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Assessment of anti-cancer property of *Syzygium cumini* seed extract in MDA-MB-231 cell line

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Abstract

Breast cancer is one of the most common cancers for women that begin in breast tissue. Every year 2.2 million women are affected by this type of cancer. Many therapies are available to treat cancer, including radiotherapy, surgery, chemotherapy and mastectomy. Biological therapy includes herbs, dietary supplements and traditional medicine systems whose advantages are applicability, affordability, therapeutic efficacy and no side effects. The present study aimed to investigate the *in vitro* anti-cancer activity of *Syzygium cumini* seed ethanol and hexane extracts against MDA-MB-231(metastatic human breast cancer cell line). We performed MTT assay to evaluate the cytotoxicity, DNA fragmentation assay to determine apoptosis, AO/PI dual staining to detect apoptotic morphological changes and mitochondrial membrane potential to assess the mitochondrial function. Our MTT results with five different concentrations (50, 100, 150, 200 and 250 µg/ml) of the hexane and ethanol extracts show promising cancer cell toxicity with IC_{50} values of 142 µg/ ml and 195 µg/ml for ethanol and hexane extract, respectively. Agarose gel electrophoresis for DNA fragmentation documents smear that indicates extensive DNA damage in the extract-treated cancer cells. AO/PI fluorescence stain in IC_{50}-treated cells exposes apoptotic changes as observed in early and late apoptotic stages and the presence of few necrotic cells. Rhodamine-123 stain with its decreased green fluorescence in IC_{50}-treated cells is evidence of the breakdown of the mitochondrial membrane due to enhanced reactive oxygen species. Our study, therefore, concludes that *S. cumini* seed can be a novel source to explore for anti-cancer therapy.

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Introduction

Cancer incidence and mortality rates worldwide are an estimated 19.3 million new cases of cancer and almost 10.0 million deaths from cancer in 2020. The most commonly diagnosed cancers were female breast cancer (2.26 million), lung cancer (2.21 million) and prostate cancer (1.41 million); the most common causes of cancer death were lung, liver and stomach cancers (Ferlay *et al.,* 2021). Breast cancer is categorized into 3 major subtypes based on molecular markers for estrogen or progesterone receptors and human epidermal growth factor 2. For non-metastatic breast cancer, therapeutic goals are tumor eradication and preventing recurrence. Systemic therapy includes either endocrine therapy, chemotherapy or targeted antibody, or small-molecule inhibitor therapy. Local therapy consists of surgical resection with postoperative radiation. For metastatic breast cancer, the goal is to prolong life and conceal symptoms with an expected overall survival of 1 to 5 years (Sharma *et al.,* 2010; Sun *et al.,* 2017; Waks and Winer, 2019).

Great advancements have been made in the treatment and control of cancer progression in the form of chemotherapy, immune therapy, radiation, hormone therapy and targeted therapy. Yet they come with possible undesired side effects, toxicity and significant deficiencies (Desai *et al.,* 2008). Natural therapies, where plant-derived products are used in cancer treatment, may reduce adverse side effects. Medicinal plants are advantageous over synthetic chemicals, and plant-derived compounds are proven to be more tolerant and safe for normal cells (Roy *et al.,* 2017). At present, a number of phyto-constituents from medicinal plants (Shoeb, 2006) show very promising anti-cancer properties *in vitro*, and few are administered to cancer patients *in vivo* to prevent and treat cancer as an alternative therapy (Kooti *et al.,* 2017). These plants find utilization due to their anti-carcinogenic and chemo-protective potentials.

Additionally, the plants have less toxic anti-cancer, anti-tumor and anti-proliferative agents than established allopathy medicines (Gezici and Sekeroglu, 2019).

Syzygium cumini (*S. cumini*) (L.) Skeels (jambolan) is one of the widely used medicinal plants in the treatment of various diseases. Various traditional practitioners in India use the different parts of the plant in the treatment of diabetes, blisters in the mouth, cancer, colic, diarrhea, digestive complaints, dysentery, piles, pimples and stomach ache. In Unani medicine, parts of jambolan act as a liver tonic, enrich blood, strengthen teeth and gums and form a good lotion for removing ringworm infection of the head. The seed extract is used to treat colds, coughs, fever and skin problems such as rashes and mouth, throat, intestines and genitourinary tract ulcers by the villagers of Tamil Nadu (Ayyanar and Subash-Babu, 2012). Different parts of jambolan are also reported for its anti-oxidant, anti-inflammatory, neuropsycho-pharmacological, anti-microbial, anti-bacterial, anti-HIV, anti-leishmanial and anti-fungal, anti-oxidant, anti-diarrheal, anorexigenic, gastro-protective, anti-ulcerogenic and radioprotective activities (Swami *et al.,* 2012; Chhikara *et al.,* 2018).

Our research was focused on the preparation of hexane and ethanol extracts of *Syzygium cumini* seeds by maceration method and to assess cytotoxicity by MTT assay, to determine apoptosis by DNA fragmentation, to detect apoptosis-associated morphological changes by AO/PI dual staining and to evaluate mitochondrial membrane potential changes in MDA-MB-231 cell, one of the most studied estrogen receptor (ER) negative breast cancer cell line (Gaglio *et al.,* 2011).

Materials and methods

Plant material and extraction

Disease-free and contamination-free *Syzygium cumini* seeds were collected from Tirunelveli District. The plant and the seeds were authenticated by a plant taxonomist in Herbal Plant Anatomy Research Centre, Chennai and a voucher specimen (PARC/2021/4520) was deposited in the herbarium. The specimen was cleansed with water to remove debris, shade-dried and ground. Extraction was performed by maceration technique (Handa, 2008) wherein 100 g powdered seed material was soaked in

hexane and ethanol solvents separately. After 72 hours, the mixture was filtered and air-dried to obtain *Syzygium cumini* seed hexane extract (*SHE*) and *Syzygium cumini* seed ethanol extract (*SEE*).

