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Article

A study to determine the anti-cancer and pro-apoptotic properties of *Amaranthus spinosus* Linn. Extract, AS20

Ishir Sharma[1], Pooja Kasture[2], Ankita Umrao[2], Jyothsna Rao[2], Gururaj Rao[2]
[1] The International School Bangalore, NAFL Valley, Whitefield – Sarjapur Road, Bangalore, KA, India, 562125
[2] iCREST-International Stem Cell Services Limited, 9/1, Mission Road, Bangalore

SUMMARY

Complementary approaches to cancer treatment seek methods that integrate into existing therapies such as chemotherapy and radiotherapy. The most common form of complementary medicine is the usage of external antioxidants, including Ayurvedic herbal extracts, which assist in the mitigation of the side effects of cancer treatment on healthy cells. Herbal extracts are usually evaluated only for their antioxidant properties for complementary use in cancer treatment. Ayurvedic products are rarely evaluated for their anti-cancer properties. In this paper, we hypothesized that AS20, a crude extract of the whole *Amaranthus spinosus* (AS) plant, would induce higher cell death to the HeLa cancer cell line compared to other extracts of individual parts of the plant. Significantly lower IC50 values were found for both 24 hours (11.56 µg/ml) and 48 hours (1.77 µg/ml) exposure compared to any of the other individual extracts of the plant part for the given exposure duration. Also, apoptosis was observed in HeLa cells treated by the IC50 concentration of AS20 using fluorescent stains such as Hoechst dye, DAPI and dual staining (Acridine Orange and Propidium Iodide). It is thus concluded that AS20 formulation exhibited cytotoxic activity on HeLa cancer cell lines and their action mechanism is via apoptosis.

INTRODUCTION

Complementary and alternative approaches to cancer treatment are premised upon relieving the symptoms of cancer treatment (1). A common complementary therapy is the usage of antioxidants (2), which assists in the mitigation of the side effects of chemotherapeutic drugs and radiotherapy on healthy cells. Hence, by complementing natural antioxidants with external herbal products, we would theoretically be able to prevent oxidative cell damage to healthy body cells, while retaining the anti-carcinogenic properties of the mainstream drug. Ayurvedic treatment is not considered a mainstream method. However, if the complement herbal product was to possess anti-cancer properties of its own which added on to those of the mainstream drug, it might be possible to reduce the dosage of the mainstream drug, still maintain the anti-cancer potential, and have the added benefit of the antioxidant properties to eliminate the side effects.

Secondary metabolites (alkaloids, saponins, flavonoids, polyphenols, tannins etc.) are organic compounds produced primarily by plants as waste products of their metabolism. These compounds are known for their medicinal properties (3). A large variety of alkaloids, for instance, have been shown to display compelling anti-carcinogenic activity and anti-cancer drugs have been created from alkaloids (4). Saponins are known for their cytotoxicity against cancer cells (5) as well as the inhibition of carcinoma growth (6). Saponins prevent the proliferation of cancer cells due to their ability to interfere with DNA replication (7). Polyphenolic compounds, including flavonoids and tannins, have been shown to possess anti-cancer activities in addition to their high antioxidant potencies (8). These metabolites are thus known to possess anticancer properties.

The *Amaranthus spinosus* (AS) Linn. plant is a medicinal plant which is found in abundance in India and around the world. This plant has been used extensively in Ayurveda (9), the traditional folk medicine of India, to treat diabetes, jaundice (10) and various other ailments. In our earlier studies (11), solvent extractions (water, ethanol, methanol, chloroform and acetone) were prepared for each part of the AS plant. We phytochemically screened the various parts of the AS plant. The phytochemical quantitative tests were conducted for total alkaloid, saponin, flavonoid, polyphenol and tannin contents. Subsequently, a crude herbal formulation, AS20 was prepared and assessed for its free-radical scavenging properties. AS20 is a crude herbal formulation consisting of the methanol extract of the leaves and the acetone extract of the inflorescence of the AS plant. This formulation was created based on maximum total alkaloid, saponin/tannin, polyphenol and flavonoid content of the entire plant since these metabolites were known to possess both antioxidant and anti-cancer properties. Some studies also suggest anti-carcinogenic properties of AS leaves in both in vivo and in vitro analyses (12). Hence, our AS20 formulation was a promising formulation to display anticancer properties.

