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Research Article

Triphala Suppresses Ovarian Cancer Cell Proliferation through Induction of Apoptosis and ROS *in vitro*

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Abstract

Background and Objectives: Breast cancer is one of the leading cancers types globally. Several strategies have been followed to control breast cancer. *Triphala* is an Ayurvedic formulation that is composed of three different plant extracts. The current work aims at evaluating the effect of *Triphala* against ovarian cancer cell line PA-1 on the proliferations and the potential molecular mechanism of action. **Materials and Methods:** Breast cancer PA-1 cell lines were cultured *in vitro* and proliferation was measured using an MTT assay. Intracellular ROS level was measured using the Dichloro–Dihydro-Fluorescein Diacetate (DCFH-DA) assay and apoptosis were observable with AO/EB staining while; the expressions of associated genes were determined by polymerase chain reaction. **Results:** Cell viability was reduced by the dose-dependent manner of *Triphala*. Further, treatment with *Triphala* induced morphological changes indicative of apoptosis, displayed elevated levels of oxidative stress and typical signs of apoptosis were observable with AO/EB staining. Most importantly, *Triphala* abolished the cell adhesion in PA-1 cells. At the molecular level, *Triphala* caused a dose-dependent increase in the expression of cMyc, an oncogene responsible for the induction and maintenance of cancer. Another apoptotic regulator Bax was over-expressed by *Triphala*. **Conclusion:** This studies have shown the possibility of exploiting *Triphala* as an anti-cancer agent, especially against ovarian cancer.

Key words: *Triphala*, ovarian cancer, apoptosis, oxidative stress, PA-1, polypeptide, anti-cancer

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Ovarian cancer is one of the cancers prevalent throughout the world with a very high mortality rate. It is most prevalent among non-Hispanic white women[1]. Ovarian cancer originates from both form epithelial and non-epithelial cells among which epithelial origin dominates[2]. A variety of factors contribute to the development of ovarian cancers which include, oral contraceptives, lack of pregnancy, delayed childbirth, pelvic inflammatory diseases, endometriosis, ovarian cysts, tubal ligation etc. A considerable number of ovarian cancers displays a germline mutation in BRCA, a gene that functions as tumour suppressor genes[3]. A variety of treatment strategies have been followed to treat ovarian cancer. Cisplatin, paclitaxel, doxorubicin is some chemotherapeutic agents being used for the treatment of ovarian cancer. However, ovarian cancer cells exhibit drug resistance as well. Drug efflux through ABC transporters and other non-ABC transporters results in the development of drug-resistant cancer cells. On the other hand, inducing apoptosis is another strategy followed to eliminate cancer cells.

Triphala churna is an *Ayurvedic* preparation in which three different fruit powders are mixed in equal proportion. The three plants include *Emblica officinalis* Gaertn., *Terminalia bellerica* Roxb. and *Terminalia chebula* Retz. Recent research on *Triphala churna* has revealed that the preparation is beneficial against a wide variety of diseases. *Triphala churna* has been shown to improve nephropathy by inhibiting TGFβ1 and recovering from oxidative stress in rodent model[4]. *Triphala churna* has been shown to have a strong anti-diabetic effect by improving the activities of pancreatic β cells. The treatment improved insulin secretion through glucose-dependent insulin-promoting polypeptide. *Triphala churna* also improves the activity of incretin-cAMP signaling[5]. *Triphala churna* has also been effective in ameliorating arthritis by suppressing the NfκB pathway in macrophages[6].

Several strategies have been followed for treating ovarian cancers. PARP inhibitors[7], aromatase inhibitors[8], MEK inhibitors[9] are some examples. In addition, anti-angiogenic agents such as monoclonal antibodies, small molecules are also effective against ovarian cancer. Radiotherapy is also being practised to control ovarian cancer. However, all these methods have a wide range of adverse effects. Natural products are being screened for their activity against ovarian cancer. Extracts of *Camptotheca acuminate*, *Allium sativum*, *Quercus tinctoria asparagus racemosus*, *Curcuma longa*, *Saraca indica*, *Azadirachta indica*, *Carica papaya*, *Geissospermum vellosii*, *Vernonia calvoana* have shown potent anti-cancer activity against ovarian cancer. Plant extracts tend to have very limited adverse effects. Therefore, this study aimed to evaluate the effect of *Triphala* on ovarian cancer.