Cell culture maintenance

MDA-MB-231 cell line was obtained from the National Centre for Cell Sciences (NCCS), Pune, India. Cells were maintained in the logarithmic phase of growth in Dulbecco's modified eagle medium (DMEM) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 100 IU/ml penicillin and 100 µg/mL streptomycin. Cells were maintained at 37 °C with 5% CO_2 in a 95% air-humidified incubator.

MTT assay

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was performed with *SHE* and *SEE* against MDA-MB-231 cell line following standard procedure with minor modifications (Mossman, 1983) to assess the cell viability. The cells were seeded in 96-well microplates (1×10^6 cells/well) and incubated at 37 °C for 48 hours in a 5% CO_2 incubator and allowed to grow 70-80% confluence. Then the medium was replaced and the cells were treated with five different concentrations of *SHE* and *SEE* (50, 100, 150, 200 and 250 µg/ml) and incubated for 24 hours. Morphological changes in the untreated (control) and the treated cells were observed under a digital inverted microscope (20X). After which, cells were washed in phosphate-buffer saline (PBS, pH: 7.4) and 20 µl MTT (5 mg/ml in PBS) was added to each well. The plates were allowed to stand at 37 °C in the dark for 2 hours. The formed formazan crystals were dissolved in 100 µl DMSO and absorbance was read at 570 nm. The percentage of cell viability was calculated using the formula,

$$\text{Cell viability (\%)} = \frac{\text{Absorbance of untreated cells - Absorbance of treated cells}}{\text{Absorbance of untreated cells}} \times 100$$

DNA ladder assay

DNA ladder assay was performed following a standard protocol (Kalinina *et al.*, 2002) with minor modifications to determine apoptosis by DNA cleavage. Briefly, MDA-MB-231 cells were grown overnight in 25 cm^2 cell culture flasks and exposed to IC_{50} concentration of the *SHE* and *SEE* for 24 hours. The cells were gently scraped and harvested by centrifugation. The cells were re-suspended in 0.5 ml lysis buffer [10 mM Tris–HCl, 0.5 mM EDTA (pH: 8.0), 2% SDS, and 1 mM NaCl)] and incubated at 37 °C for 15 minutes. Proteinase K (20 mg/ml) was added to purify the DNA and denature the protein content. The mixture was incubated at 50 °C for 45 min, followed by DNA extraction with an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) solution. The DNA was treated with 3 M sodium acetate (100 µl) for precipitation, and ice-cold 70% ethanol was used to wash the DNA pellet. The DNA was electrophoretically examined in 1.5% agarose gels containing 0.1 µg/ml ethidium bromide, and the image was captured using a gel documentation system.

AO/PI dual staining

Acridine orange/propidium iodide (AO/PI) dual staining technique following standard procedure (Nordin *et al.*, 2017) with little modifications, was performed using the IC_{50} concentrations of *SHE* and *SEE* in MDA-MB-231 cells to detect apoptosis-associated morphological changes. The MDA-MB-231 cells were cultured in a 6-well plate (1×10^5 cells per well) and treated with IC_{50} concentration of *SHE* and *SEE* for 24 hours. The untreated MDA-MB-231 cells served as a control. The extract-treated cells were washed with phosphate-buffered saline and stained with 20 µl AO/PI (100 µg/ml acridine orange and 100 µg/ml propidium iodide) for 5 minutes. The stained cells were viewed under a 40X objective of the fluorescence microscope (Invitrogen EVOS FL Cell Imaging).

Mitochondrial membrane potential (Δψm)

The changes in mitochondrial membrane potential (MMP / Δψm) level were assessed using a standard procedure (Huigsloot *et al.*, 2002) with small modifications in order to determine apoptotic alterations, mitochondrial dysfunction, and cell health. Rhodamine-123 stain was used to determine

the Δψm level. Following the treatment of MDA-MB-231 cells with IC_{50} concentrations of *SHE* and *SEE*, the cells were washed with PBS and stained with 50 µl rhodamine-123 (10 µg/ml) and kept undisturbed for 30 minutes. Morphological differences and membrane permeability were captured in a 40X objective of the fluorescence microscope.

Results

Effect of extract on cell viability

To monitor the cell viability, we used *in vitro* MTT cytotoxicity assay. The effects of the *S. cumini* seed hexane and ethanol extracts were analyzed within the concentration range of 50 to 250 µg/ml. In the presence of hexane and ethanol extract of *S. cumini* seed, the cell viability decreased in a concentration-dependent manner calculated based on the absorbance values of the MTT assay (Fig. 1). At higher concentrations, both hexane and ethanol extract caused significant loss of cell viability. At the highest concentration (250 µg/ml), the hexane extract and ethanol extract led to decreased cell viability by up to 31.29% and 12.21%, respectively (Table 1). We could determine the inhibitory concentrations causing 50% inhibitions of cell viability (IC_{50}) for hexane (Fig. 2) and ethanol (Fig. 3) extracts.

The IC_{50} obtained were 195 µg/ml and 142 µg/ml for hexane and ethanol extract, respectively. According to the IC_{50} values, *S. cumini* seed ethanol extract is more cytotoxic than *S. cumini* seed hexane extract.

Table 1. Absorbance readings and percentage cell viability of MTT assay.