HeLa is named so after Henrietta Lacks, a lady who died of cervical cancer in 1951 (13). Ever since the distribution of her cells, this cell line has been commonly used to evaluate the effects of various anticancer drugs (14). HeLa cells have been used to investigate the phytocomponents (plant-based components) and the apoptotic mechanism of the anti-cancer activity of the ethanolic extract of mango peel (15). Apoptosis is a mode of programmed cell death (16) that does not result in harm to surrounding cells. An alternate form of cell death, necrosis, is induced by chemotherapeutic drugs (17). Necrotic

cell death damages the body (18) and hence apoptosis is a more desirable mode of death, as it is an alternative mechanism of cell death which avoids the side effects seen in necrotic cell death. HeLa is an immortal cancerous cell line that is considered to be the single longest-living cell line (19).

Hence, the anti-cancer properties of the entire plant were of interest in this study. In this study, we evaluated the ability of the crude extract formulation of AS20 to exhibit a greater anti-cancer and pro-apoptotic properties than constituents and other extracts of individual parts of the plant. This, in addition to its potent free radical scavenging property (11) would make it potentially suitable complementary cancer drug. Apart from the ordinary HeLa cell line employed in this study, a 5-fluorouracil (5-FU) drug-resistant variety (HeLa-R) was developed (20) and employed in the study. The morphology of cells was also evaluated. To create this herbal formulation, we mixed extracts of different parts of the plant, which are the most potent in the desired phytocomponents, to create a crude extract that is a genuine representative of the potency of the entire plant. Thus, it was hypothesized that the crude extract, containing a greater content of the secondary metabolites, would display significant anti-cancer properties determined by the MTT assay. Crude Ayurvedic combinations have not been studied in this manner and this is an essential step in the field of integrative oncology.

RESULTS

Medicinal herbs and their derivative phytocompounds are being recognized as useful complementary and alternative medicinal treatments for cancer. *Amaranthus spinosus* (AS) Linn. plant is a medicinal plant which has been used extensively in Ayurveda. Recent studies also showed that Amaranthus spinosis has antioxidant and anticancer properties. This study was conducted to assess the cytotoxic activities using MTT assay and induced apoptosis of the formulation AS20 on HeLa cells as well as HeLa-R (5-FU drug resistant) cells.

Cytotoxicity of HeLa cells by AS20

MTT assay was used to measure cytotoxicity (loss of live cells). This is based on metabolic reduction of the soluble MTT salt, 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide which reflects the normal function of mitochondria dehydrogenase activity and cell viability, into purple coloured formazan product, which was measured spectrophotometrically.

The percentage viability of HeLa (drug-sensitive) and HeLa-R (5-FU drug-resistant) cells with an increase in the concentration of AS20 was determined by carrying out the MTT assay in a 96 well plate to determine the IC50 value of AS20. HeLa-R cell line was also employed to determine the anticancer effect of AS20 on cancer cells which are resistant to commercial anti-cancer drugs such as 5-FU. The IC50 obtained for 24 h and 48 h by using acetone extracted inflorescence was 47.3 and 22.31 µg/ml, respectively. Leaf extracted in methanol and water showed an IC50 at 23.48 µg/ml on its 24 h exposure and 13.19 and 6.28 µg/ml during 48 h treatment, respectively (**Figure 1 (a), (b), (c)** and **Figure 2**). The IC50 values at 48 h were significantly lower than that at 24 h (p<0.05 and p<0.001).

It was evident that the herbal formulation, AS20 on HeLa cells had a significantly (p<0,001) lower IC50 value of 11.56 µg/ml (24 hours) and 1.77 µg/ml (48 hours) than any of the

