



## Evaluation of cytotoxic and pro-apoptotic effects of *Annona muricata* SeNPs against the lung cancer line (A-549)

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### ABSTRACT

Cancer is the leading cause of death worldwide, with 10 million deaths reported in 2020. Current cancer treatments are inadequate, particularly for advanced-stage patients, with a high fatality rate. Multidrug resistance (MDR) is a significant barrier in cancer treatment. Plant-derived compounds have fewer side effects than chemotherapy and are being investigated for cancer prevention and treatment. The anticancer characteristics of medicinal plants and their bioactive compounds can limit cancer growth by modifying cell proliferation, differentiation, angiogenesis, death, and metastasis. In this study, we aimed to biosynthesize SeNPs from *A. muricata* extract and test their anti-cancer activity against the lung cancer cell line (A549) in vitro. The cell viability assay and dual staining assay results revealed *A. muricata* mediated SeNPs, decreases the cell growth almost 80% at 40µg/ml concentration and almost 50% of cell death in 20µg/ml concentration. Further studies in animal models and functional studies will be helpful to discovered the anticancer efficacy of AM-SeNPs.

**Keywords:** *cytotoxic, proliferation, differentiation, angiogenesis, death*

### INTRODUCTION

More than ten million people died from cancer worldwide in 2020, making it the most common cause of death. Each year, 400,000 adolescents are diagnosed with cancer. In 2018, there were reported 17.0 million new cases of cancer, according to the International Agency for Research on Cancer (IARC). The number of new cases and cancer-related deaths worldwide is predicted to reach 30 million by 2040 because of

population expansion, aging, and other factors. Besides these obstacles, the predicted burden of cancer in the future is probably going to be substantially higher because of the acceptance of cancer-risky lifestyles. The most common cancers detected in economically underdeveloped nations are lung, liver, colon, prostate, breast, and cervical/uterine cancers. Current treatment strategies for cancer do not significantly improve

patient life, especially in advanced stage cancer patients who are at high risk [1, 2, 3].

A focus on plant-derived substances lowers the incidence of adverse reactions compared with existing traditional cancer treatments, which is why medical advancements treatment and prevention of cancer are constantly needed. The major obstacle in cancer treatment is drug resistance, particularly multidrug resistance (MDR), which leads to apoptotic resistance in cancer cells. Restoration and maintenance of the robustness of signalling networks may be facilitated by natural compounds with various possible targets. Regulating MDR or activating alternative non-apoptotic mechanisms such as autophagy, pyroptosis, caspase-independent cell death, and oncosis are two strategies to elevate absorption and concentration of cancer drugs inside cells. Therefore, herbal products may be used in cancer treatment. In the majority of key domains, herbal medicine is considered the pinnacle of alternative medicine [4, 5, 6].

Research on medicinal plants is critical for promoting the proper use of herbal medicines and assessing their potential for development as novel medicinal products. Plants with therapeutic characteristics have been used to treat a wide range of diseases since the earliest days of time. There are several allusions in ancient texts on therapeutic plants and their disease-prevention properties. The chemistry of herbal plants, which cause significant changes in the human body are their primary characteristics. Researchers have looked at the extra metabolites and bioactive chemicals present in medicinal plants and determined that they possess anticancer capabilities. Mainly those medicinal plants are inhibiting cancer growth, and progression by altering cell proliferation, differentiation, angiogenesis, apoptosis, and metastasis [7, 8, 9]. Extracts of *Annona muricata* commonly called graviola are among a multitude of natural herbs that have shown potential medicinal efficacy, including anti-cancer properties, and all parts of this plant are used as natural remedies. *A. muricata* contains alkaloids, cyclopeptides, flavonol triglycosides, megastigmanes, phenolics, and essential oils. Regardless, *Annona* species, particularly *A. muricata*, are excellent sources of acetogenin. Additionally, the presence of several crucial minerals in *A. muricata* fruit, such as potassium, calcium, salt, copper, iron, and magnesium, shows that frequent fruit

consumption will aid the body's supply of vital nutrients and components. [10, 11, 28,29].