Concentration (µg/ml)	Hexane extract		Ethanol extract	
	OD	Cell viability (%)	OD	Cell viability (%)
Control	0.875±0.01	100	0.906±0.01	100
50	0.821±0.006	93.83	0.794±0.009	87.64
100	0.701±0.01	80.06	0.627±0.01	69.17
150	0.593±0.01	67.73	0.383±0.008	42.22
200	0.424±0.00	48.48	0.210±0.01	23.20
250	0.274±0.00	31.29	0.111±0.01	12.21

All values are in triplicates with Mean ± S.D.

Effect of extract on the morphology of the cells

Upon the incubation of cells with increasing concentrations of *S. cumini* seed hexane and ethanol extracts for 24 h, apoptotic and necrotic cells were more frequently seen. The most common apoptotic morphological changes observed in both included chromatin condensation, cytoplasm shrinkage, and loss of normal shape, followed by the breaking up of the nucleus into discrete fragments by budding of the cell as a whole to produce membrane-bound apoptotic bodies. Fig. 4F and 5F represent MDA-MB-231 untreated control cells revealing a normal shape, and many have large intact oval and vesicular nuclei with prominent nucleoli. Cells treated with *S. cumini* seed hexane extract (50, 100 and 150 µg/ml) have lost their normal shape and show a shrunken cytoplasm. With *S. cumini* seed hexane extract (200 and 250 µg/ml), the cells show evident signs of apoptosis, including cytoplasm and chromatin condensation, and fragmentation and formation of apoptotic bodies. It also shows signs of necrosis. The cells have more abnormal shapes with a poorly defined nucleus, clear vesicles in the cytoplasm and disrupted cell membrane with leaking cell contents (Fig. 4). Cells treated with *S. cumini* seed ethanol extract (Fig. 5) showed a significant loss of cell processes and marked changes in morphology associated with the late stage of apoptosis, such as shrinkage, irregular shape, and condensed and fragmented chromatin and cytoplasm, to produce apoptotic bodies.

A marked and visible increase in the number of necrotic cells was also observed. Necrotic cells appeared after incubation with 150 µg/ml concentration of the extract, characterized by the assemblage of numerous cytoplasmic small vesicles of unknown origin, swelling of the nucleus and lysis of plasma membrane.

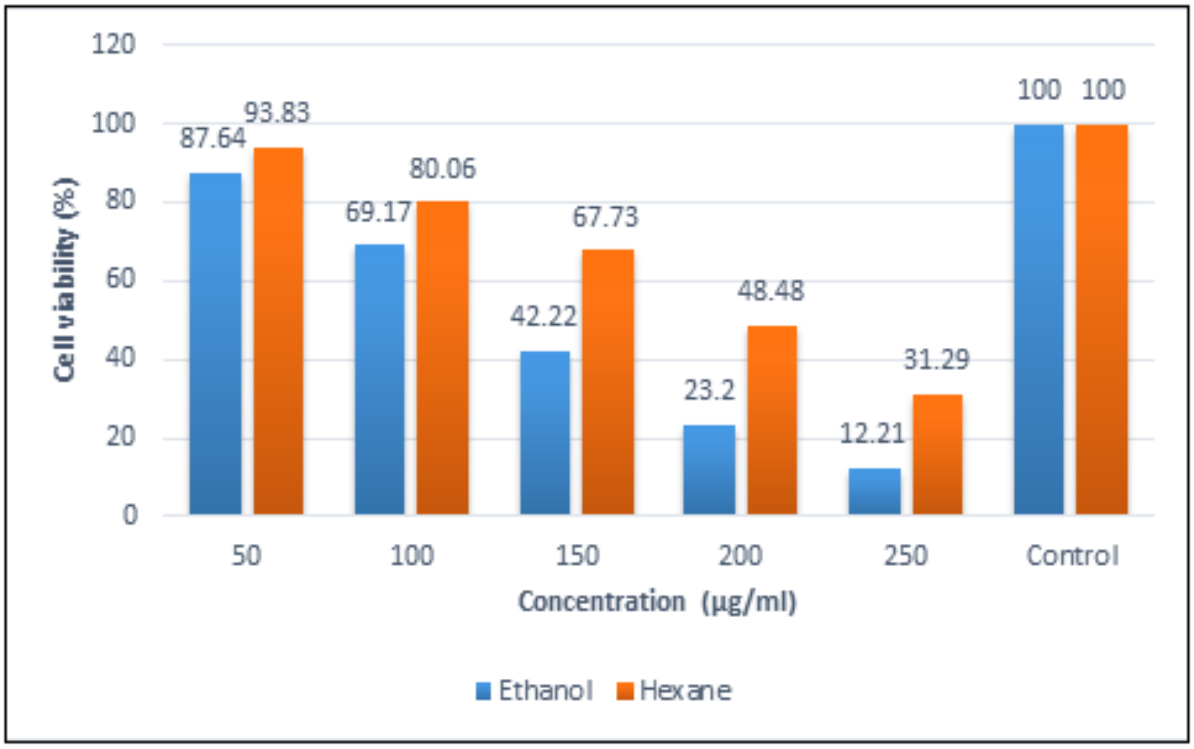

Fig. 1. Percentage cell viability as assessed by MTT assay for *S. cumini* seed hexane and ethanol extracts.