MATERIALS AND METHODS

Study area: The study was carried out at the Department of Operating Room, the People's Hospital of Jiyang, Jinan, Shandong Province and China from February-May, 2021.

Source of chemical and reagents: Dulbecco's Modified Eagle's Medium, streptomycin, penicillin-G, L-glutamine, phosphate-buffered saline, 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, 2'7'diacetyl dichlorofluorescein, sodium dodecyl sulphate, trypan blue, trypsin-EDTA, ethylene diamine tetraacetic acid, acridine orange, ethidium bromide, rhodamine-123, triton X-100, ethanol, Dimethyl Sulfoxide (DMSO) and bovine serum albumin were purchased from Sigma Aldrich Chemicals Pvt. Ltd.

Preparation of *Triphala*: A total of 10 g of each of the plant powder (*T. chebula*, *E. officinalis* and *T. bellerica*) in a ratio of 1:1:1 was dissolved in 10 mL Sterile Milli Q water, boiled in the water bath at 100°C for 1 hr. The boiled extract was cooled and centrifuged at 5000 rpm for 10 min, the pellet was discarded and the supernatant was stored at 4°C for further assays.

Cell culture maintenance: Human ovarian cancer PA-1 cell line was procured from the Cell repository of American Type Culture Collection (ATCC). Dulbecco`s Modified Eagle Media (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), Penicillin (100 U mL^{-1}) and streptomycin (100 µg mL^{-1}) was used for maintaining cell line. The cell line was maintained in a humidified environment with 5% CO_2 at 37°C.

MTT assay: The cytotoxicity of *Triphala* on PA-1 cells was determined by the method of Sylvester[10]. Cell viability assay, PA-1 viable cells were harvested and counted using a hemocytometer (Winstar Co Ltd, Shenyang, Liaoning, China) diluted in DMEM medium to a density of 1×10^4 cells mL^{-1} was seeded in 96 well plates for each well and incubated for 24 hrs to allow attachment. After PA-1 cells were treated with control and the containing different concentrations of *Triphala* (50-350 µg mL^{-1}) were applied to each well. PA-1 cells were incubated at 37°C in a humidified 95% air and 5% CO_2

incubator for 24 hrs. After incubation, the drug-containing cells wash with a fresh culture medium and the MTT (5 mg mL^{-1} in PBS) dye was added to each well, followed by incubation for another 4 hrs at 37°C. The purple formazan crystal formed was dissolved in 100 µL of DMSO was measured at 540 nm using a multi-well plate reader. The results were expressed at the percentage of viable cells to the control. The concentration of the drug that gives half-maximal inhibitory effect (IC_{50}) values were calculated and the optimum doses were analyzed at different periods.

$$\text{Inhibitory of cell proliferation (\%)} = \frac{\text{Mean absorbance of the control} - \text{Mean absorbance of the sample}}{\text{Mean absorbance of the control}} \text{ X 10}$$

The IC_{50} values were determined from the *Triphala* dose-responsive curve where inhibition of 50% cytotoxicity compared to control cells. All experiments were performed three times in triplicate.

Measurement of reactive oxygen species (ROS): Intracellular ROS level was measured by Dichloro-Dihydro-Fluorescein Diacetate (DCFH-DA) assay. DCFH-DA is a lipophilic, cell-permeable compound that is deacetylated in the cytoplasm to FDCF by cellular esterase[11]. DCF was oxidized by radicals such as hydroxyl, peroxyl, alkoxyl, nitrate and carbonate to a fluorescent molecule at excitation 530 nm and emission 485 nm. DCF was not oxidized by hydrogen peroxide or superoxide radical. After PA-1 cells were seeded in 6 well plates (2×10^6 cells $well^{-1}$) for 24 hrs before exposure and different concentrations of the *Triphala* (200 and 250 µg mL^{-1}) and untreated cells were maintained at 37°C (5% CO_2). Overnight grown PA-1 cells were transferred to 24 well plates for 24 hrs. After exposure of *Triphala* (200 and 250 µg mL^{-1}) to PA-1 cells were washed by PBS and loaded with 25 µM DCFH-DA in DMEM for 30 min at 37°C. The treated cells were washed with DMEM and fluorescent was recorded every 5 min in over 30 min at excitation 485 nm and emission 535 nm by spectrofluorimetry at 37°C. ROS increase was calculated by mean slope per min of treated cells and normalized to control.

