INVESTIGATION OF ANTI-PROLIFERATIVE AND PRO-APOPTOTIC EFFECT OF TRIPHALA ETHANOL EXTRACT AGAINST MG-63 HUMAN OSTEOSARCOMA CELLS - AN IN-VITRO STUDY

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ABSTRACT:

Introduction: Cancer is a major cause of death in developed and developing countries. Triphala is a polyherbal medicine consisting of dried fruits of three plant species. Previous studies have shown that triphala has antioxidant, radioprotective, and chemoprotective activities, which suggest that it may be able to prevent cancer development. Furthermore, Triphala has demonstrated strong anti-proliferative and apoptosis-inducing effects against various tumor cell lines and animal models, without causing damage to healthy cells. **Aim:** The study aims to analyse the anti-proliferative and pro-apoptic effect of Triphala ethanol extract against MG - 63 human osteosarcoma cells. **Materials and method:** For the study, Triphala powder from IMPCOPS (Chennai, India) was soaked in 95% ethanol for 3 days at room temperature. After filtering, the crude extract was subjected to rotary evaporation, and 3g of the material was obtained. The extract was concentrated using vacuum evaporation and stored at 4˚C. MTT[3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] assay and Apoptotic stain assay of MG-63 cells were conducted using fluorescent microscopy. **Result:** Cells were treated with Triphala extract in different concentration (20, 40, 50, 100, 200 & 300 mg) for 24 h, and cell viability was evaluated by MTT assay then Acridine orange/Ethidium bromide (AO/EtBr) staining of MG-63 cells after treatment of IC50 concentration (70mg/ml) of triphala extract for 24h and compared with untreated control cells using fluorescence microscopy. The IC50 values represent the concentration of the Triphala extract that could inhibit 50% of the cell growth. IC50 (70mg/ml) of triphala extract has shown good anti-proliferative and pro-apoptic effects. **Conclusion:** This present study concluded that the triphala ethanol extract had shown a good anti-proliferative and pro-apoptic effect against MG - 63 human osteosarcoma cells. This polyherbal combination shows a promising solution in the field of drug development for osteosarcoma. Further in-vivo studies are necessary to check the efficacy of triphala in treatment of osteosarcoma.

KEYWORDS: Ayurvedic, Osteosarcoma, Cytotoxic assay, Apoptotic assay, MTT assay, Innovation and health.

INTRODUCTION:

Cancer denotes the abnormal growth and proliferation of cells within human body. It interferes with patients quality of life and is one of the leading causes of death globally .The treatment necessitates enormous annual control expenses.[1] In addition to their high prices and shortage of widely accessible cancer medications, these therapies have a long list of adverse effects.[2] In recent years , the use of medicinal plants in place of or in addition to conventional medications as a means of controlling and preventing cancer and its complications has garnered a lot of attention.[3] Previous studies on complementary medicine worldwide have contributed in managing various illnesses and discovery of new drugs. One of the most well-known and advanced subspecialties of complementary medicine is Persian medicine (PM), which has discovered herbal based medications to treat a wide range of illnesses.[4]

In Ayurvedic medicine, triphala has long been used as a general-purpose remedy for a range of conditions, including stomach illness and tooth decay. It is also believed to promote longevity and general health. Given that it comprises a range of medicinal plants, it is categorized as a polyherbal remedy.[5] Polyherbal combination means combinations of herbs that complement one another well together and are believed to be more potent and beneficial than using any one herb by itself. These polyherbal combinations helps in illness prevention and health promotion. Triphala is prepared by combining the dried fruits of three native Indian plants: Haritaki (Terminalia chebula), Bibhitaki (Terminia bellirica), and Amla (Emblica officinalis).[6] Several studies have out in test tubes and on animals have demonstrated the protective effects of triphala against specific malignancies. For instance, it has been demonstrated to inhibit the growth of pancreatic and stomach tumors in mice, as well as lymphoma.[7] In test-tube trials, this herbal medicine also caused the death of cancer cells related to the colon and prostate.[8] Research indicates that triphala's high content of powerful antioxidants such gallic acid and polyphenols may have anti-cancer properties.