Effect of extract on chromosomal DNA

Cells treated with IC_{50} concentration of the extracts for 24 hours induced cell death which apparently was accompanied by the formation of DNA fragments, represented by a smear pattern in agarose gel with both high molecular weight DNA and smaller DNA fragments when compared with the DNA ladder. The control was untreated cells showing a clear single band of intact DNA. The smear pattern indicates damaged DNA in extract-treated cells. Fig. 6 shows agarose electrophoresis gel documentation of DNA extracted from MDA-MB-231 cells after treatment with *S. cumini* seed hexane and ethanol extracts with IC_{50} concentrations.

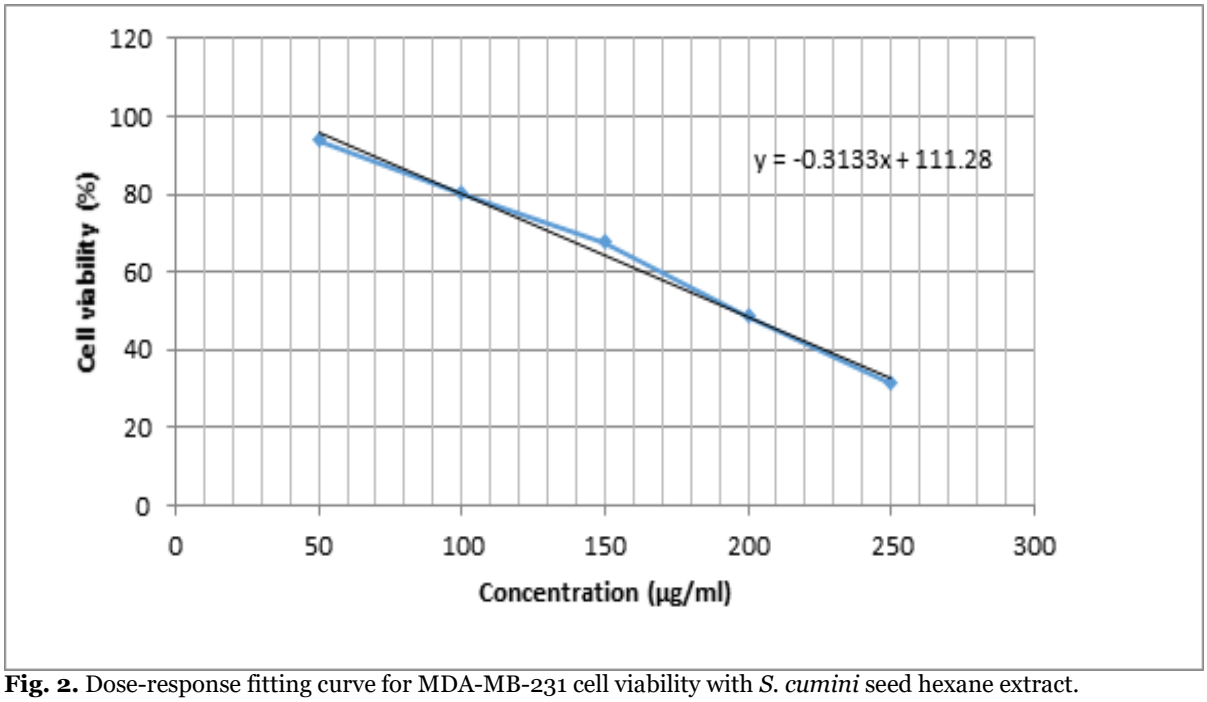

Fig. 2. Dose-response fitting curve for MDA-MB-231 cell viability with *S. cumini* seed hexane extract.

Induction of apoptosis and necrosis by extract

Fig. 7 and 8 are microphotograph collections of MDA-MB-232 cells treated with IC_{50} concentration of *S. cumini* seed hexane (Fig. 7) and ethanol (Fig. 8) extracts after 24 hours, and stained with dual staining acridine orange/propidium iodide (AO/PI). The majority of the untreated control cells emitted green fluorescence. The extract-treated cells emitted green fluorescence specifying live cells permeable to acridine orange dye.

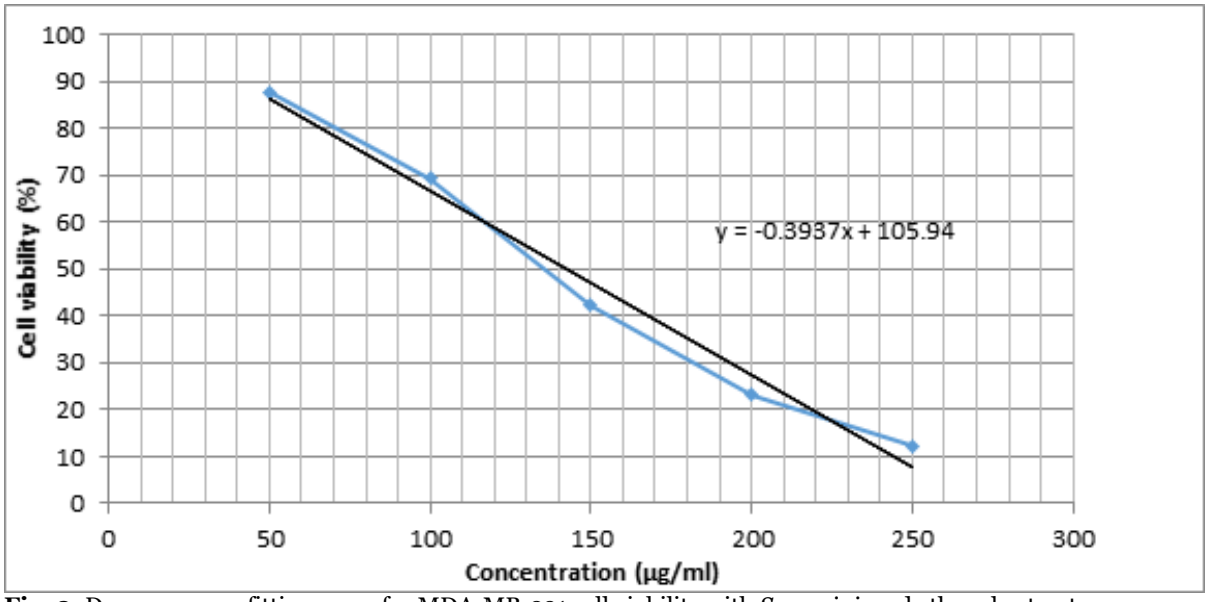

Fig. 3. Dose-response fitting curve for MDA-MB-231 cell viability with *S. cumini* seed ethanol extract.