Measurement of apoptotic induction using acridine orange/ethidium bromide (AO/EB) dual staining method: The fluorescence microscopic analysis of apoptotic cell death was carried out by combining the methods explained by Mironova[12] and Smith *et al.*[13] PA-1 cells were seeded at 5×10^4 cells $well^{-1}$ in a 6 well plate and incubated for 24 hrs. At the end of treatment with *Triphala*, the cells were collected and washed three times with PBS. The plates were stained with acridine orange/ethidium bromide (AO/EB 1:1 ratio; 100 µg mL^{-1}) for 5 min and examined immediately under fluorescent microscope 40x magnification. The numbers of apoptotic cells were counted in the field.

Table 1: List of primers and sequence

	Forward (5'-3')	Reverse (5'-3')
cMyc	TCTCCGTCCTCGGATTCTCT	TGAGCTCCCAAATCTCTCCAG
BAX	CGTGTCTGATCAATCCCCGA	GAGGCCAGAAGGCAGGATTG
B-actin	TCAAGGTGGGTGTCTTTCCTG	ATTTGCGGTGGACGATGGAG

Cell adhesion assay: Cells were treated with different doses of *Triphala* for 48 hrs and gently washed and stained with 0.2% of crystal violet in 10% formalin solution. After the removal of the staining solution, the cells were washed 3X with Phosphate-Buffered Saline (PBS) and air-dried and observed under an inverted microscope[14] (Biobase Meihua trading Co. Ltd., Jinan, Shandong, China).

Polymerase chain reaction: DNA isolation was done using a Nucleospin DNA isolation kit (MACHEREY-NAGEL GmbH and Co. KG, Duren, Germany) following the manufacturer's instructions. PCR was performed using thermal cycler gradient PCR (Hangzhou Bioer Technology Co., Ltd, Hangzhou, Zhejiang, China). Reactions were performed in a volume of 25 µL with the following constituents: 4.5 µL of template DNA, 12.5 µL of 1XPCR Emerald master mixture. About 1 µL each of the specific primers were added and made up with the remaining volume of Milli Q water. The amplification program consisted of 1 cycle at 95°C for 2 min for initial denaturation, 95°C for 1 min for DNA denaturation followed by annealing at respective temperatures (β-Actin: 54°C; cMyc: 59.6°C; Bax 54.3°C) for 30 sec for annealing and 72°C for 1 min for extension of two strands, 72°C for 5 min for a final extension. The same condition followed for 34 cycles. The list of primers and their sequence is given in Table 1. The PCR products were analysed on a 1.0 % agarose gel containing 1 mg mL^{-1} ethidium bromide, 6 µL of the PCR products with 4 µL of 6X gel loading dye were loaded into wells and electrophoresis was performed in 1 X TAE buffer at 50 V for 2 hrs. The gel was visualized under UV and photographed.

RESULTS

Cell viability: *Triphala* being an Ayurvedic preparation we attempted to evaluate its effect on ovarian cancer cells *in vitro*. To evaluate the effect of *Triphala* MTT assay was performed. Ovarian carcinoma cells were treated with various concentrations of *Triphala*. (50, 100, 150, 200, 250, 300 and