*A. muricata* extract and its active components can be employed as reducing agents in the synthesis of nanoparticles. It should be highlighted that acetogenins, notably the lactone functional group, are directly responsible for the anticancer action [12]. Lactones are chemical molecules known as cyclic esters. This functional group inhibits complex I at the mitochondrial level in cancer cells, causing protons to accumulate across the mitochondrial membrane, halting ATP synthesis and driving selective apoptosis [12, 13]. Selenium nanoparticles (SeNPs) are a type of nanomaterial that has attracted considerable attention because of their unusual physicochemical and biological features. They have been demonstrated to have high antioxidant activity and potential applications in medicine, agriculture, and environmental remediation [14, 15]. In the current study, we aimed to synthesize selenium nanoparticles (SeNPs) using *A. muricata* extract and evaluate its anti-cancer activity against lung cancer cell lines in vitro.

## MATERIALS AND METHODS

### *Preparation of extract A. muricata fruit*

Fruits of *Annona muricata* were harvested, and the species was confirmed by a botanist. The fruits were cleaned with sterile distilled water, chopped into small pieces, and dried for several days. The dried fruit was ground to a powder to prepare the extract. The dissolved solution was heated at 80°C for 3 h with constant stirring after 10 g of powdered fruit was dissolved in 100 mL of distilled water. After filtration through Whatman filter paper, the aqueous extract was stored at 4°C.

### *Biosynthesis of selenium nanoparticles*

For the green synthesis of selenium nanoparticles, we used the aqueous fruit extract of *Annona muricata* synthesized previously. Before heating at 80 °C for 3 h with continuous stirring, 10 mL of fruit extract was combined with 90 mL of a 1 mM aqueous selenium solution. The formation of the SeNPs was indicated by the shift in color from yellow to dark brown. Green nanoparticles were isolated after 20 min of centrifugation at 15,000 × g. To eliminate free silver associated with AM-SeNPs, this method was repeated three times. The final

green-synthesised selenium nanoparticles were AM-SeNPs, which were freeze-dried and stored at 4 °C until further use.

#### **Cell culture and cell viability (MTT) assay**

The Lung cancer cell line (A-549) was purchased from NCCS, Pune, India and was plated in 96-well plates at a density of  $5 \times 10^3$  cells/well in DMEM media with 1X Antibiotic Solution and 10% foetal bovine serum (Gibco) in a CO<sub>2</sub> incubator at 37 °C with 5% CO<sub>2</sub>. The cells were washed with 100 µL of 1X PBS, treated with *Annona muricata* SeNPs at different concentrations (5-40 µg/ml) and incubated in a CO<sub>2</sub> incubator at 37 °C with 5% CO<sub>2</sub> for 24 h. At the conclusion of the treatment period, the media was aspirated from the cells. The cells were then cultured at 37 °C for 4 hours in a CO<sub>2</sub> incubator with 0.5 mg/mL MTT produced in 1X PBS. Following incubation, the MTT-containing media was discarded and 100 µL of PBS was used to wash the cells. The created crystals were properly mixed and dissolved in 100 µL of DMSO. At 570 nm, the emergence of colour intensity was measured. Formazan dye took on a purple-blue hue. With the use of a microplate reader, the absorbance was determined at 570 nm.

#### **Cell Morphology**

A Lung cancer cell line ( $2 \times 10^5$ ) was plated in 6 well plate to study the effect of *A. muricata* SeNPs on cell morphological changes. The cells were treated with or without *A. muricata* SeNPs