The extract-treated cells also emitted orange color indicating apoptotic cells due to apoptosis event and red fluorescence indicating cell death due to necrosis event, whereby the propidium iodide dye remains bound to the nucleated dead cells. Besides color emission from the staining, the morphology, number, and size of the cells were also determined before and after treatment. For untreated cells, the cells were uniform in size and morphology. For *SHE* and *SEE* extract-treated cells, the size was not uniform, with a reduced number of cells, most exhibiting shrinkage and few showing swellings.

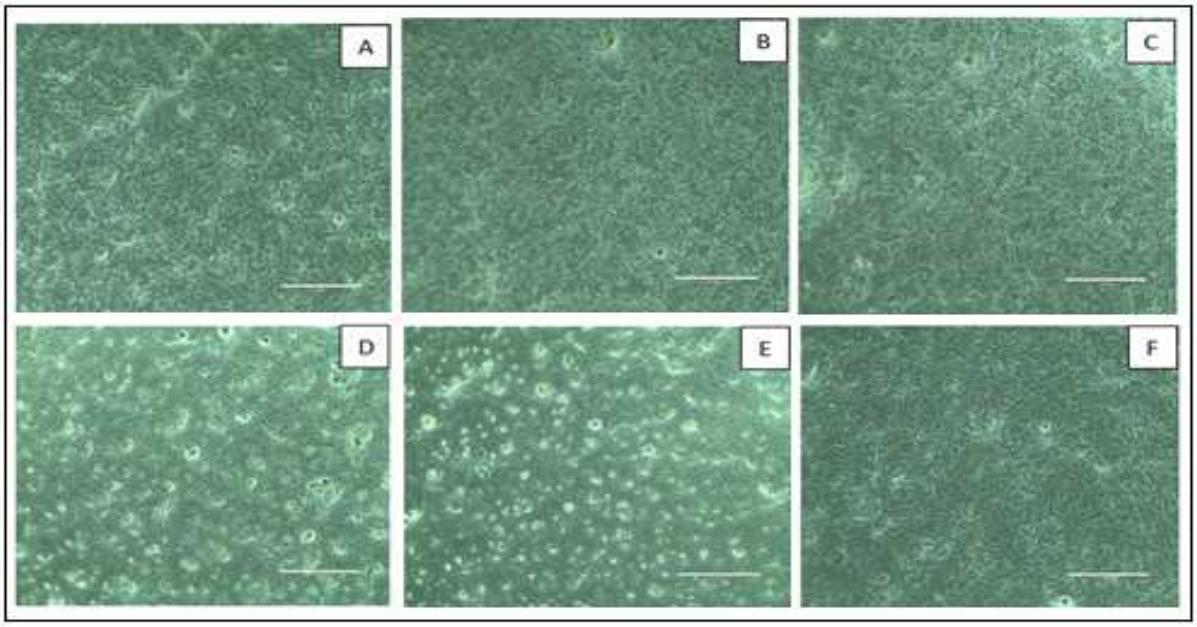

Fig. 4. MDA-MB-231 cells treated with *Syzygium cumini* seed hexane extract in various concentrations (A: 50 µg, B: 100 µg, C: 150 µg, D: 200 µg, E: 250 µg, F: control).

Effect of extract in altering mitochondrial membrane potential

The mitochondrial membrane potential (ΔΨm / MMP) was significantly altered, as demonstrated by the percentage of rhodamine-123 fluorescence measurement. After treatment of MDA-MB-231 cells with *S. cumini* seed hexane and ethanol extracts, a significant decline in mitochondrial membrane potential was observed (Fig. 9). Rhodamine-123 staining is intense and strictly located at mitochondria in the control cells, a sign of highly polarized mitochondria as depicted by bright green color. The presence of *S. cumini ethanol* extract increased the number of cells with low polarized (dim green color) mitochondria accompanied by more diffuse localization of rhodamine-123 when compared to the control. *S. cumini* hexane extract-treated cells also showed a similar characteristic change.