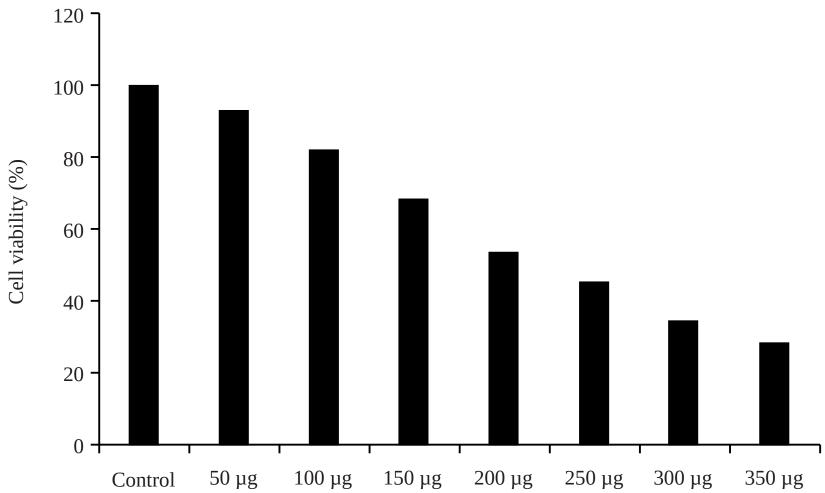

Fig. 1: Cell viability in control and *Triphala* treated human ovarian cancer PA-1 cells for 24 hrs
A dose-dependent decrease in cell viability proved the anti-cancer potential of *Triphala*

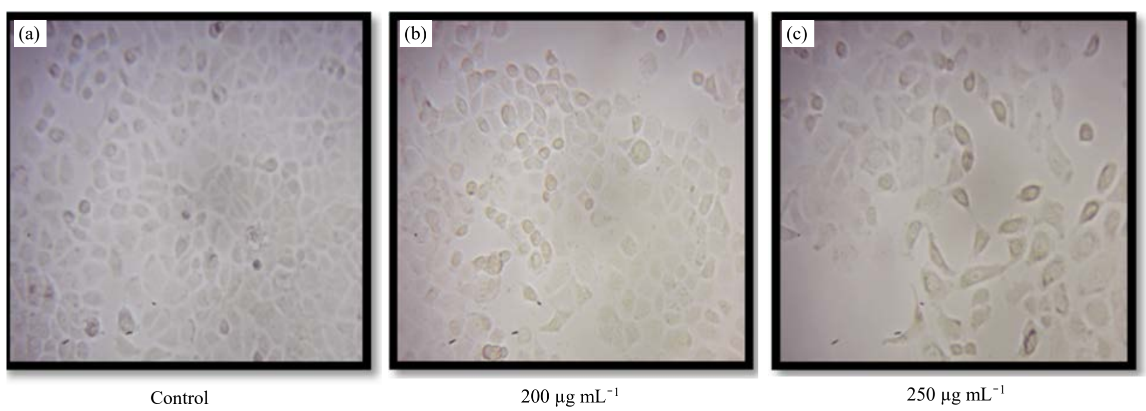

Fig. 2(a-c): Morphological changes in control and *Triphala* treated human ovarian cancer PA-1 cells for 24 hrs
Above-mentioned features are a typical characteristic of apoptosis. The same features could also be observed in the same cell lines which were treated with *Triphala* at a concentration of 200 µg mL^{-1} for 24 hrs but to a lesser extent than 250 µg mL^{-1}. The number of cells also decreased in a dose-dependent manner

350 µg) Cell viability decreased with increasing concentrations of *Triphala* (Fig. 1). A dose-dependent decrease in cell viability proved the anti-cancer potential of *Triphala*.

Morphometry: Cell viability assay revealed its strong anti-cancer activity. The anticancer activity is through induction of apoptosis. Apoptosis is apparent from the morphological changes in an ovarian cancer cell line. Treatment of PA-1 cells with *Triphala* at a concentration of 250 µg mL^{-1} for 24 hrs, could induce characteristic changes in morphology. The cells underwent shrinkage and subsequently got detached. Moreover, membrane blebbing was also visible under a light microscope (Fig. 2a-c). The above-mentioned features are typical characteristics of apoptosis. The same features could also be observed in the same cell lines which were treated with *Triphala* at a concentration of 200 µg mL^{-1} for 24 hrs but to a lesser extent than 250 µg mL^{-1}. The number of cells also decreased in a dose-dependent manner.

Measurement of ROS: To evaluate if there is any involvement of reactive oxygen species did a qualitative assay for the presence of ROS. The cells were stained with fluorescent dye DCFH-DA and visualized under a microscope. Treatment of PA-1 cells with *Triphala* induces ROS accumulation in the cells. Figure 3a-c, there was a dose-dependent increase in the intensity of fluorescence within the cells. The intensity of fluorescence is directly proportional to the amount of ROS.