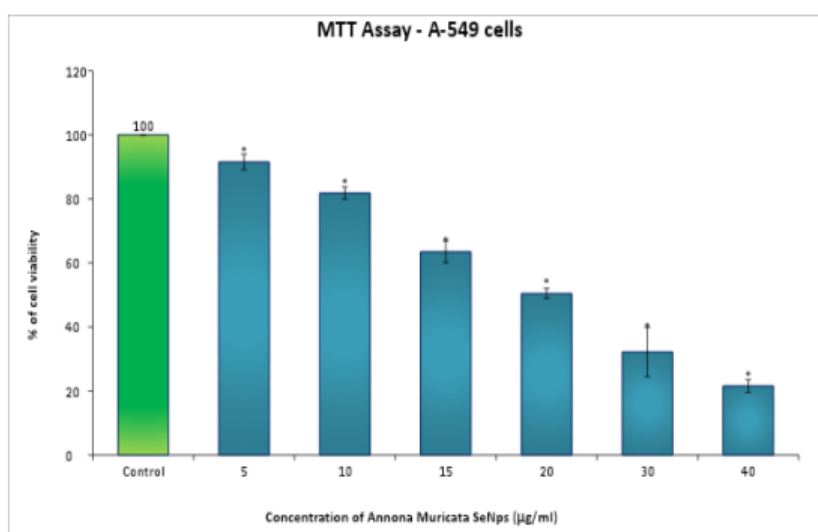
for 24h time point. After the treatment period, cells were washed with PBS and observed under an inverted phase-contrast microscope.

#### **Dual staining using acridine orange (AO) and ethidium bromide (EtBr) to determine the cell death**

By using AO/EtBr dual staining, the effects of *A. muricata* SeNPs on A-549 cell death were identified. *A. muricata* SeNPs was applied to the cells for 24 hours before they were collected and rinsed with ice-cold PBS. The pellets were redissolved in a mixture of 5 µL of EtBr and 1 mg/mL acridine orange. The stained cells were examined under a fluorescence microscope to detect apoptosis.

## **RESULTS**

In this study, we observed the cytotoxicity level of *A. muricata* SeNPs through MTT assay and revealed that 40 µg/ml of AM-SeNPs inhibited almost 80% of the lung cancer cells (A-549) in 24 h. Furthermore, we observed the cell morphology of AM-SENPs (20 µg/ml) treated lung cancer cells under an inverted microscope, which showed reduced lung cell growth with cell structural changes. In order to detect cell death, we also stained the cells with acridine orange (AO)/ethidium bromide (EtBr). The outcomes demonstrated that most lung cancer cells in the AM-SeNP-treated plate died under a fluorescence microscope, indicating apoptotic cell death.



**FIG 1:** Cytotoxic effect of *Annona muricata* SeNPs on lung cancer cells (A-549)

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**Morphology – Phase contrast image**