Recent clinical studies on triphala demonstrated to have a wide range of biological activities, including laxative, immunomodulatory, antibacterial, and antioxidative ones.[9-13] It is also useful in treating a number of other conditions and diseases, such as gingivitis, arthritis, cataracts, and constipation. Furthermore, a rising amount of experimental research suggests that triphala is a herbal cancer therapy that may be useful. According to previous studies, triphala exhibits potent anti-proliferative and apoptosis-inducing properties against various tumour cell lines and animal models without harming healthy cells. It also shows oxidant, radioactive, and chemoprotective activities, suggesting that it may be able to stop oncogenesis.[14-16] In addition, triphala can inhibit tumor invasion and metastasis via controlling angiogenesis and the epithelial-to-mesenchymal transition.[17]

Metastatic cancers occurs when cancer cells spread from the organ where they started to the distant part of the body. Bone loss is common in patients with metastatic cancer who also have multiple

myeloma, breast, prostate, primary colon, lung, and kidney malignancies. The most prevalent primary malignant bone tumours are Ewing sarcoma (ES), osteosarcoma (OS), and chondrosarcoma (CS), accounting for about 70% of all malignancies. Of the three cancer forms discussed above, osteosarcoma is more common in adults and teenagers, and over 80% of patients experience lung metastases.[18,19] Twenty to thirty percent of people with osteosarcoma survive in the long run following surgery. Furthermore, osteosarcoma is a lytic tumour that spreads quickly and frequently affects the lung in humans.

Osteosarcoma is the most frequent primary solid cancer of bone; it is characterized by the presence of malignant mesenchymal cells that create osteoid and/or immature bone. Adjuvant chemotherapy has somewhat improved osteosarcoma treatment, but during the past thirty years, little has changed most likely as a result of drug resistance. Therefore, there is a need for novel innovative for osteosarcoma treatment methods.[20]

The rationale of the study was that integrating the natural remedies as part of a holistic approach to health is beneficial since they not only treat symptoms as well as promoting general wellbeing, natural therapies are advantageous when used in conjunction with a holistic approach to health. To improve the overall therapeutic effect, people may combine it with allopathic medications to enhance the overall treatment effect.
The study aimed to evaluate the anti-proliferative and pro-apoptic effect of Triphala ethanol extract against MG - 63 human osteosarcoma cells.

MATERIALS AND METHODS:

Extract Preparation:

Stem powder of Triphala Powder obtained from IMPCOPS (Chennai, India) was used for the present study. About 100 g of Triphala powder was soaked in 1000 mL of 95% Ethanol and kept in room temperature for 3 days in a static condition (Figure 1). Then the solution was filtered with crude filter paper followed by Whatman filter paper. Fine filtrate aqueous crude extract was subjected to rotary evaporation after that 3g of the material was obtained. The total ethanol extract of Triphala was concentrated in a vacuum evaporate and immediately stored at 4˚C.

Figure 1: Schematic representation of the steps in the methodology

Cytotoxicity Assay:

MTT Assay [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide]:

To assess the cytotoxicity with different concentration (20, 40, 50, 100, 200 & 300 mg/ml) Triphala extract along with positive and negative control treated on human breast cancer MG-63 cells was determined over 24hr by MTT assay as we previously described (Koka P et al, 2018.). Briefly, Triphala crude extract in different concentration was incubated on MG-63 cells were seeded on 96 well culture plate for 24hr respectively. To determine percent viability, the post incubated cells were replaced with 10 μl of stock MTT dye (10 mg/ml) was added in each well and plate was incubated again at 37 °C for 4 h. The medium was replaced with 100 µl DMSO in each well to dissolve the formazan crystals and absorbance was recorded at 570 nm. with Synergy hybrid Multi-Mode Reader (BioTek, Winooski, VT, US). The following equation was used to determine the percentage of cell viability:

$$\text{Cell viability (\%)} = \frac{OD(\text{test sample}) - OD(\text{blank})}{OD(PC) - OD(\text{blank})} \times 100$$

Acridine Orange/Ethidium Bromide Staining:

The MG-63 cells were seeded in 6 well culture plates, at a concentration of 1×10^6 cells/mL, and incubated for 24 h. Following incubation, the cells were treated with 70 mg/ml (IC50) concentration of Triphala crude. After the treatment, the supernatant was collected and transferred into a falcon tube, whereas, the remaining cells in the flask were trypsinized and collected, using a scraper. The cells were mixed with 2 mL PBS, resuspended, and mixed with the supernatant in the falcon tube. The cells were centrifuged at 300×g for 5 min. The supernatant was discarded and cells were washed again, using 5 mL PBS. The centrifugation step was repeated and the falcon tube was put on ice. One ml Acridine Orange/Ethidium Bromide (AO/EtBr) solution (Sigma-Aldrich, St. Louis, MO, USA) was diluted with PBS (1 mL), the process being carried out under dark conditions, due to the sensitivity of both solutions to light. AO and EtBr solutions (10 mg/mL of each) were transferred into an Eppendorf tube and the mixture was resuspended, repeatedly,

until a homogeneous solution was obtained. An aliquot of the cell preparation (10 µL) was mixed with the prepared AO/EtBr solution. The mixture (10 µL) was transferred on to a slide and viewed under fluorescent live cell microscope with a 20x objective (EVOS FLoid Imaging System, Thermo Fisher Scientific - USA).

