

Full Length Research Paper

Anti-cancer efficacy of ethanolic extracts from various parts of *Annona Squamosa* on MCF-7 cell line

Sneha Veerakumar¹, Safreen Shaikh Dawood Amanulla¹ and Kumaresan Ramanathan^{1,2*}

¹Department of Biotechnology, Periyar Maniammai University, Vallam, Thanjavur-613 403, India.

²Department of Biochemistry, Institute of Biomedical Sciences, College of Health Sciences, Mekelle University (Ayder Campus), Mekelle, Ethiopia.

Received 26 April, 2016; Accepted 21 June, 2016

Medicinal plant extracts are known to possess breast cancer antidote. The present investigation is focused on anticancer efficacy of various parts of *Annona squamosa*. The organic (ethanol) extracts from various parts of *Annona squamosa* were prepared using soxhlet apparatus and tested for *in vitro* anticancer efficacy on Breast cancer cell line MCF-7 by MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. The results obtained from MTT assay showed that the inhibitory concentration values of bark, peel and seed were found to be approximately 20, 30 and 10 µg/ml, respectively. The ethanolic seed extract had high anticancer activity with IC₅₀ value of 10 µg/ml, reveals that *A. squamosa* inhibits the proliferation of MCF-7 by inducing apoptosis. The plant investigated has anti-cancer activity; hence further studies should be carried out for the isolation of the lead molecules from the parts of the plant to treat the breast cancer.

Key words: *Annona squamosa*, MCF-7 cell line, 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, anti-cancer activity.

INTRODUCTION

Breast cancer remains pre-eminent in the scientific, clinical and societal challenge, frequently diagnosed in women, with an estimated 1.38 million new cases per year. There were 458,000 deaths per year from breast cancer worldwide being leading cause in female mortality in both developed and developing world (Suzanne et al., 2013; Ferlay et al., 2010). Even though great advancement has been made in treatment and control, still there is a scope

for improvement, due to the toxicity of chemotherapy drugs and the side effect of current treatment that ultimately necessitates the need for alternative therapeutic strategy (Avni et al., 2008). Medicinal plants have been gaining popularity as natural therapies from plant-derived products in cancer treatment may reduce the risk of side effects. Botanical extracts from plants like *Abrus precatorius L*, *Allium sativum L*, *Alstonia scholaris*

*Corresponding author. E-mail: kumaresanramanatha@gmail.com. Tel: +251966968532.

L and *Annona reticulata* *L* exhibit shown anticancer property (Sumitra and Krunal, 2013). Annonaceae, the custard apple family is a family of flowering plants consisting of trees and shrubs. There are about 2300 to 2500 species and more than 130 genera in the Annonaceae family. The genus name, '*Annona*' is from the Latin word 'anon', meaning 'yearly produce', referring to the production of fruits of the various species in this genus seasonally. The family is concentrated in the tropics, with few species found in temperate regions (Iba et al., 2014; Rajsekhar, 2011).

The taxonomical classification of *Annona squamosa* is:

Kingdom: Plantae
Order: Magnoliales
Family: Annonaceae
Genus: *Annona*
Species: *squamosa*

A. squamosa is a small well-branched tree or shrub that bears edible fruits which are commonly called custard apple or sugar apple. The tree is more willing to grow at lower altitudes. The plant parts are used for treating various diseases. Leaves are used to treat hysteria, fainting spells. Leaf decoction is employed in the treatment of cold, cough, intestinal infections and acidity condition. A bark decoction is used in diarrhoea. Roots are used to treat dysentery. Fruit is used in making of ice creams and milk beverages (Neha and Dushyant, 2011). Some of the novel chemical constituent isolated from *A. squamosa* showed anti-cancer, anti-HIV and anti-diabetic properties. This medicinal plant exists with diverse pharmacological spectrum (Martino-Roaro et al., 2008; Biba et al., 2014). Few other *Annona* species (*A. triloba*) have also showed anti-cancer property (Jerry, 2008). Malignant sores were treated by seeds of *A. squamosa* as a traditional remedy in the south of China (Guangdong Food and Drug Administration, 2004). Botanical extracts can be easy and effective approach for treating breast cancer. The aim of this study is to determine the anticancer efficacy of *A. squamosa* and to predict the lead molecule from a natural source with fewer side effects that could be extracted and purified further for the prevention or cure of breast cancer.

MATERIALS AND METHODS

Collection of plant samples

Different plant parts namely bark and leaves were isolated from *A. squamosa* while its fruits were collected from the local market.

Preparation of sample and extracts

The collected plant parts such as bark, leaves, peel, pulp and seeds were shade dried for ten days. The dried samples were grounded separately into a fine powder and stored at 4°C for further use. The powdered samples (20 g each) were subjected to Soxhlet

extraction individually with organic solvent ethanol (200 ml) and the temperature was set at 70°C (boiling point). The extracts of different plant parts were collected and stored in different sterile Petri dish. The excess solvent present in the extracts were removed by oven drying for 10 to 15 min at 50°C.