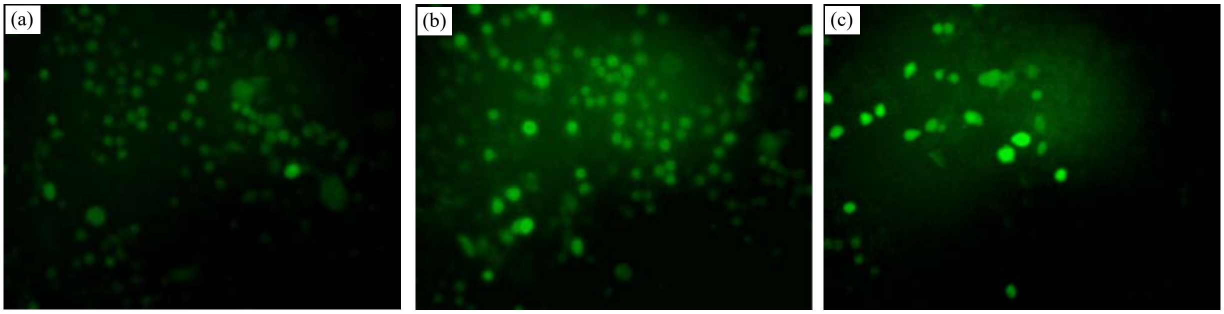

Fig. 3(a-c): Effects of *Triphala* on the intracellular ROS generation in PA-1 cells by DCFH-DA staining assay
There was a dose-dependent increase in the intensity of fluorescence within the cells. The intensity of fluorescence is directly proportional to the amount of ROS

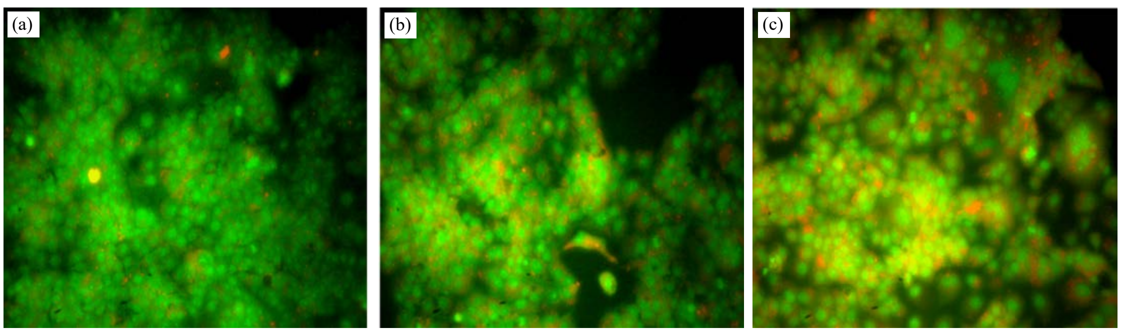

Fig. 4(a-c): Effect of *Triphala* on on the apoptotic incidence in PA-1 cells
Dense green spots indicate nuclear condensation which is an indication of apoptosis

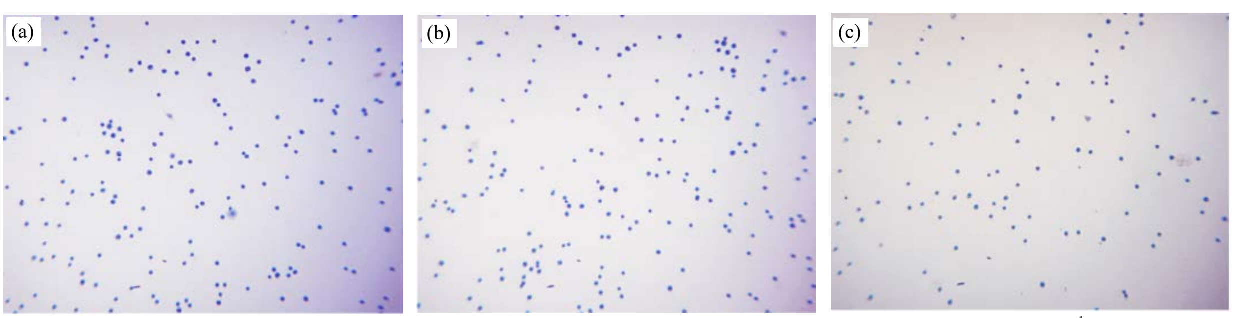

Fig. 5(a-c): Effects of *Triphala* on the Cell adhesion of PA-1 cells
These results imply that the adherence of cells was abrogated by the treatment with *Triphala*

