Evaluation of cytotoxic and anti-migratory potential of Aegle marmelos fruit extract on osteosarcoma cell line.

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ABSTRACT:

Background: An osteosarcoma is a cancerous tumor in a bone and is an aggressive malignant neoplasm that arises from primitive transformed cells of mesenchymal, origin and produces malignant osteoid.Although a number of anticancer drugs have been discovered, apart from being expensive they have some serious side effects as well. So, it is important to develop safe and economical treatment of the disease.The use of herbal medicine for prevention and cure of various ailments has been practiced by humans since antiquity, and it was the main source of treatment before the evolution of modern allopathic, or synthetic medicine. Thus the aim of the study is to evaluate the anti-migratory and cytotoxic properties of Aegle marmelos fruit extract on osteosarcoma cell line.

Methodology: The cytotoxic effect of aegle marmelos was assessed by an MTT assay. MG-63 cells were plated in 96 well plates at a concentration of $5x10^3$ cells/well 24 hours after plating, cells were washed twice with 100µl of serum-free medium and starved by incubating the cells in serum-free medium for 3 hours at 37ºC. After starvation, cells were treated with Aegle marmelos

fruit extract at different concentrations for 24 hours. Analysis of changes in the cell morphology is examined by phase contrast microscope.

Results: In our study cells were treated with aegle marmelos fruit extract 40µg/ml for 24 hours compared with the control group. These results revealed that aegle marmelos inhibited the proliferation of MG63 cells, changed the cytoplasmic morphology, reduced the migration ability of MG-63 cells and exhibited anti osteosarcoma effects.

Conclusion: As a result of the study it is concluded that the aegle marmelos fruit extract possesses cytotoxic and anti migratory potential on osteosarcoma cell line.

Keywords: Aegle marmelos, Osteosarcoma, Cytotoxicity, Cell Migration, Metastasis.

Introduction

Cancer is an uncontrollable proliferation of cells which leads to tumor formation and spreads from one organ to another (1). Cancer is the main cause of death and acts as an important barrier to increasing lifespan.Generally a cancer cell grows more and then breaks down from the original mass of cells and then travels through blood and lymph systems and attaches to other organs and again starts its abnormal growth cycle (2,3). This process of leaving an organ and going to another organ and continuing its abnormal growth cycle is called metastasis. Such kind of tumor spreads all over the body is called malignant tumor (2).

Osteosarcoma is a type of bone cancer that usually develops in the osteoblast cells that form bone (4). It happens most often in children, adolescents, and young adults. Osteosarcoma most commonly happens in the long bones around the knee. Other sites for osteosarcoma include the upper leg, or thigh bone and lower leg. Osteosarcoma may grow into nearby tissues, such as tendons or muscles. It may also spread, or metastasize, through the bloodstream to other organs or bones in the body (4,5). The use of herbal medicine for prevention and cure of various ailments

has been practiced by humans since antiquity, and it was the main source of treatment before the evolution of modern allopathic, or synthetic medicine (6). According to evidence from the centers of civilization, plants have been employed as a source of cure for a variety of health concerns in almost all civilizations from the dawn of human civilization. They are a fantastic source of exogenous antioxidants, with activity ranging from incredibly low to highly high (7).

A. marmelos (commonly known as "bael") is often called Indian quince, Bengal quince and golden apple in English. It is a slow-growing, tough subtropical tree and the only plant belonging to the genus Aegle (8). The tree grows in the wild in well-drained soil up to about 12 to 15 m in height, even in the harsh and dry climates. They have spiny branches and alternate leaves with three to five oval, pointed, shallowly toothed leaflets (9). The flowers are fragrant and are found in clusters along the young branches (9).

Materials and Methods

Cell line maintenance

Osteosarcoma cancer cell line (MG-630 were obtained from the NCCS, Pune. The cells were grown in T25 culture flasks containing DMEM supplemented with 10% FBS and 1% antibiotics. Cells were maintained at 37◦C in a humidified atmosphere containing 5% CO2. Upon reaching confluency, the cells were trypsinized and passaged.

