

# Anticancer and anti-inflammatory effects of polyherbal drug AS20 on HeLa cells resistant to 5-Fluorouracil

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## SUMMARY

Cancer treatment that involves chemotherapy often results in developed drug resistance which can be a major deterrent for the success of a treatment protocol. Increased inflammation, due to the drug's presence, contributes to disease progression and increased Cyclooxygenase-2 (COX2) expression (inflammatory marker). Integrative oncology is a field that focuses on combining natural herbal formulations with standard care to provide optimal clinical outcomes in a holistic manner, to minimize side effects, and to overcome drug resistance, including inflammation. 5-Fluorouracil (5-FU) is a chemotherapeutic medication used to treat cancers. We studied drug resistance by deriving 5-FU-resistant HeLa cells (HeLa R) and evaluating the efficacy of AS20 in vitro experiments. AS20 is an herbal formulation derived from the plant *Amaranthus spinosus*, an indigenous plant found in India. It contains different types of secondary metabolites such as saponins, polyphenols, alkaloids, terpenoids, and flavonoids. We hypothesized that AS20 has anti-inflammatory and anticancer properties on HeLa R. We investigated if AS20 could downregulate increased inflammatory markers such as COX2 in HeLa R. Also, we studied anti-apoptotic and pro-apoptotic genes in AS20 treated HeLa R cells. Our results demonstrated that the inhibitory concentration at 50% (IC50) values of HeLa wild type cells with AS20 treatment was significantly less than HeLa R cells. Also, AS20 brought down induced COX2 expression in HeLa R cells. AS20 treatment on HeLa R cells showed upregulation of BAX (pro-apoptotic) but no significant change in BAD (pro-apoptotic) and downregulation of BCL2 and BCL2L1 (anti-apoptotic) in HeLa R cells. Our results showed that AS20 has anti-inflammatory and pro-apoptotic properties when treated on HeLa R cells.

## INTRODUCTION

Worldwide, cancer kills one out of every six people (1). Cancer is a disease where homeostasis is disrupted due to uncontrollable cell proliferation, and cancer cells have the potency to spread to other parts of the body by invading adjacent tissue (2). Chemotherapy is one of the most common methods of treating cancer. Chemotherapy drugs kill tumor cells through various mechanisms (3, 4). 5-Fluorouracil (5-FU), for example, kills cancer cells by preventing DNA replication and repair (5). However, chemotherapy drugs induce various undesirable side effects in patients. Nausea, hair loss, and loss of appetite are the most widely observed

effects (6). Many chemotherapy drugs are also intrinsically problematic. 5-FU has caused myelotoxicity and cardiotoxicity (7, 8). Another major problem associated with these drugs is chemotherapy resistance. An estimated 90% of failures in chemotherapy treatment are a direct result of drug resistance (9). This makes chemotherapy resistance one of the most pressing issues in cancer treatment.

Additionally, cancer is regarded as an inflammatory disease, which is demonstrated by increased levels of Cyclooxygenase-2 (COX2) leading to chronic inflammation (10). COX2 can be induced and is often overexpressed in inflammatory tissues and in cancer (11). A family of inducible transcription factors known as nuclear factor kappa B (NF- $\kappa$ B) controls a wide range of genes involved in various immunological and inflammatory response pathways. Innate immunity is regulated, activated, and differentiated by NF- $\kappa$ B (12). NF- $\kappa$ B also controls a variety of immunological processes and plays a key role as a mediator of inflammatory reactions (13). It triggers the expression of several pro-inflammatory genes, including COX2, as well as certain cytokines such as IL1, IL-6, and IL-23 (14). Although COX2 is usually undetectable in most tissues under normal conditions, it is highly inducible and rapidly upregulated in response to various inflammatory stimulations (15). Modulating this expression of COX2 could lead to resolution of inflammation, induce apoptosis, and result in favorable outcomes.