Induction of apoptosis by *Triphala*: To evaluate whether *Triphala* induces apoptosis we performed AO/EB staining. The control cells stained uniformly green with sporadic orange colours indicating that most of the cells are alive and healthy with occasional necrotic cells. At a 200 µg mL^{-1} concentration of *Triphala* more cells stained with dense green spots could be observed. Further, at 250 µg mL^{-1} concentration of *Triphala*, intensely stained green coloured cells could be seen with a few orange colour stained cells (Fig. 4a-c). Dense green spots indicate nuclear condensation which is an indication of apoptosis.

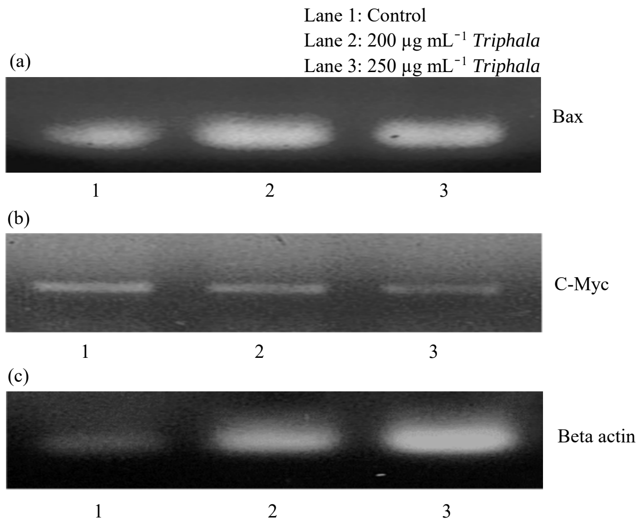

Fig. 6(a-c): Effect of *Triphala* on apoptotic related gene expression of PA-01 cell line, (a) Bax gene, (b) c-Myc and (c) Beta-actin.
The expression cMyc was inhibited by *Triphala* in a dose-dependent manner. On the other hand, pro-apoptotic gene Bax was overexpressed by *Triphala* in a dose-dependent manner

Effect of *Triphala* on cell adhesion: The cells upon treatment with *Triphala* lost the ability to adhere to the surface of the wells in 96 well plates. The number of cells attached to the surface of the plate decreased in a dose-dependent manner. Less number of cells stained with crystal violet was observable in the cells treated with *Triphala* at a concentration of 200 μg mL^{-1}. The cells treated with 250 μg mL^{-1} of *Triphala* had a much lesser number of stained cells (Fig. 5a-c). These results imply that the adherence of cells was abrogated by the treatment with *Triphala*.

Evaluation of molecular mechanism of apoptosis by *Triphala*: Assessment of the mechanism of anti-cancer activity and induction of apoptosis was done by PCR. Gene-specific primers were used for amplifying the genes. The expression pro-apoptotic gene Bax was overexpressed by *Triphala* in a dose-dependent manner (Fig. 6a). On the other hand, cMyc was inhibited by *Triphala* in a dose-dependent manner (Fig. 6b).

DISCUSSION

Ovarian cancer is one of the most prevalent cancers. Though a variety of treatment strategies are being followed, adverse effects limit the effective clinical applications of the current treatment regimen. Plant extracts and preparations of folk medicines are being attempted for re-purposing against cancers and other diseases since they have limited adverse effects[15,16]. Ayurveda is one of the oldest medical practices having its origin in India. *Triphala* is one of the Ayurvedic preparations which are a combination of three different plants which has a wide application. In the current work, we attempted to evaluate the effect of *Triphala* against ovarian cancer. We used PA-1, an ovarian cancer cell line to study the effect of the preparation, for its anti-cancer potential.

MTT assay revealed that *Triphala* had strong inhibitory activity against the PA-1 cancer cell line. *Triphala* preparation showed inhibitory activity in a dose-dependent manner (Fig. 1). Only 28% of the cells were viable at a concentration of 350 μg mL^{-1}. At concentrations of 200 and 250 μg mL^{-1} 54 and 46% of the cells were viable receptively. Therefore, we took these two concentrations for our further studies as these two concentrations are near to IC_{50}.