RESULTS:

Cytotoxicity Assay:

Figure 2 represents bar graph with the cytotoxic effects of Triphala ethanol crude extract on MG-63 osteosarcoma cancer cells. The X axis indicates percentage of concentration (mg/dl) and Y axis indicates percentage of cell viability. Cells were treated with Triphala extract in different concentration (20, 40, 50, 100, 200 & 300 mg) for 24 h, and cell viability was evaluated by MTT assay. The IC50 values represent the concentration of the Triphala extract that could inhibit 50% of the cell growth.

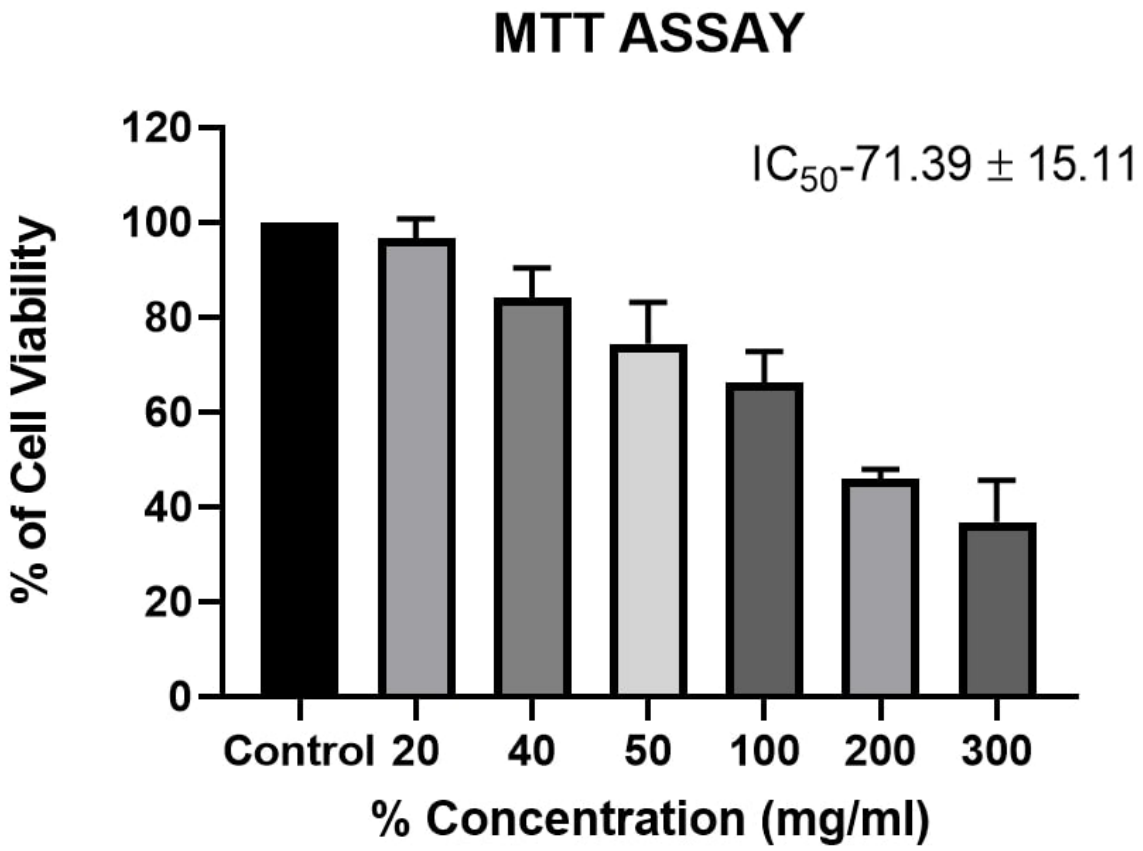

Figure 2: The cytotoxic effects of Triphala ethanol crude extract on MG-63 osteosarcoma cancer cells, Cells were treated with Triphala extract in different concentration (20, 40, 50, 100, 200 & 300 mg) for 24 h, and cell viability was evaluated by MTT assay. The IC50 values represent the concentration of the Triphala extract that could inhibit 50% of the cell growth. Data are shown as means ± SD (n = 3).