Modulating the expression of COX2 can be done using traditional medicine (16). According to the World Health Organization, an estimated 80% of the world's population depends mainly on indigenous medicine (17). In India, Ayurveda is the most widely used system of traditional medicine (18). Ayurveda combines an abundance of knowledge from various traditional systems of medicine can open up new possibilities for the discovery of herbal drugs (18). As modern medicine progresses, it is the duty of scientists to bridge the gap between traditional and modern ideologies (19). Herbal drugs are a significant aspect of complementary and alternative medicine (CAM), which involves the use of a non-mainstream approaches in conjunction with conventional medicine (20). In recent years, more patients are choosing methods of treating cancer that involve CAM (21). CAM is a platform based on integrative oncology, where the standard line of therapy is simultaneously administered with natural products of medicinal value to complement their benefits (22). This complementary approach could reduce the side effects of conventional cancer treatments and also have various physiological benefits (23).

Polyherbal drugs are formulations made using compounds from multiple parts of multiple plants. Traditional medicine practices like Ayurveda extensively use polyherbal drugs for treatments like dashamoola (24). Dashamoola is an

important polyherbal substance comprising of 10 medicinal roots used to treat various clinical conditions, including lower back pain, fever, and lung problems (25). Due to their multi-target approach, the efficacy of polyherbal drugs is usually lesser than pharmacological single target drugs, but leads to less toxicity and drug resistance (26). Single target drugs demonstrate higher efficacy, but lead to drug resistance, as observed in cancer therapy (27). Such an approach provides a unique method to tackle the shortcomings of chemotherapy (28).

AS20 is a polyherbal drug formulated from the inflorescence and leaf extracts of the *Amaranthus spinosus* plant (29, 30). Previous studies from our group have shown that AS20 has antioxidant and anticancer properties (29, 30). *A. spinosus* is a plant native to South America but has been introduced all over the world (31). In South Asian countries, it is commonly consumed as a vegetable. *A. spinosus* is also extensively used in indigenous Asian medicine and Ayurveda. Recent studies showed that the chemotherapeutic drug 5-fluorouracil 5-FU can cause drug resistance in different cancer cell lines (32, 33). Studies have also shown increased COX2 expression due to 5-FU chemotherapy, showing its therapeutic inefficiency (34).

In this paper we aimed to explore the efficacy of AS20 formulation in HeLa R, as compared to HeLa cells. AS20 induced cell death as measured by a cell counting kit-8 (CCK-8) assay in HeLa R cells, demonstrating the efficacy of AS20 to overcome resistance in 5-FU-resistant cells. HeLa R cells expressed higher levels of COX2, indicating a direct association between resistance and inflammation. Additional 5-FU was added to HeLa R cells, to probe for a further increase in the COX2 levels when compared to the basal level in HeLa R cells. Additional 5-FU did not result in further increase in COX2 expression, possibly indicating the involvement of the Multi Drug Resistance-2 (MDR-2) and the p-glycoprotein pump (p-GP). Phorbol 12-myristate 13-acetate (PMA) was used as a positive control to induce COX2 gene induction. PMA induces COX2 expression by upregulating NF-kB. We found that AS20 brought down expression in constitutive and induced conditions. AS20 induced cell death via apoptosis as observed by up-regulation of *BAX* and down-regulation of *BCL2* and *BCL2L1*, suggesting its promise as a complimentary polyherbal compound, which could be used alone with standard chemotherapeutic drugs.