Microscopic examination was done for the above-mentioned concentrations for the morphological changes. In line with the results from the MTT assay, there were morphological changes indicative of apoptosis was observable. The number of cells declined in a dose-dependent manner and parallel morphological changes could also be observed. Shrinkage of cells, blebbing of the cell membrane, cellular detachment and distorted cell shape were the changes in a cell in response to *Triphala* treatment. Overall, these morphological changes indicated the possible involvement of apoptosis. In addition, we also observed the loss of adhesion of PA-1 cells upon treatment with *Triphala* (Fig. 5). Loss of adhesion could also trigger apoptosis.

To further confirm the involvement of apoptosis we performed AO/EB staining. Acridine orange stains the nucleic acid and reveals the integrity of the nucleus of the target cell. The control cells displayed a normal staining pattern which could be interpreted that the cells are alive and healthy. On the other hand, cells undergoing apoptosis loses the integrity of the nuclear membrane and chromatin condensation. *Triphala* treatment-induced chromatin condensation and allowed the passage of ethidium bromide which was stained in orange colour which was much pronounced in 250 μg mL^{-1} of *Triphala*. Therefore, it is confirmed that the *Triphala* induced cell death through triggering apoptosis.

Since *Triphala* induced cell death via apoptosis we investigated the cause. Accumulation of ROS is one of the triggering factors for apoptosis. Therefore, we assessed the involvement of ROS by staining the cells with DCHF-DA and observed them under the fluorescence microscope.

Interestingly, we observed moderate fluorescence at 200 μM of *Triphala* and a much stronger green fluorescence at 250 μM of *Triphala*. Overall, staining with DCHF-DA revealed the involvement of reactive oxygen species in a dose-dependent manner. *Triphala*-induced apoptosis in ovarian cancer cells could likely have been mediated by elevated reactive oxygen species. This view is in line with earlier findings that ROS could mediate apoptosis[17-19].

We further evaluated the mechanism of *Triphala* mediated cancer arrest. Gene expression analysis was done to elucidate the molecular mechanism of action of *Triphala* on ovarian cancer. cMyc is a proto-oncogene and activates several transcription factors. This gene is involved in triggering several cancers including ovarian cancer. This gene is also involved in the maintenance of cancer[20]. One of the strategies of targeting cancer is through suppression of cMyc[21]. Therefore, we checked for the expression level of the cMyc gene by PCR. Our results revealed the dose-dependent suppression of cMyc. cMyc was strongly down-regulated by a higher dose of *Triphala* whereas a milder suppression of the gene was observable in a low dose of *Triphala*.

To further evaluate if *Triphala* triggers apoptosis in cancer cells, we estimated the expression of the Bax (Bcl-2 associated protein X) gene. Bax is one of the pro-apoptotic genes belonging to the Bcl2 gene family. Bax interacts with Bid (BH3 interacting-domain death agonist) to result in apoptosis[22]. Triggering of apoptosis is essential for an anti-cancer agent since it does not trigger an inflammatory response. Our results indicate that *Triphala* induces Bax to cause apoptosis.

CONCLUSION

Our results indicate that *Triphala* could be a potential anti-cancer drug, especially for ovarian cancer. Our MTT assay revealed that the Ayurvedic preparation causes cell death in ovarian cancer cells. Further, morphological changes indicated the involvement of apoptosis and fluorescence imaging demonstrated the induction of oxidative stress in a dose-dependent manner. AO/EB staining confirmed the involvement of typical signs of induction of apoptosis. Moreover, treatment with *Triphala* inhibited cell adhesion as revealed by the light microscopy. At the molecular level, suppression of cMyc by *Triphala* was confirmed while induction of Bax. Overall, our study reveals *Triphala* could be used for treating ovarian cancer.

SIGNIFICANCE STATEMENT

The present work explored the effect of *Triphala*, an Ayurvedic preparation, on ovarian cancer. *Triphala* significantly induced apoptosis in ovarian cancer cells *in vitro* as evidenced by elevated expression of Bax. *Triphala*-mediated oxidative stress contributed to the induction of apoptosis.

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