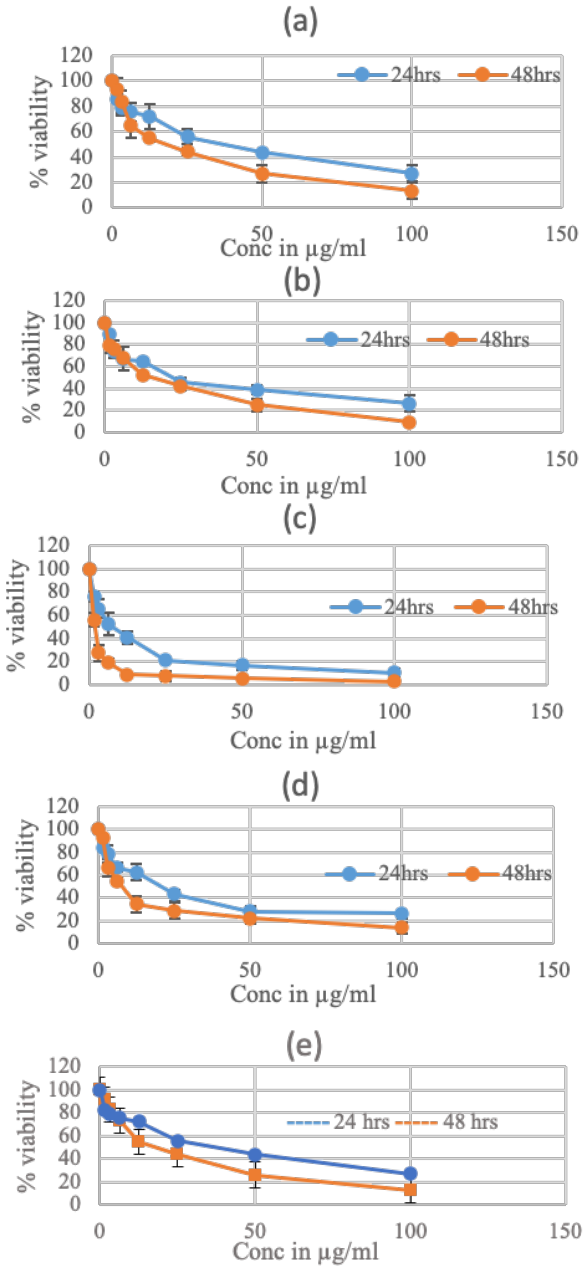

Figure 1: Graph displaying decrease of percentage viability of HeLa cells with increase in concentration of *Amaranthus spinosus*-derived extracts using MTT assay: (a) AS Inflorescence Acetone extraction, (b) AS Leaves Methanol extraction, (c) AS Leaves Water extraction, (d) AS20, and (e) AS20 on HeLa-R cells, following 24 hours and 48 hours of drug exposure. Results are expressed as percentage (%) at each concentration in µg/ml (N=3, mean±SD).

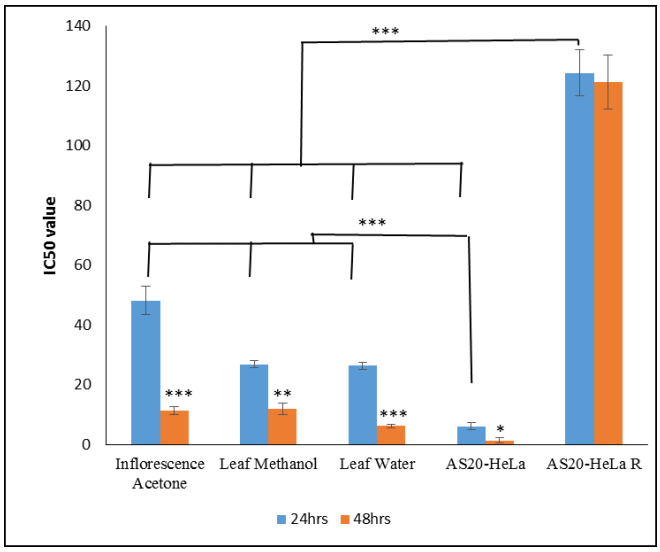

Figure 2: Bar graph showing the IC50 values for the MTT cell viability assay for the five groups tested. The concentration was expressed in µg/ml (N=3, mean±SD). HeLa cell viability decreases with increasing concentrations of *Amaranthus spinosus*-derived extracts using MTT assay. Effect of the extracts and formulation on 24 h and 48 h was compared. AS20 effect on HeLa and HeLa-R was compared with other groups. Groups were considered statistically significant if $p<0.05$ (*), $p<0.01$ (**) and $p<0.001$ (***).

other individual component groups for the given exposure duration (**Figure 1 (d)** and **Figure 2**). Similarly, for the HeLa-R cells, AS20 had IC50 values of 124.28 µg/ml at 24 hours and decreased to 99.82 µg/ml at 48 hours ($p<0.001$) (**Figure 1 (e)** and **Figure 2**).