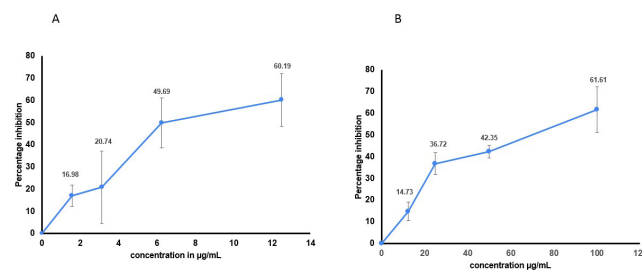
## RESULTS

### Cytotoxicity by using CCK-8 assay

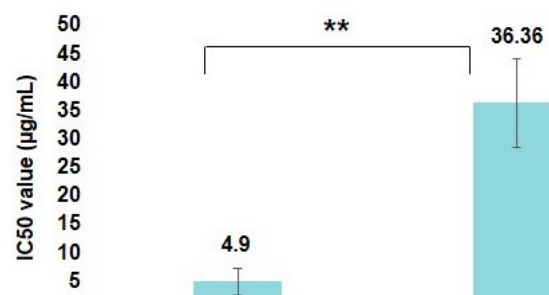
First, we sought to determine whether AS20 induces cell cytotoxicity in HeLa R cells when compared to HeLa wildtype (WT) cells. This was accomplished by the CCK-8 assay which measures cell cytotoxicity. The CCK-8 assay is a sensitive and quick way to measure NAD(P)H levels and dehydrogenase enzyme activity. It uses highly water-soluble tetrazolium salt to provide convenient tests. IC<sub>50</sub> values obtained from such assays help determine the concentration of formulation required to obtain 50% cell death, which could be further extrapolated to determine dosage required for *in vivo* models.

We found that a higher concentration of AS20 was required to induce cell death in HeLa R, confirming its resistant state. The IC<sub>50</sub> value of AS20 obtained for HeLa R cells (36.36 μg/

mL) was significantly higher compared to that for HeLa cells WT (4.9 μg/mL) (n=3, p<0.05) (Figure 1 and 2).



**Figure 1. AS20 induced cytotoxicity to HeLa WT and HeLa R.** All data presented as mean ± standard deviation, n=3 for all groups. **A)** The CCK-8 assay kit revealed cytotoxicity by calculating percentage inhibition for HeLa WT after 48 hours of incubation with different concentrations of AS20. **B)** The CCK-8 assay kit revealed cytotoxicity by calculating percentage inhibition for HeLa R after 48 hours of incubation with different concentrations of AS20.



**Figure 2. CCK-8 assay for cytotoxicity (IC<sub>50</sub> value).** The CCK-8 assay kit revealed IC<sub>50</sub> for HeLa WT and HeLa R after 48 hours of incubation with different doses of AS20. The data is presented as mean ± standard deviation, with n = 3. \*\*p < 0.01

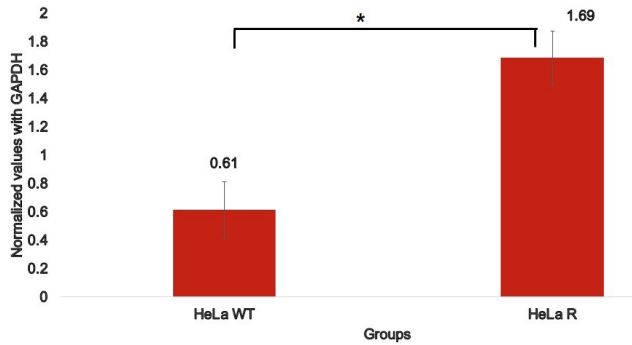
### Inflammatory gene expression

COX2 is implicated in the inflammatory cascade driven by NF-kB. Increased COX2 expression suggests chronic inflammation and a refractory phenotype of malignancy. COX2 expression study was carried out using polymerase chain reaction (PCR). HeLa R cells showed higher constitutive COX2 expression when compared to HeLa WT cells (data not shown) (Figure 3). However, PMA induction (positive control) resulted in increased COX2 expression by 2 fold which was brought down by AS20 treatment in HeLa R (2-fold decrease) (n=3, p< 0.05) (Figure 4A). This indicates that AS20 is showing an anti-inflammatory property.