Preparation of the Herbal Extract:

Fruit extract of Aegle marmelos Fruit powder obtained from IMPCOPS (Chennai, India) was used for the present study. About 50g of aegle marmelos powder was soaked in 500 mL of 95% ethanol and kept at room temperature for 3 days in a static condition. Then the solution was filtered with crude filter paper followed by whatman paper. Fine filtrate was subjected to rota evaporation after that 3g of the material was obtained. The total ethanol extract was concentrated in a vacuum and immediately stored at 4˚C.

Cell viability (MTT) assay

The cell viability of Aegle marmelos fruit extract treated MG-63 cells was assessed by MTT assay. The assay is based on the reduction of soluble yellow tetrazolium salt to insoluble purple formazan crystals by metabolically active cells. MG-63 cells were plated in 96 well plates at a concentration of $5x10^3$ cells/well 24 hours after plating, cells were washed twice with 100µl of serum-free medium and starved by incubating the cells in serum-free medium for 3 hours at 37ºC. After starvation, cells were treated with Aegle marmelos fruit extract at different concentrations for 24 hours. At the end of treatment, the medium from control and *Aegle* marmelos fruit extract treated cells were discarded and 100µl of MTT containing DMEM (0.5 mg/ml) was added to each well. The cells were then incubated for 4h at 37ºC in the CO_2 incubator.

The MTT containing medium was then discarded and the cells were washed with 1x PBS. Then the formazan crystals formed were dissolved in dimethyl sulfoxide (100µl) and incubated in dark for an hour. Then the intensity of the color developed was assayed using a Micro ELISA plate reader at 570 nm. The number of viable cells was expressed as the percentage of control cells cultured in serum-free medium. Cell viability in the control medium without any treatment was represented as 100%. The cell viability is calculated using the formula: % cell viability = [A570 nm of treated cells/A570 nm of control cells]×100.

Morphology study

Based on MTT assay we selected the optimal doses for further studies. Analysis of cell morphology changes by a phase contrast microscope. 2×10^5 cells were seeded in 6 well plates and treated with Aegle marmelos fruit extract (40 µg/ml for MG-63 cells) for 24h. At the end of the incubation period, the medium was removed and cells were washed once with a phosphate buffer saline (PBS pH 7.4). The plates were observed under a phase contrast microscope.

Cell migration analyzed by scratch wound healing assay

Human osteosarcoma cell lines (2×10^5 cells/well) were seeded onto six-well culture plates. The cell monolayer was scratched using a 200µl tip to create a wound. The detached cells were removed by washing with 1X PBS and add fresh culture medium with Aegle marmelos Fruit extract (40µg/ml for MG63 cells) for 24 h along with control group for 24 h. After incubation,

the wells were washed and fixed in 4% paraformaldehyde. Photographs were taken using an inverted microscope (Euromex, The Netherlands).

Statistical analysis

All data obtained were analyzed by One way ANOVA followed by Student's-t-test using SPSS, represented as mean ± SD for triplicates. The level of statistical significance was set at $p<0.05$.

