

Apoptosis induction and anti-inflammatory activity of polyherbal drug AS20 on cervical cancer cell lines

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SUMMARY

Cancer occurs when normal cells convert into tumor cells as a result of the interaction between a patient's genes and physical, chemical, or biological carcinogens. There are more than 100 types of cancer and each type requires a specific treatment regimen. The traditional treatment regimen for most types of cancer includes surgery, radiotherapy, and chemotherapy. However, treatment methods such as chemotherapy not only kill the cancer cells but also kill healthy cells that divide quickly. Chemotherapy induces widespread senescence, which contributes to local and systemic inflammation. To overcome these problems herbal drug treatments can be used as complementary and alternative medicines (CAMs). We obtained the polyherbal drug AS20 using *Amaranthus spinosus* leaves and inflorescences, which contain phytochemicals such as saponins, polyphenols, flavonoids, and alkaloids that are known to have anticancer and anti-oxidant activities. We hypothesized that AS20 would show anti-inflammation and induce DNA fragmentation, which is a hallmark of apoptosis in HeLa cells. Furthermore, this study explored the effects of AS20 on the expression of COX2, a marker of inflammation. We found that treatment with AS20 suppressed phorbol 12-myristate 13-acetate (PMA) and 5-fluorouracil (5-FU) induction of COX2 expression. We also observed AS20 treated cells showed DNA fragmentation in HeLa cells. This research looks into AS20's possible anti-inflammatory effect on cervical cancer cell lines and its capacity to trigger apoptosis, a programmed cell death mechanism that can restrict cancer cell proliferation. These elements are examined in order to offer light on the therapeutic potential of AS20 and aiding the development of safer and more efficient cervical cancer treatment plans.

INTRODUCTION

Cancer is one of the key contributors to the global public health crisis, with 10 million deaths reported in 2020 alone (1). Studies based on cervical cancer have lately become more relevant as a result of its extremely high occurrence rate, which ranges from 10% to 40%. (2). While the optimal treatment options are heavily contested, some of the most frequently employed routes include radiotherapy, chemotherapy, and surgery (3). These intervention methods target malignancies, causing them to shrink enough to be suitable for surgical tumor removal. Among many other chemotherapeutic drug

options, 5-fluorouracil (5-FU) is one of the most common antineoplastics employed (4). Although these standard-of-care treatments have demonstrated significant efficacy in the past, they pose a high risk of creating chemoresistant cancer cells (5). Furthermore, these treatments have demonstrated both desirable and undesirable effects when applied to *in vivo* studies, ultimately diminishing their impact (6). Moreover, chemotherapy has been correlated with poor patient quality of life and high cancer recurrence rate (7).

The downsides of conventional cancer treatments have provoked discourse in seeking more viable alternatives and highlight the need for a more nuanced understanding of cancer mechanisms. Approaches based on the seed and soil theory show promise (8, 9). The theory maintains that the extent of tumor metastasis depends on crosstalk between the cancer cells and organ microenvironments, giving rise to a tumor microenvironment (TME) that facilitates cancer progression (10). The therapeutic impact on healthy cells may be mitigated by TME changes, which would lessen the burden of chemotherapy (11).

Integrative oncology studies consider the efficacy of natural products alongside conventional treatments. Medicinal plants are a key component in complementary and alternative medicines (CAM) studies due to their anti-cancer and anti-inflammatory effects (12, 13). These aspects can be attributed to plant secondary metabolites that cannot be synthesized chemically, such as saponins, flavonoids, and polyphenols (14). *Amaranthus spinosus* is one such plant that is widely used in traditional medicine for viral diseases, malarial, bacterial, and helminthic infections, and as a potent snake venom antidote, particularly in south Asia (15, 16).

Traditional anticancer medications can cause apoptosis, also known as programmed cell death (17). In this mechanism, cells break down into apoptotic bodies with well-preserved organelles. In addition, cell condensation and budding are some morphological alterations that occur (18). Apoptosis induction is one of the most crucial indicators of efficacy for cytotoxic anticancer drugs. DNA fragmentation is a hallmark of apoptosis (19). DNA laddering or DNA fragmentation is a characteristic of DNA where caspase-activated DNase (CAD) plays a crucial part in the apoptosis process and breaks down DNA at certain internucleosomal linker sites (20).