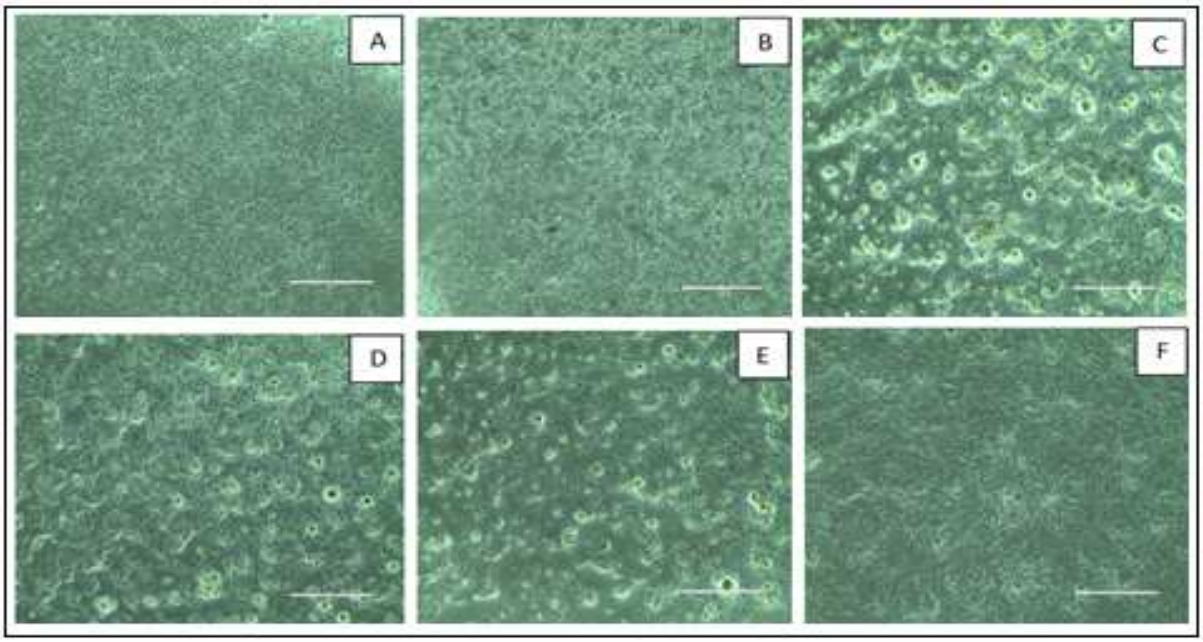

Fig. 5. MDA-MB-231 cells treated with *Syzygium cumini* seed ethanol extract in various concentrations (A: 50 µg, B: 100 µg, C: 150 µg, D: 200 µg, E: 250 µg, F: control).

Discussion

Medicinal plants play a key role in cancer prevention and therapy. They provide new active chemo-preventive molecules and can ease side effects (Bachrach, 2012). In this study, we explored the anti-cancer property of *Syzygium cumini* seed hexane and ethanol extract in human metastatic breast cancer cell line MDA-MB-231 (Wang *et al.,* 2004) using standard screening procedures to establish scientifically its claim by traditional practitioners to possess numerous therapeutic benefits. The treatment regimen for cancer is to kill the proliferating cancerous cells by altering the morphology, reducing its growth and its subsequent disintegration. The monitoring of alterations in mitochondrial activity can be detected with the use of MTT, which is a non-expensive and commonly used screening method to measure cell viability.

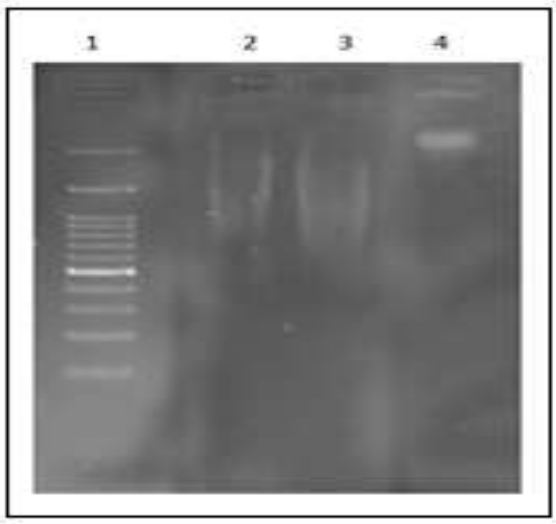

Fig. 6. Photograph of agarose gel from DNA ladder assay (Lane 1: DNA marker; Lane 2: DNA from MDA-MB-231 cells treated with *SEE*; Lane 3: DNA from MDA-MB-231 cells treated with *SHE*; Lane 4: DNA from control (untreated MDA-MB-231 cell).

It is believed to be based on the reduction of tetrazolium salt by mitochondrial succinic dehydrogenases in viable cells yielding purple formazan crystals that are not soluble in aqueous solutions typical for cell environment (Boncler *et al.,* 2014). Our results reveal reduced cell viability in *S. cumini* seed hexane and ethanol extract-treated cells.

A similar MTT assay study with methanol *S. cumini* seed extract at various concentration ranges (1-300 µg/ml) caused a dose-dependent growth inhibitory effect in oral squamous carcinoma cells, presumed to be induced by reactive oxygen species-mediated apoptosis (Ezhilarasan *et al.,* 2019). A prominent growth inhibitory effect of *Syzygium cumini* extract on colorectal cell line (Khodavirdipour *et al.,* 2021) and fruit peel extract on two cervical cell lines have also been observed, contributed by a gradual increase in the apoptotic index following increased extract-treatment duration (Barh and Viswanathan, 2008). These support the fact that extracts in the study are cytotoxic at the tested range of concentration.