Apoptotic stain assay using fluorescent microscopy:

In the Figure 3, the picture represents Acridine orange/Ethidium bromide (AO/EtBr) staining of MG-63 cells after treatment of IC50 concentration (70mg/ml) of triphala extract for 24h and compared with untreated control cells using fluorescence microscopy. When compared to control cells that exhibit green fluorescence, those that display intense orange fluorescence are indicative of apoptosis. When the control and drug-treated groups were compared, the drug-treated group had fewer cells overall and more cells that were developing orange flourosis, a sign of apoptosis. Consequently, the results demonstrate the potency of triphala ethanol extract as an antiproliferative and proapoptotic agent.

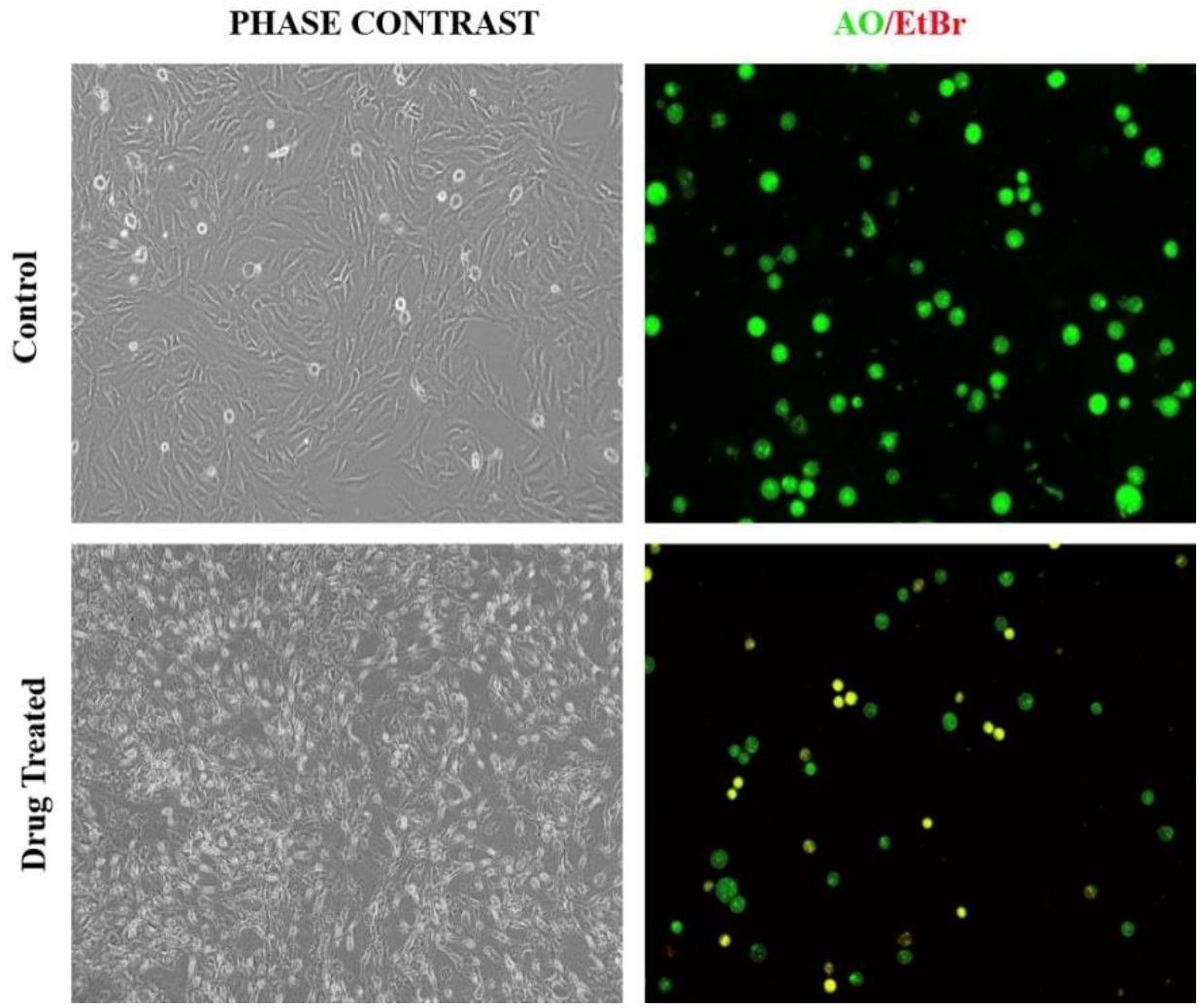

Figure 3: Acridine orange/Ethidium bromide (AO/EtBr) staining of MG-63 cells after treatment of IC50 concentration (70mg/ml) of triphala extract for 24h and compared with untreated control cells using fluorescence microscopy. Cells showing bright orange fluorescence indicate apoptosis in comparison to control cells showing green fluorescence.