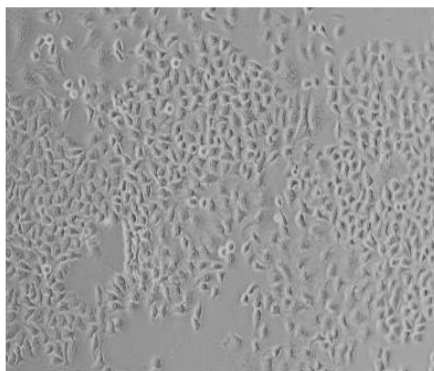


Fig 2.1 Control

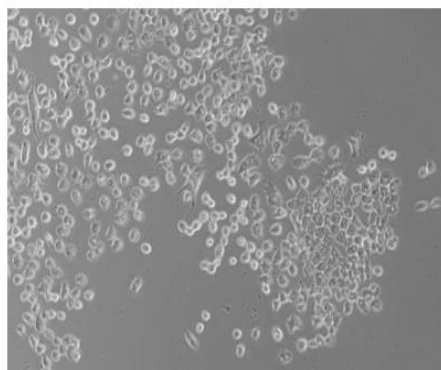


Fig 2.2 *Annona muricata* Senps(20ug/ml)Control

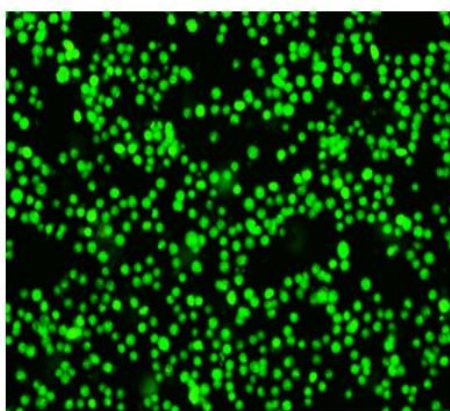


Fig 3.1 Control

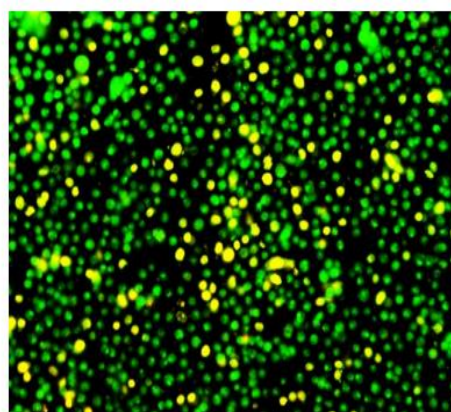


Fig 3.2 *Annona muricata* Senps(20ug/ml)Control

**DISCUSSION**

The Annonaceae family has been extensively studied recently because of its potential as a therapeutic plant, and it has a long history of documented medicinal uses. This particular species has recently drawn attention owing to its bioactivity and long-standing use. The treatment of chronic degenerative illnesses has received clinical attention since they have reached widespread proportions and are now recognized as major health issues. Fruit and leaf/bark extracts or infusions have been used to treat fever, respiratory diseases, heart diseases, malaria, liver illnesses, gastrointestinal disorders, and renal issues. This has been frequently utilized in recent years for hypoglycemic, hypotensive, and cancer treatment [11, 16, 17].

Acetogenins also been demonstrated to inhibit ATP generation in mitochondria. This type of action has been proven effective against cancer

cells that produce more ATP than normal cells, restricting the development of cancer cells. Interestingly, AGE toxicity was observed in cancer cells, but had little effect on normal cells. Acetogenin analogs synthesized in vitro exhibit toxicity against colon adenocarcinoma cell lines (HCT-8 and HT29), with negative toxicity towards normal human cell lines, according to studies using AA mimetics [18]. Additionally, *A. muricata* leaf extract exhibits strong toxicity against breast cancer cell lines (MCF-7 and MDA-MB-231) and low toxicity in normal breast cells (MCF10A) [19]. The ability of acetogenins to inhibit NADH oxidase has also been proven to be significant for their antitumor effects. Furthermore, exposure to acetogenin bullatacin decreased NADH oxidase enzyme functions in HeLa cells as well as HL-60 cancer cells [20].

Previously, Meenakshisundaram et al. (2020) discovered that *A. muricata*-mediated biogenic

silver nanoparticles (AM-AgNPs) inhibited the human lung cancer cell line (A549) at 6µg/ml (IC50). Furthermore, western blot analysis confirmed the regulation of cell cycle arrest by AM-AgNPs, increased apoptotic protein expression, and decreased anti-apoptotic protein expression [21]. Moreover, another study found that AM-AgNPs reduced cancer cell proliferation by controlling the apoptosis, inflammasome, and autophagy pathways [22,23]. These results indicate that biosynthesis of *A. muricata* with nanoparticles is a good approach to achieve enhanced anticancer properties. Interestingly, biosynthesis of *A. muricata* fruit extract mediates the antioxidant and antimicrobial properties of SeNPs. Therefore, the use of AM-SeNPs might help treat multiple diseases, including infectious and non-infectious diseases [24-28].

The extract of *A. muricata* leaf shows substantial cytotoxic action, with IC50 values of 21.05 0.42 g/mL, producing cell cycle arrest at the G0/G1 phase and apoptosis, according to an in vitro study by Moghadamtousi et al. on human lung adenocarcinoma (A549) cell lines. In a different investigation, *A. muricata* fruit extract improved the Bax/Bcl-2 ratio-mediated inhibition of mitochondrial membrane potential, caspase-3/9 activation, and cytosolic cytochrome c activation in the A549 cell line.[35-38] It also repressed nuclear factor-B (NF-B) signalling and increased ROS generation. Several studies have shown potential therapeutic anticancer effects of photochemical agents [39-42].

### CONCLUSION

The findings of this study show that lung cancer cell lines are significantly cytotoxic when SeNP is produced through green synthesis by *A. muricata*. Additionally, AM-SeNP-treated cells underwent apoptosis after dual labelling. The effectiveness of AM-SeNPs as anticancer medications for lung and other malignancies may be improved by more research employing in vivo models and functional tests.

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