HeLa R cells showed higher constitutive COX2 expression, which could not be further induced significantly by 5-FU which was brought down by AS20 (n = 3, p<0.05) (Figure 4B). HeLa R cells treated with DMSO (vehicle control) showed no significant change in COX2 expression when compared to HeLa R cells. AS20 alone was able to bring down COX2 expression in HeLa R cells (n=3, p<0.05) (Figure 4C).

### Pro- and anti-apoptotic gene expression

Apoptosis is widely acknowledged as one of the primary



**Figure 3. Normalized COX2 expression with GAPDH in HeLa WT compared with HeLa R.** COX2 was significantly higher in HeLa R cells compared to HeLa WT cells. The data is presented as mean  $\pm$  standard error of the mean (SEM),  $n = 3$ . \* $p < 0.05$

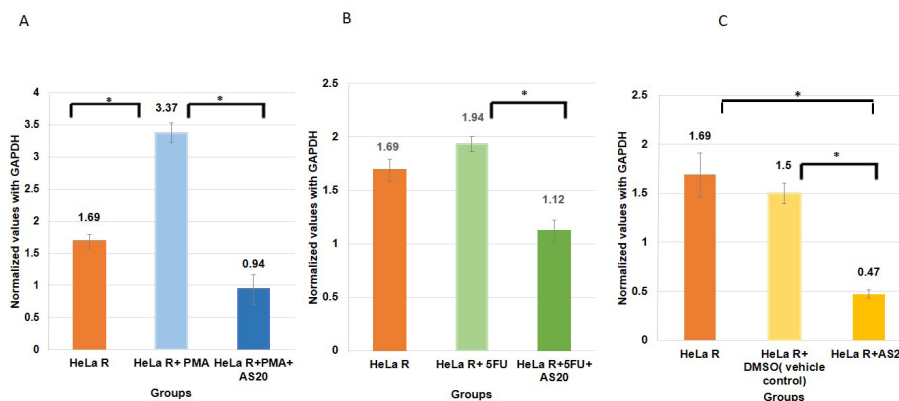
mechanisms of programmed cell death (35). The promoter of the protein that regulates apoptosis, the BCL2 family, is significantly regulated by NF- $\kappa$ B (36). A balance of anti-apoptotic and apoptotic molecules signifies cellular homeostasis. In cancer, this loss of homeostasis results in an increase of anti-apoptotic markers as opposed to pro-apoptotic markers (37). Intervention at the cellular level is considered beneficial when it results in decreased anti-apoptotic markers, thereby restoring homeostasis (38). AS20 treatment decreased expression of anti-apoptotic genes, *BCL2* ( $n=3$ ,  $p < 0.05$ ) and *BCL2L1* ( $n=3$ ,  $p < 0.05$ ) and increased expression of *BAX* (pro-apoptotic) significantly ( $n = 3$ ,  $p < 0.05$ ). Although we did not observe a significant change in *BAD* (pro-apoptotic) expression ( $n = 3$ ,  $p < 0.08$ , suggesting its attributes as a promising anticancer herbal formulation (Figure 5).

As a result, this study showed that AS20 has showed cytotoxic effect on both HeLa WT and HeLa R. AS20 was able to brought down COX2 on HeLa R cells which demonstrates that AS20 has anti-inflammatory effect of HeLa R. It also showed that AS20 has pro-apoptotic properties.

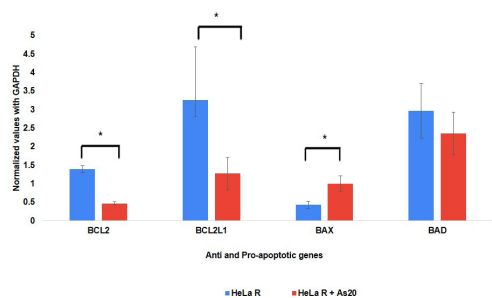
## DISCUSSION

Developing drug resistance as a consequence of chemotherapy is a major obstacle in cancer therapy (39). Inflammation, which is an intrinsic aspect of cancer and drug resistance, aggravates the prognosis, triggers the NF- $\kappa$ B pathway and epithelial-to-mesenchymal transition (EMT) leading to metastasis (40). Approaches to mitigate this inflammation associated with cancer, and importantly with drug resistance, will pave way for better clinical outcomes. Our initial work with AS20 formulation has provided promising results to establish its role as a CAM, on the integrative oncology platform (29,30). This approach to integrate natural products with therapeutic value, in conjunction with single target chemotherapeutic drugs, could mitigate deleterious side effects including drug resistance associated with chemotherapy.

