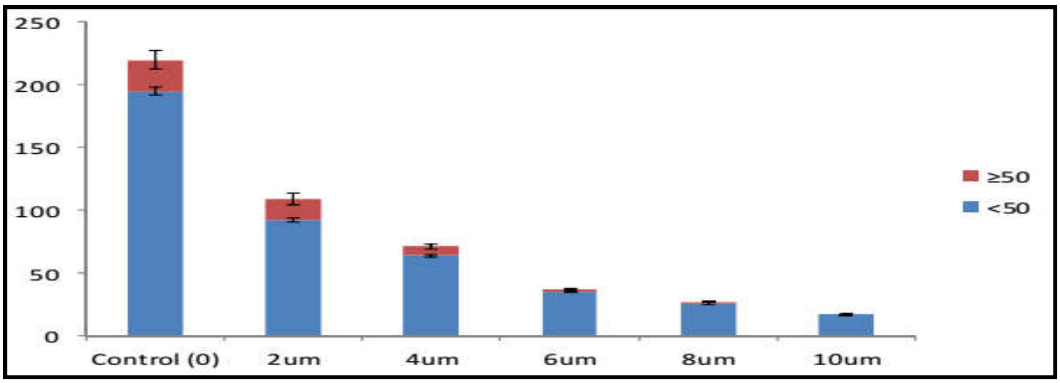


Figure No.5B: The large (>50 cells) and small (<50 cells) OC spheres formed by each cell line were counted and average value presented

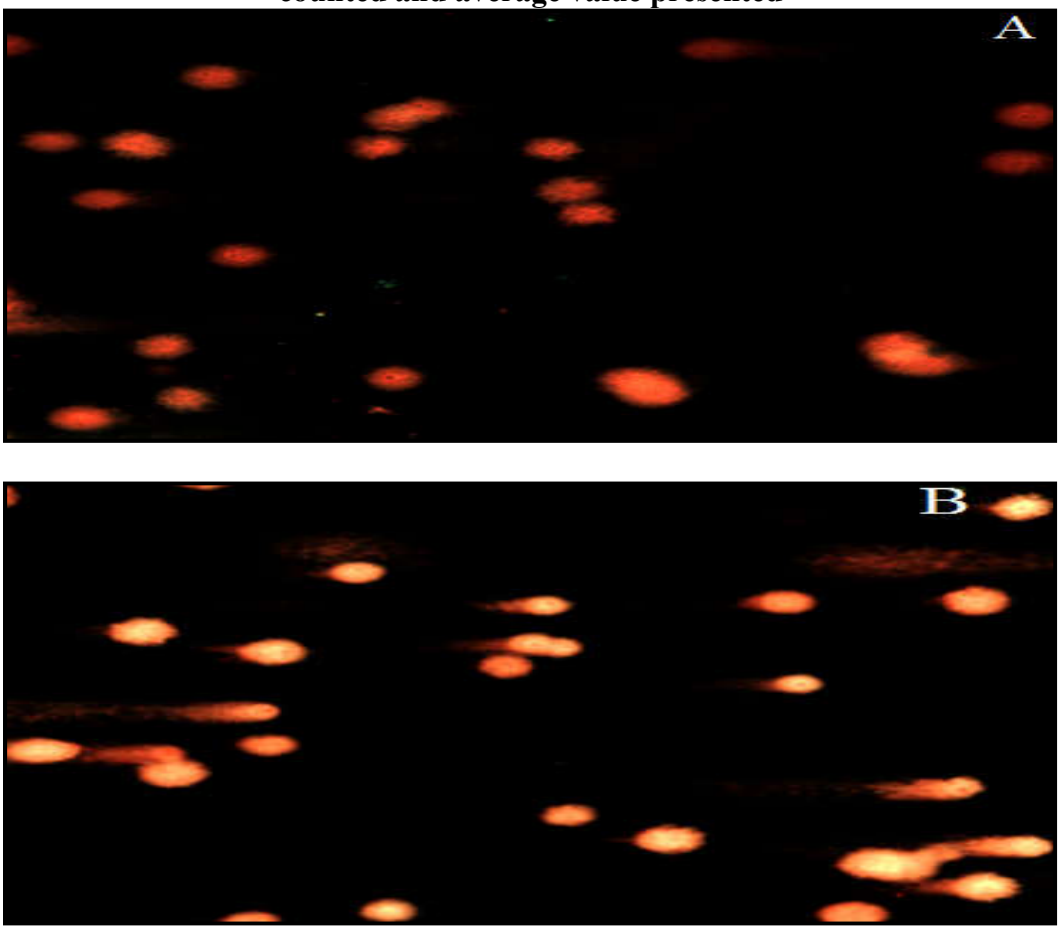


Figure No.6: Comets were observed in the cells untreated (A) and treated (B) with andrographolide indicating the single and double strand DNA breaks in the cells thereby suggesting genotoxic nature

CONCLUSION

The study indicates that andrographolide might be useful as a possible chemotherapeutic agent for ovarian cancer.

CONFLICT OF INTEREST

None to declare.

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Please cite this article in press as: Venkateshwari Ananthapur *et al*. *In vitro* evaluation of anticancer properties of natural compound andrographolide in ovarian cancer cell lines, *Asian Journal of Phytomedicine and Clinical Research,* 11(1), 2023, 1-13.

Available online: www.uptodateresearchpublication.com January – March13