DISCUSSION:

Many patients seek complementary or alternative therapies due to the high death rate from cancer and the numerous adverse effects of medications used in radiation and chemotherapy.[21] The most crucial cancer prevention strategies are treating inflammatory conditions, stopping smoking, altering one's diet, and taking immune-stimulating vitamins. In light of recent advancements in the field of cell biology, scientists are currently searching for novel, effective chemotherapeutic approaches to cure cancer with negligible or nonexistent side effects. Chemotherapy is the primary therapeutic approach for managing malignant tumors in their advanced stages. It also serves as a preventive measure against potential metastases, which can pose a major risk to the body's normal cells. Many animal and human ailments have long been treated using plants. In addition to healing illnesses, plants help people stay healthy and strong physically. They can eradicate cancer, for instance, without being hazardous. Over 50% of currently used pharmaceuticals are derived from naturally occurring substances that have anti-cancer properties.[22] According to WHO estimates, primary care is provided by traditional medicine to over 80% of the population in underdeveloped nations. Herbal and traditional medicine has gained global acceptance in recent decades, impacting both international trade and the medical community at the same time. Herbal remedies are integral parts of many people's global health systems.[23] Apoptosis is a type of genetically regulated programmed cell death that eliminates physiologically unnecessary, physically damaged, and faulty cells, regulating the evolution of multicellular organisms and tissues.[24]

Numerous herbs, like T. chebula and P. emblica, have tonic properties and can strengthen the body's immunity, according to reports from Persian medicine. However, recent studies support its qualities, which include immune system stimulation, antioxidants, anticancer, and antibacterial actions.[25,26] A mixture of T. chebula, T. bellirica, and P. emblica is known as Triphala in traditional Indian medical materials. Triphala has been proven in earlier research to exhibit anticancer properties on MCF-7, PC-3, S115, DU-145, T47D, and Barcl-95 cell lines.[27] Tannins, which are potent primary constituents of these plants, might be essential to this strategy. T. chebula contains the tannin compounds gallic acid, ellagic acid, chebulic acid, chebulinic acid, and chebulagic acid. Ellagic acid, gallic acid, ethyl gallate, galloyl glucose, and chebulagic acid are among those found in T. bellirica, while ellagic acid and gallic acid are found in P. emblica.[28] Whether in the form of pure molecules or total tannins, tannins are vital in both preventing and treating cancer. Tannins have excellent potential as preventative and therapeutic agents in the development of novel anticancer medications.[29] In a study done by Ali et al., the HepG2 cancer cell line was found to be sensitive to all concentrations of Triphala and its constituents' cytotoxic action, which may dramatically lower the survival rate as demonstrated by the MTT experiment.[30]

Limitations of the study:

The study is limited to in-vitro settings, which may not adequately replicate the complex interactions that occur in the human body. To confirm the observed effects in a more complete

biological setting, in-vivo studies are necessary. Furthermore, harvesting methods, regional climate, and geographic location can generate variabilities in the content of plant extracts, which could jeopardize the findings' repeatability. The synergistic effects of many compounds in the extracts may also go unnoticed. These disadvantages support extending the research scope in the future.

Future perspectives of the study:

The future scope is to conduct studies to determine the active components responsible for this traditional product's cytotoxic action, in-vivo studies and clinical trials can be conducted to determine the clinical efficacy of the Triphala for osteosarcoma treatment.

CONCLUSION:

This present study concluded that the Triphala ethanol extract had shown good anti-proliferative and pro-apoptotic effects against MG - 63 human osteosarcoma cells. Triphala can be employed as adjunct to synthetic medications, potentially mitigating drug resistance and reducing overall costs while minimizing side effects. The comparison between the herbal formulation and the standard showed that triphala has good anti-proliferative and pro-apoptotic effect. Further research is required to determine the clinical effectiveness in the field of drug development in treating osteosarcoma.

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