There are very few instances of CAM in general practice, and fewer clinical trials to substantiate this claim, which has made it harder to weave it in modern therapeutic medicine. Standalone treatment using *Withania somnifera* (Ashvagandha), *Tinospora cordifolia* (Amruthaballi), *Selaginella bryopteris*, *Boswellia serrata*, *Garcinia mangostana*, and *Punica granatum* have been studied in various parts of the world in *in vivo* experiments and demonstrated cytotoxic effects (41–43). In our own study with Swastha Rakshak (HF1), a polyherbal substance with green tea and turmeric demonstrated efficacy in minimizing drug resistance in cancer cells (44). In a study using the Dalton Lymphoma Ascites (DLA) tumor mouse model, the anti-tumor activity of four herbs : *Curcuma longa* L., *Ocimum sanctum* L., *Tinospora cordifolia* (wild), and *Zizyphus mauritiana* L. was evaluated, with 5-FU as a control. All four herbs demonstrated cytotoxicity with *Tinospora cordifolia* being the most potent (45). A study with *Tinospora cordifolia* has elucidated Berberine (active component) to contribute to its anti-proliferative activity (46). The antiproliferative properties of Triphala, an Ayurvedic polyherbal consisting of three medicinal fruits: *Phyllanthus emblica* L. or *Embilica officinalis Gaertn*, *Terminalia chebula* Retz, and *Terminalia bellerica* Retz. Triphala was tested on three cell lines: SK-OV -3, cervical cancer cell line HeLa and endometrial cancer



**Figure 4. Normalized COX2 gene expression with GAPDH in HeLa R.** All data presented as mean  $\pm$  SEM,  $n = 3$ . \* $p < 0.05$ . **A)** COX2 was significantly upregulated when HeLa R cells were treated with PMA and lowered when this group was treated with AS20. **B)** COX2 expression was not induced when HeLa R cells were treated with additional 5-FU but lowered when treated with AS20. **C)** COX2 was not significantly changed when HeLa R cells were treated with DMSO (vehicle control). When HeLa R was treated with AS20 alone, COX2 expression was significantly decreased.



**Figure 5. Anti-apoptotic and pro-apoptotic gene expression.** HeLa R cells as a control and HeLa R cells treated with AS20 were compared. The AS20 group showed down-regulation of *BCL2* and *BCL2L1* and upregulation of *BAX* but no significant change in *BAD*. The data are presented as mean  $\pm$  SEM, n = 3. \*p < 0.05

cell line HEC-1-B (47). It proved to be effective, making it an attractive candidate for the treatment of gynecological cancers. Medicinal plants that have anticancer properties and have found their way to standard formulations are vinca alkaloids, taxanes, and podophyllotoxin (48). However, these drugs are single targeted drugs, which are associated with drug resistance and inflammation as a side effect. The WHO has recognized the importance of CAM and has emphasized that this approach may lead to better clinical outcomes, especially in the field of cancer (49).

Emphasizing the connection between inflammation and cancer, studies in colorectal cancer cell lines suggest anti-tumor activities of non-steroidal anti-inflammatory drugs (NSAIDs), as they inhibit COX enzymes and induce apoptosis (50). Previous studies have shown that AS20 contains saponins, tannins, polyphenols, flavonoids, and alkaloids (29, 30). Polyphenols have been shown to regulate anti-metastasis, anti-carcinogenesis, and anti-angiogenesis (51). Saponins showed anti-proliferative properties and many alkaloids have been incorporated into anticancer and anti-inflammatory drugs (52, 53).