Results

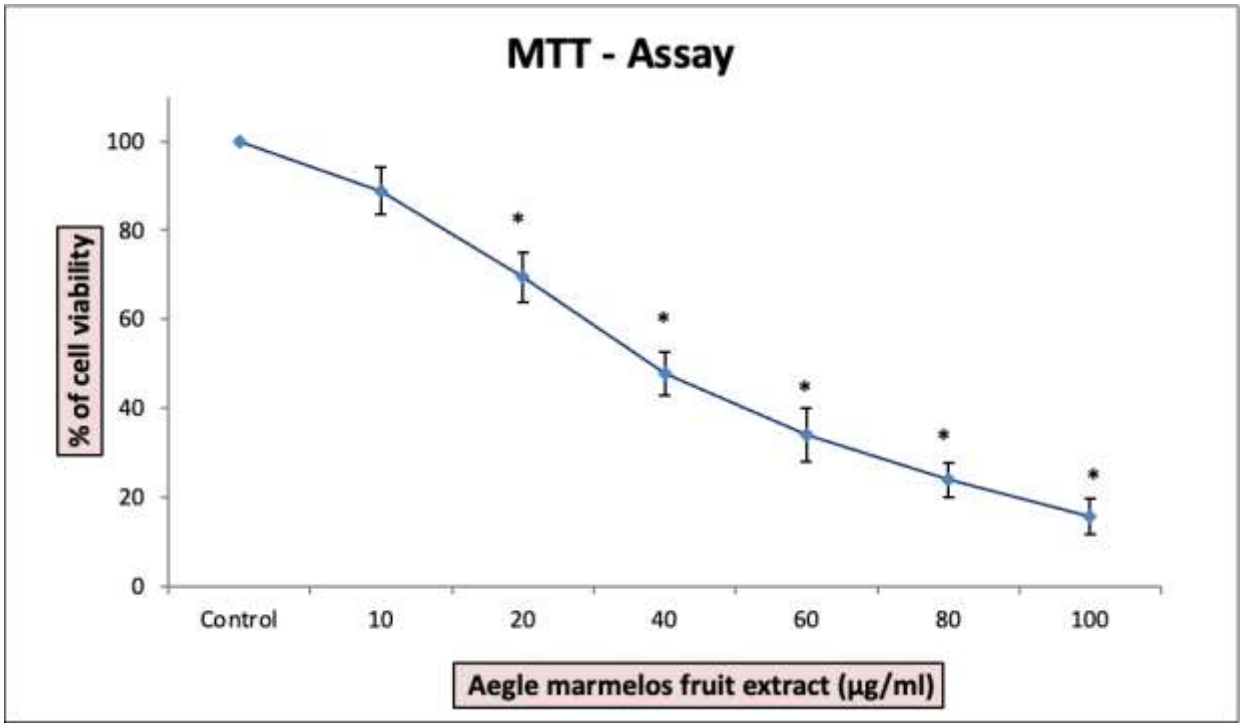

Figure 1: The cytotoxic effects of Aegle marmelos fruit extract on osteosarcoma cell lines. Cells were treated with aegle marmelos (10, 20, 40, 60, 80 and 100 µg/ml) for 24 h, and cell viability was evaluated by MTT assay. Data are shown as means ± SD ($n = 3$). * compared with the control blank group, $p < 0.05$.