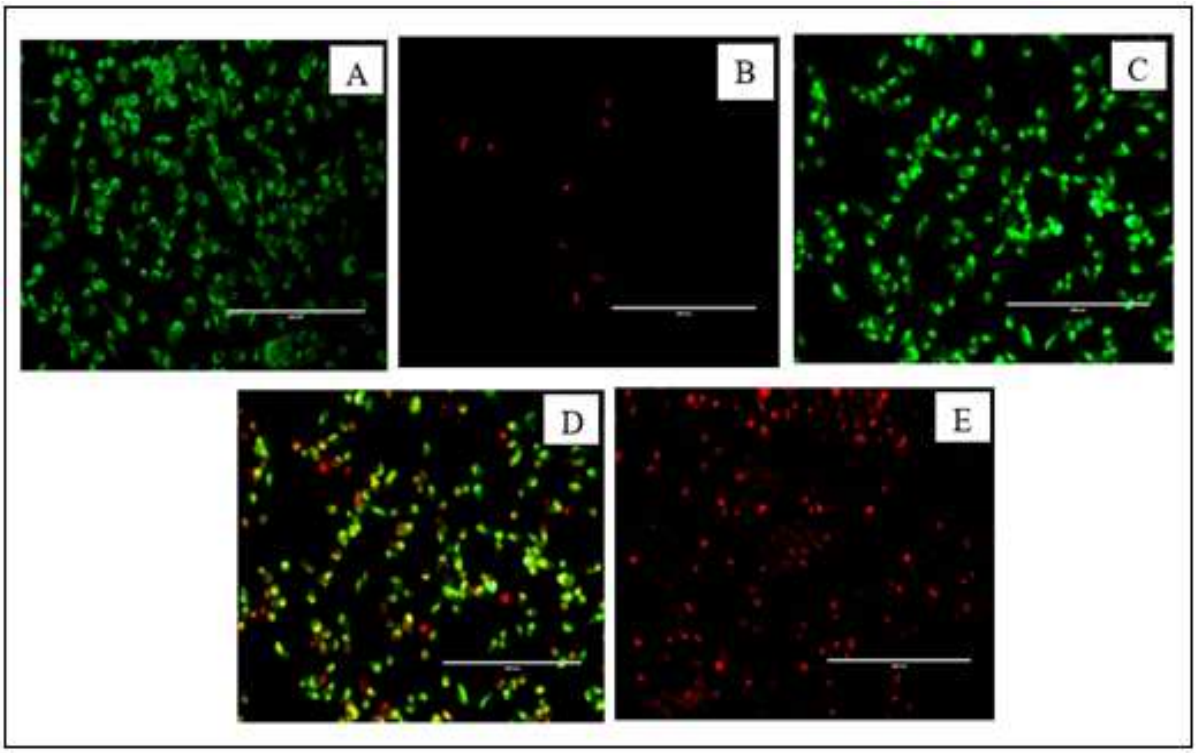

Fig. 7. Apoptotic/necrotic detection by AO/PI staining of *SHE*-treated MDA-MB-231 cells (A & B: control, C: *SHE*-treated live cells, D: *SHE*-treated early and later apoptotic cells, E: *SHE*-treated necrotic cells).

DNA fragmentation is a hallmark feature of apoptosis. In our results, a prominent DNA ladder pattern was not apparent in the *S. cumini* seed extract-treated cells. This is in accordance with an earlier study on the apoptosis and necrosis of human breast cancer cells by *Lepidium sativum* seeds, where the research indicates that DNA damage in dying cells is not only a peculiar feature of apoptosis but also of necrosis. Apoptotic cells usually exhibit a ladder pattern, and visible DNA smear in the agarose gel indicates that the apoptotic cells enter into late apoptosis, also called secondary necrosis, suggesting the absence of phagocytosis or lack of caspase-3 or long incubation leading to lysis of cells (Mahassni and Al-Reemi, 2013). The antimicrobial action of methanol seed extracts of *Syzygium cumini* against *Bacillus subtilis* is also proven to be the result of DNA fragmentation made visible by the formation of a DNA smear (Yadav *et al.,* 2017). The anti-cancer activity of *S. cumini* seed hexane and ethanol extracts was further supported with microscopic apoptosis evaluation by AO/PI dual fluorescent staining. Extract-treated cells showed a collection of viable cells, apoptotic cells and necrotic cells.