Induced apoptosis by AS20

Subsequently, the cell and nuclear morphology were observed before and after drug action, under the fluorescent microscope using dual staining with Acridine orange and Propidium iodide (**Figure 3**), Hoechst staining and DAPI staining to determine the possible mode of cell death. Acridine orange stains live cells green, propidium iodide stains dead cells red. Hoechst's and DAPI staining fluoresces the nucleus blue when it binds to dsDNA, which is usually present in apoptotic cells (21). Thus, DAPI positive cells are a possible indicator of the apoptotic cell death. In the estimated cell counts, 0.23% and 0.33% death was obtained in the untreated control group of HeLa and HeLa-R cells, the percentage of apoptotic cells in the vehicle-treated group (dimethyl sulfoxide) of HeLa and HeLa-R cells was found to be 5.88% and 3.17%, respectively, whereas that in the treated group was 53.25% and 51.61% suggesting a notable impact of the IC50 AS20 on apoptotic death of HeLa and HeLa-R cells, respectively (**Figure 4**). The cells undergoing apoptosis, represented by the morphological changes of apoptotic nuclei were observed in AS20 treated group (**Figure 3**). The apoptotic process seems to be a bit slower in AS20 treated HeLa-R cells when compared with that in HeLa cells.

DISCUSSION

The results obtained have implications in the field of integrative oncology as AS20 is suggestive of the amplified in vitro anti-cancer and pro-apoptotic properties of the AS plant. As a result, this formulation can be further studied to work as a complementary and alternative approach to mainstream cancer therapies both to enhance the anti-cancer properties of the mainstream treatment and to alleviate its undesirable repercussions on the body.

Figures 1 and **2** support our hypothesis that AS20 possessed greater anti-cancer properties than extractions of individual parts of the plant. Specifically, the formulation displayed a far greater anti-cancer property than its different components and the positive control group (leaves water extract), since a lower concentration was required to kill 50% of the cancerous HeLa cells. Furthermore, a significant difference existed between the IC50 value for 24 hours and 48 hours, which showed the substantial impact of exposure time of AS20 on killing cancerous cells. A similar trend in the results was obtained for HeLa-R cells with the use of AS20. It showed that AS20 also has the potential to destroy cancerous cells despite their resistance to anti-cancer drugs.

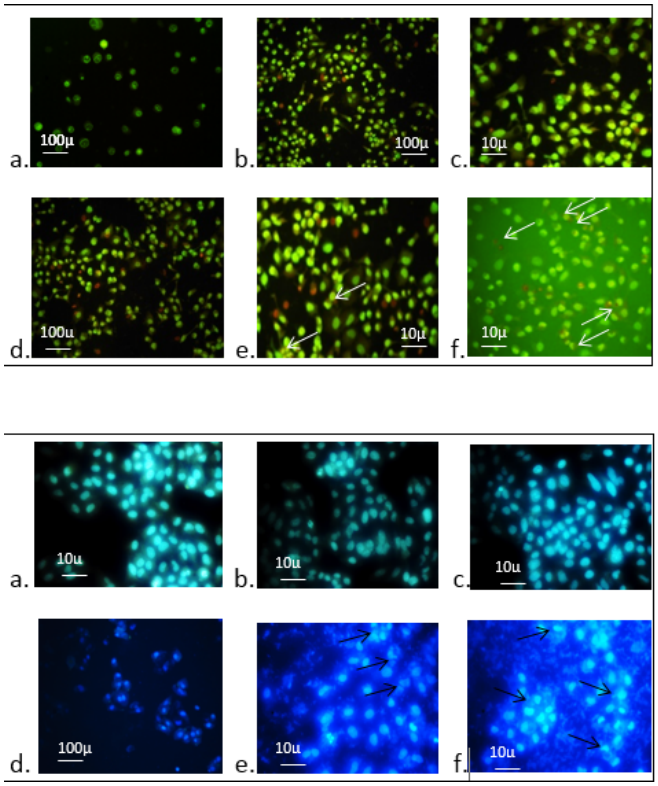

Figure 3: Apoptosis caused by AS20 on treated groups: (a) HeLa control, (b) HeLa-R control, (c) HeLa vehicle control (DMSO), (d) HeLa-R vehicle control (DMSO), (e) HeLa treated, and (f) HeLa-R treated cells were observed under fluorescent microscope using Acridine Orange and Propidium Iodide (dual) staining (20x) (top) and Hoechst and DAPI (dual) staining (20x) (bottom) with NA of 0.34. Presence of fluorescent nuclear fragmentation and blebs is indicative of apoptosis marked as white arrow in Acridine Orange and Propidium Iodide (dual) staining and as black in Hoechst and DAPI (dual) staining. Scale bar: 10µ and 100µ.