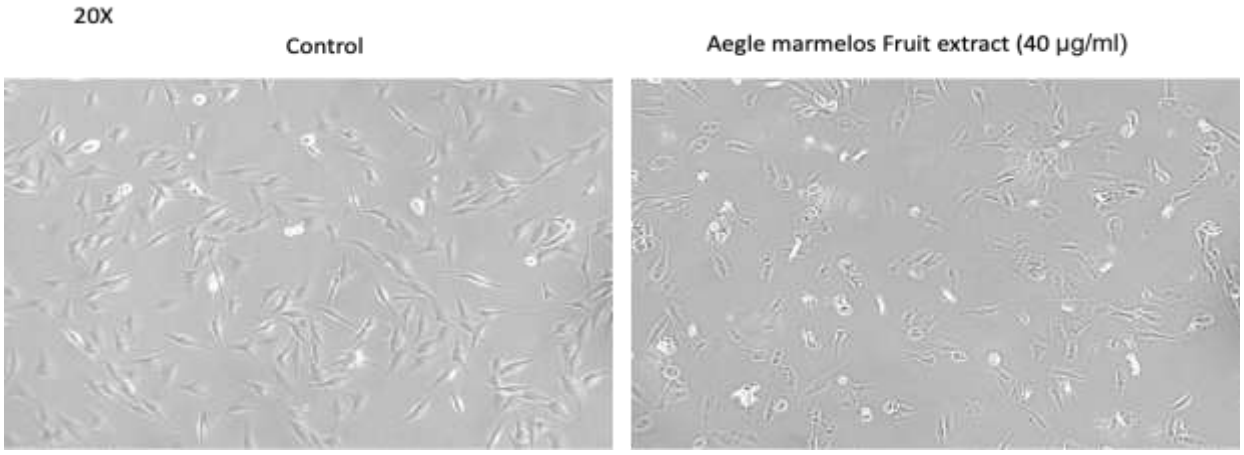

Figure 2: Effect of Aegle marmelos Fruit extract (40 µg/ml) on cell morphology of human osteosarcoma cells (MG-63). Cells were treated with Aegle marmelos Fruit extract (40 µg/ml) for 24 h and cells were observed under an inverted phase contrast microscope. The number of cells decreased after Aegle marmelos Fruit extract (40 µg/ml) treatment and the cells exhibited cell shrinkage and cytoplasmic membrane blebbing.