COX2 is an enzyme that is a key mediator of inflammatory pathways (21). Induced COX2 expression has been found in several cancers (21). Some studies showed that phorbol 12-myristate 13-acetate (PMA) is a tumor promoter that can induce inflammation. Possible effects of PMA include activation of PKC, activation of the Ras/Raf-1/ERK1/2 pathway, activation of NF- κ B, and induction of COX2 expression (22, 23). 5-fluorouracil (5-FU) is a chemotherapeutic drug used

to treat various cancers. 5-FU releases prostaglandin E2 (PGE2) which upregulates COX2 (24, 25).

The rationale of using 5-FU and PMA was to see the induction of COX2. COX2 was chosen as a particular gene of interest due to its potent roles in tumor progression and inflammation *in vitro* and *in vivo*, which made it an apt target for determining the efficacy of AS20 (26, 27). AS20, herbal formulation made up of leaves and inflorescence of *A. spinosus* (28, 29). In this study, we used AS20, the cytotoxic effects of which on HeLa cells have been previously reported (28, 29). We hypothesized that AS20 would cause DNA fragmentation in HeLa cells, a hallmark of apoptosis, and would have anti-inflammatory characteristics measured by COX2 expression level. We observed that HeLa cells treated with AS20 had DNA fragmentation. We also observed that AS20, has demonstrated promising results in reducing COX2 expression that has been brought on by the drugs 5-FU and PMA. Our findings substantiated impacts of AS20 in restricting cancer cell growth, we hope to elucidate the mechanisms in greater detail in future investigations.

RESULTS

Cell viability

Mesenchymal stem cells (MSCs) were used as a healthy cell line control for checking the percentage viability (cell health) using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Under specific conditions, NAD(P)H-dependent cellular oxidoreductase enzymes may serve as an indicator of the quantity of live cells. The tetrazolium dye MTT can be converted by these enzymes to its insoluble, purple form. After the incubation, MSCs showed more than 90% cell viability using different concentrations of AS20 (n=3) (Figure 1).

DNA fragmentation assay

DNA was extracted from treated and non-treated HeLa cells. DNA laddering was observed in HeLa cells after treatment with AS20 drug. However, no laddering was observed in untreated HeLa cells by using gel electrophoresis (Figure 2).

Inflammatory gene expression using semi-quantitative PCR

COX2 is implicated in the NF-κB-driven inflammatory cascade (30). A refractory phenotype of cancer and chronic inflammation are both suggested by increased

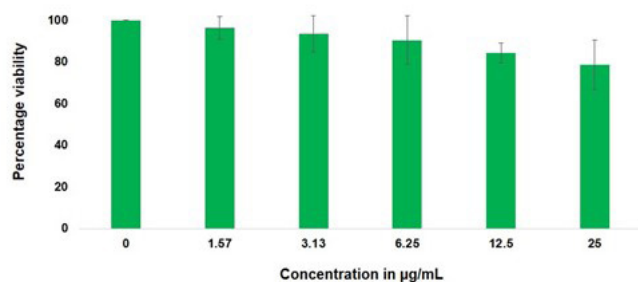


Figure 1. MTT assay for cell viability. MSCs treated with AS20 showed more than 90% of cell viability, regardless of treatment concentration. The data is presented as mean ± standard deviation, with n = 3.

COX2 expression (26). COX2 expression in HeLa cells was observed following treatment with AS20 or no treatment by normalizing to *GAPDH* using PCR. As per our hypothesis, COX2 was induced by PMA as well as 5-FU and was reduced after the treatment with at concentration 0.89µg/mL which is half of IC50 for AS20.

When HeLa cells were treated with PMA, COX2 expression was significantly upregulated; while in the AS20 treatment group, COX2 expression was downregulated (n = 3, one-way ANOVA, $p < 0.05$) (Figure 3A). HeLa cells treated with 5-FU had significantly upregulated COX2 expression, while COX2 expression was downregulated with AS20 treatment (n = 3, one-way ANOVA, $p < 0.05$) (Figure 3B). DMSO was used as a vehicle control and did not result in a significant change in COX2 expression. AS20 treated cells demonstrated the lowest COX2 expressions across all groups (n = 3, one-way ANOVA, $p < 0.01$) (Figure 3C).