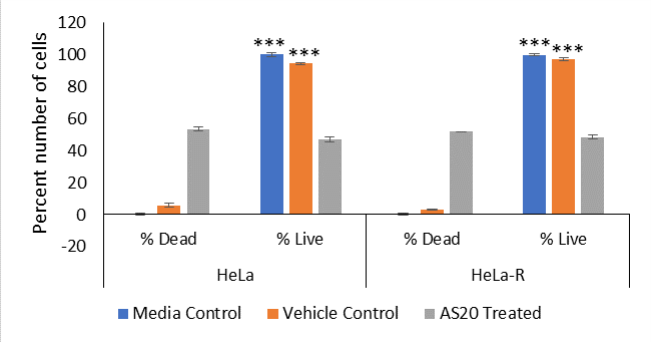

Figure 4: Bar graph showing dead and living cells obtained from fluorescent staining of HeLa and HeLa-R cells in media control, vehicle control and with the treatment of AS20 (N=3, mean±SD). The graph shows significant difference (p<0.001) between living and dead cells in media control group of both the cell types, as well, in the vehicle (DMSO) control group. There was no significance found in the groups treated with AS20.

As the IC50 of AS20 on HeLa-R cells was significantly higher, it could be used in a complementary fashion.

From the cell morphology obtained in Figure 3 and 4, it is evident that there was a higher number of DAPI positive cells in the treated HeLa than in control. In the treated group, there is evidence of both live and dead cells since the HeLa was treated with the IC50 of the drug (50% of cell death), hence suggesting the efficacy of drug action in killing cancer cells. There was an increase in apoptotic cells by 48% (Hoechst's stain) in the group treated with AS20, indicating a notable impact of the IC50 of AS20 on the apoptotic death of cancerous cells. The stained images of HeLa-R after the treatment also displayed higher DAPI positive cells.

As the experiment involved several steps, there was scope for human error. For instance, the serial dilution procedure required pipetting minimal volumes from well to well, which could have introduced errors. For the MTT assay, the incubation start time was assumed to be the same, even the addition of MTT was done sequentially. Besides, the cell counting in Hoechst's staining was carried out manually, to prevent errors.

While our results for the MTT assay on HeLa cells compared the anti-cancer property of the AS20 formulation with that of its constituents, the same was not done for the HeLa-R as it assumed to show similar characteristics. Additionally, a limitation of our study was that quantitative cell counting was done only for Hoechst's staining method as there was no difference in observation between Hoechst's and DAPI methods. Future studies could: 1) compare AS20 with its constituents for MTT assay against the HeLa R cell line 2) carry out quantitative cell counting based on morphology using DAPI as well 3) explore additional staining methods to confirm that it is indeed apoptosis that is occurring on addition of AS20. An example of a method which can be explored is the usage of an antibody for cleaved caspase-3, a more accurate apoptotic marker which is activated at the start of apoptosis (22).

Future experimentation in this field could expand on other pharmacological properties of AS20, such as antimicrobial, antifungal and anti-inflammatory. Other species in the Amaranthus genus can be assessed for their anti-carcinogenic properties in a similar manner. Furthermore, AS20 can be combined with other crude extracts and explored for enhanced anti-cancer properties. Other cancerous cell lines may also be explored, which could be an exciting future direction.

MATERIALS AND METHODS

Cell viability assay

The parameters chosen for MTT assay (4, 5-dimethyl tetrazolium-2-yl, 2, 5-diphenyl tetrazolium bromide assay) were based on the high presence of phytochemicals from previously conducted phytochemical screening (11). From the phytochemical studies, the constituent groups of AS20 were of particular interest. A positive control group (leaves water extract) which contained all the phytochemical groups was included in this evaluation (Table 1). Dimethyl sulfoxide (DMSO) was used as a vehicle control.