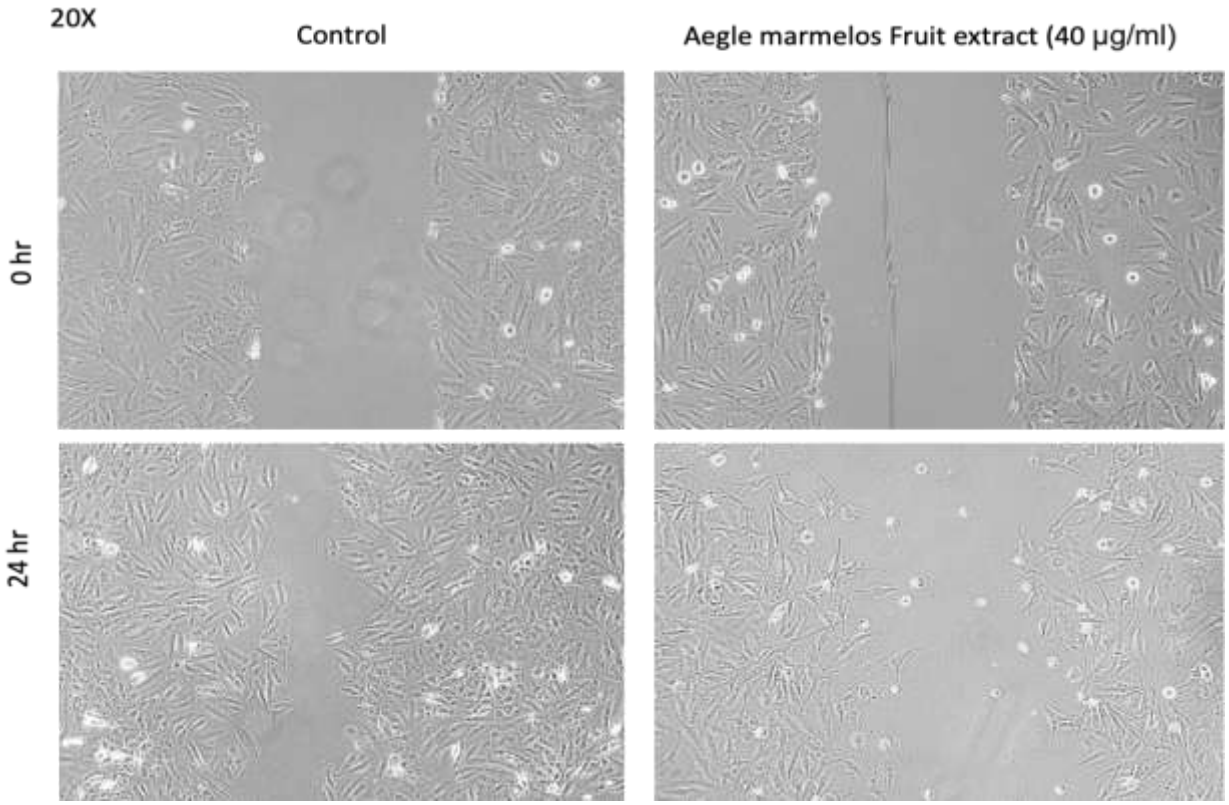

Figure.3. Anti-migratory potential of Aegle marmelos Fruit extract (40 µg/ml) in osteosarcoma cells. In vitro scratch wound healing assay. Osteosarcoma cells (MG-63) were injured and cell migration assay with and without treatment was performed at 24h and 40 µg/ml concentrations of Aegle marmelos Fruit extract. Images were obtained using an inverted Phase contrast microscope.

3.1. Effect of Aegle marmelos fruit extract (40 µg/ml) on cell viability of breast cancer cell line

The cytotoxic potential of Aegle marmelos fruit extract (40 µg/ml) was assessed by MTT assay. The cells were treated with different concentrations of the plant extract for 24 hours. The Aegle marmelos fruit extract significantly decreased the viability of osteosarcoma cancer cells compared to control at 24 hour time point. The percentage of viability of cells gradually reduced with increase in concentration of leaf extract. It was observed that 50% of growth inhibition occurred at a dose of 40µg/ml (Fig.1). Hence, the above mentioned dose was used for further experiments.

3.2. The cell morphological analysis of Aegle marmelos fruit extract treated osteosarcoma cells were observed through a phase-contrast microscope

Osteosarcoma cell line was treated with Aegle marmelos fruit extract (40 µg/ml) for a duration of 24 hrs and compared with the untreated cells, the treated cells demonstrated significant morphological changes, such as cell shrinkage and reduced cell density which are characteristic of apoptotic cells were observed in the Aegle marmelos fruit extract treated cells. In addition to it, cells undergoing apoptosis also exhibited other types of morphological changes such as rounded up cells that shrink and lose contact with neighboring cells. Few sensitive cells were also detached from the surface of the plates (Fig.2).

3.3. **Aegle marmelos Fruit extract inhibits the migratory potential of osteosarcoma cells.**

A scratch test was performed to evaluate the effect of Aegle marmelos fruit extract on the migration of osteosarcoma cells. The results showed that Aegle marmelos Fruit extract inhibits the cell migration rate when compared to control cells. The following observations were made: In the control group, untreated cancer cells migrated to the scratched area and were almost completely scratched after 24 hours. Treatment with Aegle marmelos Fruit extract at

concentration of 40µg/ml significantly inhibited the migration of osteosarcoma cells compared to the control group. Aegle marmelos fruit extract has a concentration-dependent inhibitory effect on cell migration. The higher the Aegle marmelos fruit extract concentration, the more pronounced the inhibitory effect on cell migration (Fig.3).

Discussion

Aegle marmelos belong to the family of Rutaceae. Bael is a slow growing, tough, medium sized subtropical tree and the tree produces fruits once in a year. According to ayurveda it is a healing tree as all its parts cure all kinds of diseases and are edible in nature(10). The Aegle marmelos leaf extract is said to be very effective against various cancer cell lines (11) (12) (12). The Aegle marmelos extract is found to have anti inflammatory and anti proliferative activity (13). In a study examining the medicinal potential of the dry and ripe A. marmelos fruit, it was revealed that the chloroform and aqueous extracts had significant free radical-scavenging and lipoxygenase inhibitory activity at different concentrations. In another study, methanolic extract from unripe A. marmelos significantly prevented the decline of enzymatic and non-enzymatic antioxidants at various concentrations in the Sprague-Dawley rat gastric system. Another study, Aegle marmelos ethanolic fruit pulp extract possesses anti-proliferative activity by suppressing the progression of breast tumors in rat model (14). The plant extract also possesses hepato-renal protective effect. Hence, it can be targeted as novel and safe anti-cancer drug against breast cancer. Cytotoxic effect of A. marmelos extract on MCF-7 and MDA-MB-231 breast cancer cells was also confirmed by an investigation of in vitro cytotoxicity of Bangladeshi medicinal plants on breast tumor cells. The results of this study showed that Aegle marmelos fruit extract treatment decreased the cell viability in a dose dependent manner, alteres the cell morphology and consequently reduced the migration potential of osteosarcoma cancer cells.