In our study, the CCK-8 assays demonstrated that a higher amount of AS20 was required to achieve IC50 value in HeLa R cells compared to HeLa WT. A high dose of 5-FU is toxic and finding ways to break this resistance, in combination with AS20, could widen its therapeutic window. COX2 is found to be responsible for inducing cancer stem cell-like activity and promoting apoptotic resistance in cancer cells (54, 55). Basal COX2 level was higher in HeLa R than in HeLa WT cells, demonstrating a direct correlation between resistance and COX2, which is effectively brought down by AS20. Increased COX2 expression mediated by PMA is also reduced with AS20 treatment. Also, AS20 resulted in increased expression of *BAX* (pro-apoptotic) but no significant change in *BAD* (pro-apoptotic) and decreased in expression of *BCL2* (anti-apoptotic) and *BCL2L1* (anti-apoptotic), suggesting its potential as an anticancer formulation.

Taken altogether, our study confirms selective cytotoxic activities of AS20, a polyherbal formulation on HeLa R cell lines. These key observations pave way for AS20 to be used as potential CAMs in combating the resistance of current chemotherapeutic drugs, in order to achieve maximum therapeutic efficacy. Our future work will explore the synergistic properties of AS20 with standard interventions to reduce drug resistance in HeLa R cells.

## MATERIALS AND METHODS

### Polyherbal Drug Formulation

The IC50 of acetonic inflorescence extract and methanolic leaf extract of *A. spinosus* were prepared by dissolving in dimethyl sulfoxide (DMSO) at a concentration of 0.01 g/mL. AS20 was prepared using the protocol described by an earlier study (29, 30). IC50 of the inflorescence and leaf extracts were combined to form AS20. A single batch was used to perform this experiment to avoid variability in the results.

### Cell Culture

HeLa cells were obtained from NCCS Pune, India. HeLa R cells were prepared by culturing escalating doses of 5-FU until it reached its IC50 value (2 $\mu$ g/mL) in HeLa cells. HeLa R cells emerging from this protocol displayed no cell death when grown in IC50 of 5-FU.

Passage 8 of HeLa R cells were rapidly thawed in a 37°C water bath and cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% Fetal Bovine Serum (FBS) in cell culture dishes at 37°C with 5% CO2. Cells were allowed to proliferate until 80% confluency. After which, the cells were passaged and stored for further use.

### CCK-8 Assay

The CCK-8 assay was used to assess the cytotoxicity of the polyherbal medication AS20 on HeLa and HeLa R cell lines. Sigma provided the CCK-8 assay methodology (Cat. No. 96992-100tests-F). For 48 hours, cells were treated with 100, 50, 25, or 12.5  $\mu$ g/mL concentrations of AS20 for HeLa R cells and 12.5, 6.25, 3.13 and 1.57  $\mu$ g/mL AS20 for HeLa cells. Ten microliter of the CCK-8 solution was added after the drug action and incubated for 4 hours. Microplate readers were used to take absorbance readings at 450nm wavelength.

The percentage cell cytotoxicity of AS20 was calculated by the formula:

$$\frac{Abs_{Control} - Abs_{Sample}}{Abs_{Control}} \times 100$$

The cell viability values for each concentration of each sample were calculated using the absorbance readings. For all four groups, percentage viability graphs were plotted against sample concentration. After 48 hours of drug activity, the IC50 value for AS20 using the CCK-8 assay was obtained using the IC50.tk software for each group (56).