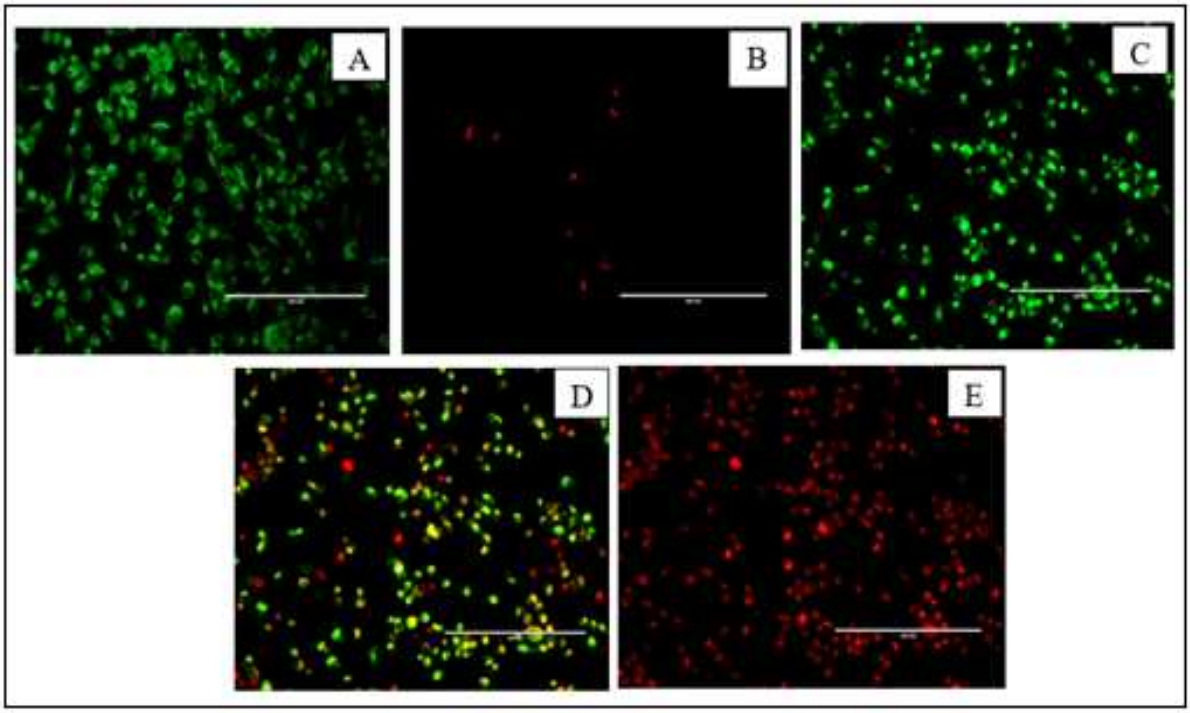

Fig. 8. Apoptotic/necrotic detection by AO/PI staining of *SEE*-treated MDA-MB-231 cells (A & B: control, C: *SEE*-treated live cells, D: *SEE*-treated early and later apoptotic cells, E: *SEE*-treated necrotic cells).

Interestingly, asynchrony of events in certain cells would at any one time demonstrate a mixture of apoptotic events together with the terminal necrosis-like events of cell lysis at the end of apoptosis (Collins *et al.,* 1997), as observed in our study. Hence further gene expression and gene regulatory cell signaling studies could reveal the exact mechanism involved in mediating the cytotoxic effect of the cancer cells. No studies have been conducted on the apoptosis pathway, specifically from the seeds of *S. cumini*. Therefore, an exhaustive study is required to elucidate its true potential.

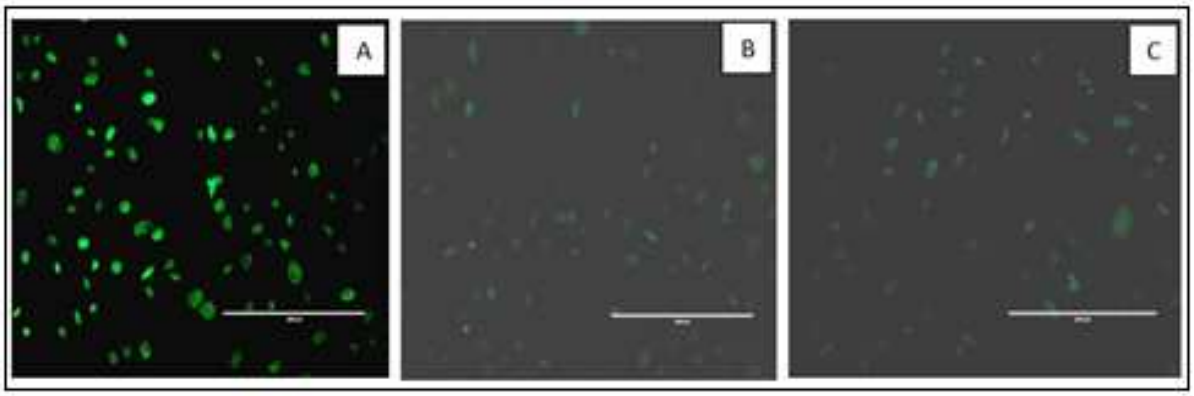

Fig. 9. Alterations in mitochondrial membrane potential induced by *S. cumini* seed extract on MDA-MB-231 cells obtained by fluorescence microscopy and rhodamine-123 (A: control cells, B: *SEE*-treated cells, C: *SHE*-treated cells).

In *S. cumini* extract-treated cells, a decline in the membrane potential was noticed. An essential manifestation in the intrinsic apoptotic pathway is depolarization of the mitochondrial membrane and the subsequent increase in permeability of the outer mitochondrial membrane, accompanied by the release of cytochrome c through pores (Zhu *et al.,* 2015). The fluorescent dye, rhodamine-123, detects

alterations in mitochondrial membrane potential. Our present results demonstrate that *S. cumini* seed hexane and ethanol extracts induced a significant reduction in the number of cells with intact membrane potential and increased the number of cells with low ΔΨm after 24 hours. This is congruent to a report on *S. cumini* seed methanol extract that induced apoptosis in HepG2 cells through the decrease in MMP and down-regulation of HFN-1α (Prakash and Devaraj, 2019). The anti-cancer effect of *S. cumini* seed hexane and ethanol extracts can certainly be attributed to the rich profile of bioactive compounds documented in the seeds (Kumar *et al.,* 2009; Ramya *et al.,* 2012; Parimala and Salomi, 2021), which have been shown to exert diverse biological effects, including reduction in cell viability, abnormal cell morphology, chromosomal DNA fragmentation, induction of apoptosis and/or necrosis and alteration in the mitochondrial membrane potential of MDA-MB-231 human metastatic breast cancer cell line.

Conclusion

The present study aimed to investigate the anti-cancer potential of *Syzygium cumini* seed hexane and ethanol extracts in MDA-MB-231 (human breast cancer cell line). MTT results of the extracts showed promising cancer cell toxicity. DNA fragmentation analysis portrayed smear indicating extensive DNA damage in the extract-treated cancer cells. AO/PI dual staining of IC_{50} extract-treated cells exposed apoptotic changes as observed in early and late apoptotic stages and necrotic cells. Evidence of the breakdown of the mitochondrial membrane was notable by lowering the membrane potential. Hence, our study concludes that *S. cumini* seed can be utilized for discovering anti-cancer lead molecules. Further bioactive compound identification and molecular signaling pathway studies are highly recommended to substantiate its role in cancer treatment and prevention.

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