The MTT assay results showed that the inhibitory concentration (IC-50) dose value found in our study from the flower extracts of Aegle marmelos fruit extract was 40µg/ml. A similar study evaluated the cytotoxicity of Aegle marmelos fruit extract on the lung cancer cell lines and the IC50 value was determined to be 40 µg/ml.

Apoptosis or programmed cell death is represented by cell shrinkage, condensation of chromatin, DNA fragmentation and the activation of specific enzymes known as caspases (15). The process of apoptosis is arrested during cancer progression. Induction of apoptosis in tumour cells is the most established anticancer mechanism and is employed in many cancer therapies (15,16). To further investigate the mechanism of cell death induced by Pycnogenol, we examined apoptosis, a process of programmed cell death.

In our current study, the morphological changes in oral cancer cell line upon treatment of Aegle marmelos fruit extract at 40 µg/ml for 24hrs has been observed, and the number of cells decreased after treatment, also the cells exhibited cell shrinkage and cytoplasmic membrane blebbing. The morphological changes observed in cancer cells indicate that Aegle marmelos fruit extract treatment induces apoptosis, including cell shrinkage, nuclear condensation and fragmentation. This finding is consistent with previous studies showing pro apoptotic effects of Aegle marmelos fruit extract cancer cells. Induction of apoptosis by Aegle marmelos fruit extract may contribute to its antitumor effects, suggesting that Aegle marmelos fruit has potential as a therapeutic agent in cancer therapy.

Migration in cancer involves the movement of cells from one location to another, affecting tumor invasion and metastasis (17). Cancer cells acquire this ability through changes in cytoskeleton and adhesion properties, allowing them to detach from the tumor and invade surrounding tissues. Studying metastasis and migration in cell culture helps identify therapeutic targets and develop novel anti-cancer strategies (18). Complementing cell culture studies with animal models and clinical research is crucial for a comprehensive understanding of cancer biology. Scratch test showed that Aegle marmelos fruit extrac inhibits the migration of cancer cells in the body depending on the concentration. This Observation is consistent with previous studies showing the anti-migration effect of pycnogenol on cancer cells. Inhibition of cell migration is an important step in preventing cancer metastasis, and Pycnogenol's ability to inhibit this process demonstrates its potential to treat cancer. Our finding suggests that Aegle marmelos fruit extract may have potential benefits against osteosarcoma cells by inhibiting the cell migration.

Conclusion

These findings suggest that Pycnogenol has the potential to be an anti-metastatic agent in lung cancer by inhibiting vimentin gene expression. Further studies are needed to elucidate the underlying mechanisms and to evaluate the efficacy and safety of Pycnogenol in-vivo. Overall, our study provides a good starting point for the development of new treatments for osteosarcoma using natural products.

Limitation of the study

In vitro anticancer activity studies of Aegle marmelos fruit extract have limitations, primarily stemming from their isolated and simplified laboratory conditions. These studies often fail to replicate the complexities of the human body's environment, including interactions with the immune system and various organs. Consequently, promising results observed in vitro may not accurately predict the efficacy and safety of Aegle marmelos fruit extracts when used in living organisms. Other concerns include a lack of information on bioavailability, selectivity, and the potential for toxic effects on healthy cells. For a more comprehensive understanding of the therapeutic potential of Aegle marmelos fruit extract, further research involving in understanding the molecular mechanism of anti-cancer activity and in vivo studies are essential to bridge the gap between laboratory findings and real-world application in cancer treatment.

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