DISCUSSION

The rationale behind this work was to test AS20, a phytochemical derivate from *A. spinosis*, on its capacity to promote HeLa anti-inflammatory and pro-apoptotic properties for AS20 drug. Our investigation demonstrated the efficacy of AS20 showed anti-inflammatory and apoptotic properties by downregulating 5-FU-induced and PMA-induced COX2 and DNA fragmentation on HeLa cell lines. The results of these experiments have proven the hypothesis that AS20 promotes anti-inflammation and pro-apoptosis.

Studies on colorectal cancer cell lines indicate that NSAIDs have anti-tumor properties because they suppress COX enzymes and cause apoptosis, highlighting the link between inflammation and cancer (31). AS20 contains saponins, tannins, polyphenols, flavonoids, and alkaloids, according to earlier investigations (28, 29). The control of anti-metastasis, anti-carcinogenesis, and anti-angiogenesis by polyphenols has been demonstrated (32). Many alkaloids have been used in anticancer and anti-inflammatory medicines, while saponins show anti-proliferative effects (33, 34).

A. spinosus has been demonstrated to bear the potent antioxidants β-cyanin, β-xanthin, and betalain alongside a host of other pigments which have been implicated in free radical species scavenging including carotenoids and amarantine colorants (35, 36). Being rich in phenylpropanoids, including

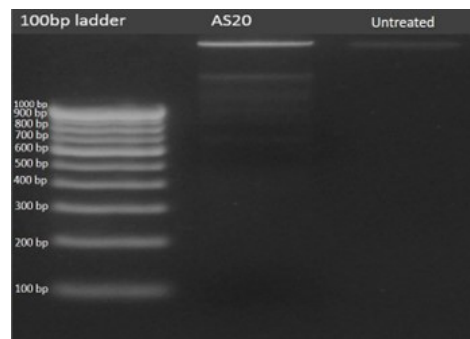


Figure 2. DNA Fragmentation using gel electrophoresis. HeLa cells treated with AS20 polyherbal drug showed DNA fragmentation i.e., DNA laddering, while untreated HeLa cells did not show DNA laddering. A standard 100bp DNA ladder was used as a positive control as it is designed for quantification and sizing of DNA with the range of 100bp-1000bp.

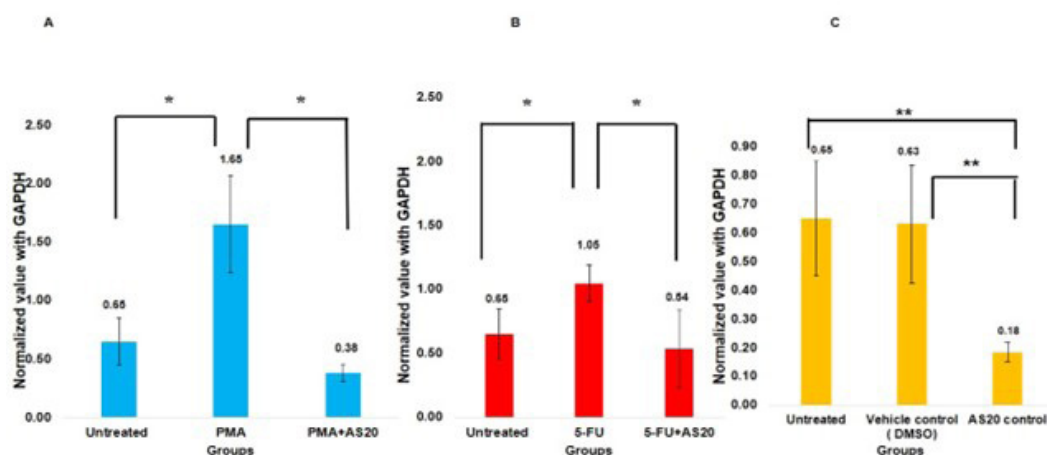


Figure 3. Normalized COX2 expression with GAPDH. Gene expression was measured using RT-PCR. **A)** When HeLa cells were treated with PMA, COX2 expression was significantly upregulated, while in the AS20 treatment group, COX2 expression was downregulated. **B)** When HeLa cells were treated with 5-FU, COX2 expression was significantly upregulated, while in the AS20 treatment group, COX2 expression was downregulated. **C)** When HeLa cells were treated with DMSO, which was used as a vehicle control, COX2 expression did not show any significant change. COX2 expression was significantly downregulated in the AS20 treatment group. All data is presented as mean \pm standard deviation, with $n = 3$ for each group. A one-way ANOVA was performed: * $p < 0.05$, ** $p < 0.01$.