### Groups used for gene expression studies

Previously cultured passage 8 HeLa R cells were trypsinized and cells were counted. One million cells were cultured and incubated for 24 hours. After incubation, PMA, 5-FU, and half of the IC50 value AS20 was added to the cell medium in respective groups and further incubated for 48 hours to observe the anti-inflammatory and pro-apoptotic effects of AS20. Seven groups of cells were cultured such as HeLa R (Control), HeLa R + PMA(6.25ng/mL), HeLa R + PMA(6.25ng/mL) + AS20(18.18 $\mu$ g/mL), HeLa R + 5-FU(2 $\mu$ g/mL), HeLa R + 5-FU(2 $\mu$ g/mL) + AS20(18.18 $\mu$ g/mL), HeLa R + DMSO (Vehicle control) and HeLa R + AS20(18.18 $\mu$ g/mL) respectively.

### mRNA isolation

After 48 hours of incubation, treated cells were trypsinized

and RNA was extracted from the cells using the RNAiso reagent (Takara: Cat.No.-9108Q) as per manufacturer's instructions. Spectrophotometric analysis at 260 and 280 nm was used to assess the concentration and purity of isolated RNA. For calculating the concentration of RNA following formula was used:

$$\text{Concentration of RNA } (\mu\text{g/mL}) = \text{Absorbance}@260\text{nm} \times \text{dilution factor} \times 40$$

### cDNA synthesis

Extracted mRNA was appropriately diluted to 1000 ng/ $\mu$ L using nuclease free water. The standard protocol was used to synthesize cDNA. The steps included such as the addition of 2  $\mu$ L cDNA buffer, 0.5  $\mu$ L oligo dT, and 0.5  $\mu$ L reverse transcriptase enzyme and 2  $\mu$ L random hexamer to 5  $\mu$ L of mRNA sample. The mixture was incubated at 37°C for 1 hour, heated at 90°C for 1 minute, and then stored at -80°C until further use.

### Semi-quantitative PCR

55 ng of cDNA was used, and PCR was carried out using JumpStart™ Taq DNA Polymerase (6  $\mu$ L) (Sigma-Aldrich) with 1 pmol (0.5 $\mu$ L) of forward and reverse primer for each gene (Juniper Life Sciences). A final volume of 12.5  $\mu$ L PCR reaction was obtained (Table 1). Five genes were looked at: BAX, BAD, BCL2, BCL2L1 and COX2. GAPDH, a housekeeping gene, was used to normalize gene expression values (Table 1).

Gene	Forward (5' - 3')	Reverse (5' - 3')
GAPDH	GGTCGGAGTCAACGGATTTGGTCG	CCTCCGACGCGCTTCACCAC'
COX2	CCACTTCAAGGGATTTTGGGA	GAGAAGGCTCCAGCTTTT
BAX	GCTGGACATTGGACTTCCTC	CTCAGCCCATCTTCTCCAG
BCL2	ATTGGGAAGTTTCAAATCAGC	TGCATTCTGGACGAGGG
BAD	CCTCAGGCCTATGCAAAAAG	AAACCCAAAACCTCCGATGG
BCL2L1	GGCTGGGATACTTTTGTGGA	AAGAGTGAGCCAGCAGAAC

Table 1. Forward and reverse primers sequences.

### Gel electrophoresis

10  $\mu$ L of PCR product was mixed with 2  $\mu$ L of 2.5X gel loading dye and then loaded in 2% agarose gel. A 100 base pair DNA ladder was added and the gel ran for 1.5 hrs at 50V. Image J software was used to analyze images of the gel. The intensity of the bands was used to plot the peaks. The area under the curve was measured and normalized with the area under the curve of GAPDH.

### Statistical analysis

To check experimental reliability, three independent sets of the same experiment were performed. For the CCK-8 assay, the experimental data is expressed as mean and standard deviation (SD). A p-value of less than 0.05 was considered significant.

COX2, BCL2, BCL2L1, Bax, and BAD mRNA levels were normalized with GAPDH levels. The experimental data is expressed as mean and standard error of the mean (SEM). A p-value of less than 0.05 was considered significant.

Statistical significance between the three groups was assessed by a t-test using Excel software, followed by the Bonferroni correction method.

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