The MTT assay protocol was adapted from that described by Morgan D.M.L (23). 0.3 x 106 HeLa cells were cultured in a 96 well plate with Dulbecco's Modified Eagle Medium (DMEM) in 10% Fetal Bovine Serum (FBS) (100 µl, 3000-5000 cells per well). The cells were left for 24 hours of incubation for cell adhering. The samples were then treated with two-fold serial dilutions of AS inflorescence acetone, AS leaves methanol, AS leaves water and the AS20 (10% of the original stock solution was used as first concentration for all groups). Incubation was carried out for 48 hours and 24 hours of drug action. After these durations, 20 µl of MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) was added and the plates were incubated for 4 hours to allow for action of MTT on the mitochondria of live cells. Subsequently, the media from each well was discarded and 100 µl DMSO was added to dissolve the purple formazan crystals. The presence of purple formazan crystal indicated the presence of live cells. The absorbance readings were taken at a wavelength of 545nm in the spectrophotometer.

Table 1: Groups considered for MTT cell viability assay. Range (+++) represented concentrations in the range 1001-2000 µg/ml.

Samples	Phytochemicals	Range
Inflorescence Acetone	Flavonoids	+++
Leaf methanol	Polyphenols Saponins Alkaloids	+++
Leaf water	Flavonoids Polyphenols Alkaloids Saponins	+++
AS20 (Combination of acetone extracted inflorescence and leaf extract in methanol)	Flavonoids Polyphenols Alkaloids Saponins	+++

Three independent sets of experiments were conducted in triplicates to increase accuracy. The following formula determined the percentage of cell viability:

$$Abs_{Sample} / Abs_{Control} \times 100$$

The formula calculated the percentage cell cytotoxicity:

$$(Abs_{Control} - Abs_{Sample}) / Abs_{Control} \times 100$$

From the absorbance readings obtained, the cell viability values were obtained for each concentration of each sample. The percentage viability graphs were plotted against the concentration of the sample for all four groups. The IC50.tk software (24) was used to determine the IC50 value for MTT assay for each of the four groups (after both 24 and 48 hours of drug action). Similar protocol was performed to determine the IC50 value of AS20 on HeLa-R cells.

Cell Staining and Microscopy

HeLa cells were cultured in a 24 well plate with Dulbecco's Modified Eagle Medium (DMEM) in 10% Fetal Bovine Serum (FBS) (1 ml, 0.1 x 106 cells per well) and were treated with the IC50 concentration of AS20 as was carried out for the MTT assay. After 24 hours of drug action, the media was discarded and the cells were washed with Phosphate Buffer Saline (PBS), which was maintained at a pH of 7.4 using Hydrochloric acid (HCl), Sodium Hydroxide (NaOH) and litmus paper. After the addition of PBS, the well plate was incubated for 5 minutes at room temperature. After this incubation, fixative Methanol was added to the wells and incubated for a further 5 minutes. Subsequently, the cells were washed once more with ice-cold PBS. The fluorescent stains were used in the dark and were covered with aluminium foil to prevent exposure of the wells to ambient light. The respective dyes were added after fixation and the wells were observed immediately under an Olympus fluorescent microscope (25), to prevent the possibility of cell death over time.

Dual staining: Dual staining method was adapted from that used by Mascotti *et al*. (26). 5 µl of (1 mg/ml) Acridine Orange and 5 µl of (1 mg/ml) Propidium Iodide were added to the cells and the treated sample, as well as the control sample, was observed under the fluorescent microscope in a dark room at 488-493 nm (excitation), 630 nm (emission).

Hoechst's staining: Hoechst's staining method was adapted from the protocol, followed by Crowley *et al*. (27). 50 µl-100 µl (0.5 µg/ml) Hoechst's stain was added to the cells and the treated sample, as well as the control sample, was observed under the fluorescent microscope in a dark room at 350-460 nm. For the Hoechst's staining, an approximated quantitative analysis was also carried out and cells were counted under the fluorescent microscope using a manual counter.

DAPI staining: The Hoechst's staining method used was adapted for DAPI (4′,6-diamidino-2-phenylindole). 50 µl-100 µl (1000 mg/ml) DAPI stain was added to the cells and the treated sample, as well as the control sample, was viewed under the fluorescent microscope in a dark room at 359-461 nm.

The images were captured by ProgRes® Capture Pro software (28) using Olympus microscope under 20X magnification and 0.34 numerical aperture (NA). These staining protocols indicated the possibility of the apoptotic mode of cell death.

Statistical analysis

GraphPad Prism software was used to analyze the data. ANOVA was used to analyze the statistical difference between groups, which was followed by Tukey's test. All the experiments were performed three times independently in triplicates. $P<0.05$ was considered as significant, denoted by single asterisk, $p<0.01$ was denoted by double asterisk and $p<0.001$ by triple asterisk.

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