flavonoids, phenolic acids, and their related amides, followed by alkaloids and terpenoids, *A. spinosus* can be considered essentially an anticancer agent (37). These oxidant-modulatory properties elevate AS20 in the context of CAM when applied to integrative oncological studies have been proven to be rich in free radicals and ROS, e.g., superoxides, hydrogen peroxide, oxygen-containing radicals, and others due to the prevalence of inflammatory reactions (38).

Numerous investigations have noted the anti-inflammatory properties of natural substances, and many preclinical studies have confirmed these findings (39). The results of anti-inflammatory studies have demonstrated that bioactive extracts and their natural compounds exert their biological effects by obstructing two key signaling pathways, nuclear factor kappa B (NF- κ B) and mitogen-activated protein kinases (MAPKs), which are crucial in the production of a variety of proinflammatory mediators (40). COX2 is secreted into the tumor microenvironment (TME) by cancer cells, macrophage type 2 (M2) cells, and cancer-associated fibroblasts (CAFs) (41). In addition to encouraging apoptotic resistance, cancer cell proliferation, angiogenesis, inflammation, invasion, and metastasis, COX2 also generates behavior resembling that of cancer stem cells (CSCs) (41).

In the current context of chemotherapy's negative side-effects such as chronic fatigue, thrombocytopenia, and alopecia threatening to overpower it, CAMs can be key in supplementing standard approaches to cancer treatment (41). Herbal medicines are a key component of CAM, which combines traditional therapy with non-mainstream methods (42). Poly-herbal medications typically have lower efficacy than pharmacological single target drugs due to their multi-target approach, but they have lower toxicity and drug resistance (43). Drug resistance develops as a result of single target medications, as seen in cancer therapy, despite their better efficacy. The integrative oncology platform provides combination therapy strategies that pair widely used single-target chemotherapeutic medicines with multi-target herbal formulations. Such a strategy offers a novel way to address

chemotherapy's drawbacks (44).

Overall, our research supports the anti-inflammatory and pro-apoptotic effects of the polyherbal formulation AS20 on HeLa cell lines. These important findings open the door for the use of AS20 as prospective CAMs and reach the highest level of therapeutic efficacy. Our future work will investigate the synergistic capabilities of AS20 with conventional therapies. Our investigation can be said to be limited by the non-consideration of various other interacting factors including multiple cytokine signaling pathways and gene expressions which could have contributed considerably to the scope of this study and provided further substantiation to our results. This will be the primary preoccupation of our work in the future while also allowing us to map the exact methods of action of the drug against cancer.

MATERIALS AND METHODS

Preparation of AS20 phytochemical extract

Based on qualitative and quantitative analysis of various solvents, acetone and methanol were employed to create the initial inflorescence and leaf extracts of *A. spinosus* plant (organic market, Bangalore, India). The final derivate was formed by combining 0.01 g of each dried extract and dissolving in 1 mL of dimethyl sulfoxide (DMSO). This final solution was named AS20 and employed for further studies (28, 29).

Cell Culture

HeLa cell cryovials were obtained from National Centre for Cell Science, Pune. We utilized passage seven for this study. Cells were cultured in Dulbecco's Modified Eagle Media (DMEM/F-12) with 10% Fetal Bovine serum (Himedia) post-rapid thawing using distilled water at 37°C. Hemocytometry was performed to determine cell count after centrifugation (1500 rpm for 10 minutes). The cells were then put into culture and maintained at 37°C with 5% CO₂.

MTT assay for percent cell viability

The 4, 5-dimethyl tetrazolium-2-yl, 2, 5-diphenyl tetrazolium bromide (MTT) test was done to check the percentage of cell viability. MSCs were cultured (0.3x10⁶) in a 96-well plate and incubated for 24 hours. After 24 hours, cells were treated with different concentrations of AS20 (25, 12.5, 6.25, 3.13, 1.57, and 0 µg/mL) for 48 hours. Twenty microliters of MTT was added to the wells after 48 hours of incubation and 100 µL of DMSO was used to dissolve the purple formazan crystals which formed depending on the presence of live cells. The absorbance was measured at 545 nm using a spectrophotometer (Thermo Scientific).

DNA Fragmentation

DNA fragmentation was performed using HeLa cell lines after 48 hours of incubation with and without AS20. Cells were harvested after trypsinization and lysed using 1% sodium dodecyl sulfate (SDS) and Tris - ethylenediamine tetraacetic acid (EDTA) buffer (pH 8.0). DNA was extracted using the phenol, chloroform, and isoamyl alcohol (PCI) method (45).

Gel Electrophoresis

To prepare the sample for gel electrophoresis, 5 µL of the DNA sample was combined with 2 µL of 2.5X gel loading dye (Sigma). The sample was then loaded into wells formed in a 1.2% agarose gel alongside a 100 bp ladder to observe the extent of DNA fragmentation. The electrophoresis assay was conducted for 1.5 hours at 50 V.

mRNA Isolation

Both control and treated HeLa cells underwent trypsinization and were centrifuged at 1500 rpm for 10 minutes. RNAiso plus (Takara, 9108Q) solution along with chloroform was added to the cell pellet and vortexed. The cell suspension was then incubated for 15 minutes at room temperature prior to being centrifuged at 12,000rpm for 15 minutes.

The aqueous phase containing the mRNA was collected into new tube, after which 500 µL of 100% isopropyl alcohol was added to it. After 10 minutes, the incubation mixture was centrifuged using a microfuge for 10 minutes at 12,000 rpm. The supernatant was discarded, and 75% ice-cold ethanol was added and centrifuged at 7500 rpm for 7 minutes. Following this, the mRNA pellet was dried in order to remove all contaminants. After 45 minutes, RNase free water was added to the dried pellet. The mRNA was stored at -20°C for further use.

Quantification of RNA

The purity of the RNA was determined by dividing the absorbance at 260 nm by the absorbance at 280 nm. After RNA quantification, the concentration of the RNA was determined by adjusting the absorbance by a dilution factor of 600 and a constant 40.

cDNA Synthesis

cDNA was prepared using DEPC-treated tubes via the Takara PrimeScript RT Reagent Kit (RR037A). Samples were incubated at 37°C for an hour, followed by 2 min at 90°C in a water bath. Finally, the cDNA obtained was stored at -80°C for further use.

Evaluation of COX2 Expression

After calculating the appropriate cDNA volumes, polymerase chain reactions (PCR) were set up with 6 µL of Jumpstart mix (Ct.No. -P2893-100 Rxn) and 1 µM of the desired genes (*GAPDH*, *COX2*) forward and reverse primers (Eurofins). Primer sequences and melting temperatures are given in **Table 1**.

Target	Forward (5' - 3')	Reverse (5' - 3')	Melting temp (°C)
<i>GAPDH</i>	GGTCGGAGTCAACGGATTTGGTCG	CCTCCGACGCCTGCTTCACCAC	61.9
<i>COX2</i>	CCACTTCAAGGGATTTTGGGA	GAGAAGGCTTCCAGCTTTT	51

Table 1. Forward and reverse primers used for PCR.

Gel electrophoresis to analyze gene expression in mRNA level

10 µL of each PCR product was mixed with 2 µL of 2.5X gel loading dye (Sigma) before loading the wells in 2% agarose gel with a 100bp ladder. The gel was run for 1 hour at 100 V. Each gel image was exported and analyzed using ImageJ software.

Statistical analysis

To check the experimental reliability, three independent sets of each experiment were performed. *COX2* mRNA levels were normalized with *GAPDH* levels. Statistical significance between the three groups was assessed by one-way ANOVA using Microsoft Excel software. P-values of less than 0.05 were considered significant.

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