Different concentrations of plant extracts

The plant extracts were made at different concentrations by dissolving them in any of the following solvents such as water, ethanol or incomplete media. The bark and seed extracts were dissolved in ethanol and the peel extract was dissolved in incomplete media.

Each milligram of the plant extract was dissolved in its respective solvent of 1 ml. Further, the concentrations were taken and added to the 96 well plates.

MTT assay

Collection of MCF-7 cell lines

The breast cancer cell lines, MCF-7 cells were purchased from National Centre for Cell Science (NCCS), Pune. MCF-7 cells were maintained in DMEM medium (Gibco, Gaithersburg, MD) supplemented with 10% of Fetal Bovine Serum (FBS: Gibco) and 2% of Penicillin-Streptomycin Antibiotics (Gibco). The cells along with the medium were maintained in a CO₂ incubator at 37°C at 5% of CO₂. Doubling time of MCF-7 cell line is about 24 h. The cells were quantified using hemocytometer.

Incubation of cells

The purchased cells were sub cultured in a T25 animal tissue culture flask (Sigmaaldrich) for further use. Cultures were kept in a CO₂ incubator at 37°C in a 5% CO₂ incubator and cells were harvested by centrifugation.

Cytotoxicity assay

The cytotoxicity assay was performed by the MTT method (Edmondson et al., 1998). MCF-7 (1×10⁴) cells were seeded into two 96-well plates and incubated overnight. The first plate was divided into two sections; the first section was treated with ethanolic extracts of *A. squamosa* peel at 5, 10, 20, 30, 40, 50, 100 and 200 µg per well and in the second section the cells were treated with the ethanolic extracts of bark at 5, 10, 20, 30, 40, 50 and 100 µg per well. The second plate was treated with the ethanol extract of seed at 5, 10, 20, 30, 40 and 50 µg concentrations. Each concentration of the extract treated 2 plates were incubated at 37°C for 24 h in a CO₂ incubator. An untreated group (without extracts from different parts of *A. squamosa*) was used as a control in both of the 96-well plates. A vehicles control was added to the plates and ethanol was used as a control for the cells since, the bark, and seed samples were dissolved in ethanol. The cells were incubated for 12 h. Thereafter, 5 mg/ml of the MTT dye (Thiazolyl Blue Tetrazolium Bromide: (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) was added to each well of the micro-titre plates and the plates were further incubated for 3 h in a dark cupboard. The formazan crystal products were formed. To dissolve the formazan 100 µl of Dimethyl Sulfoxide (DMSO) was added. After 15 min, the amount of purple formazan formed was determined by measuring the optical density (OD) using the ELISA micro plate reader at a wavelength of 595 nm. The amount of formazan formed at each concentration of the extract was measured at 595 nm using a (Thermoscientific) of a

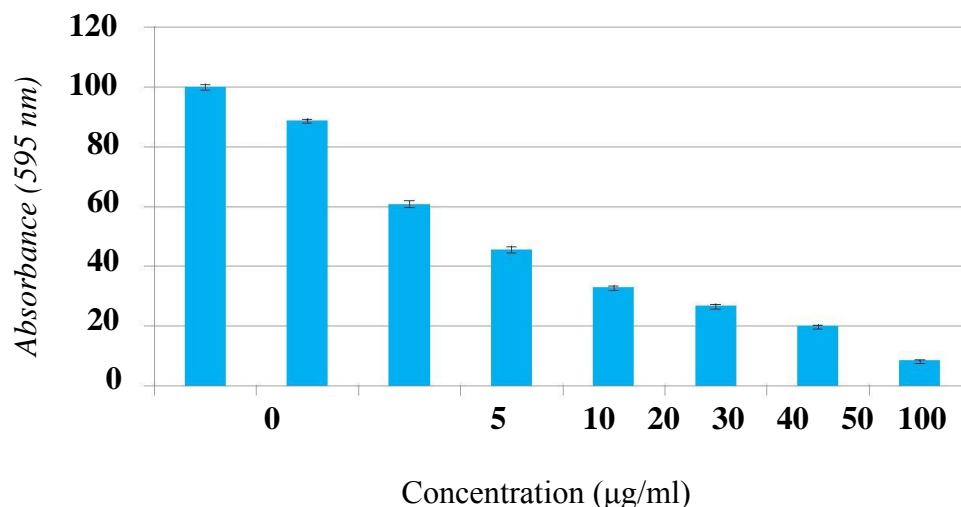


Chart 1. Percentage of cell viability in bark extract treated cells.

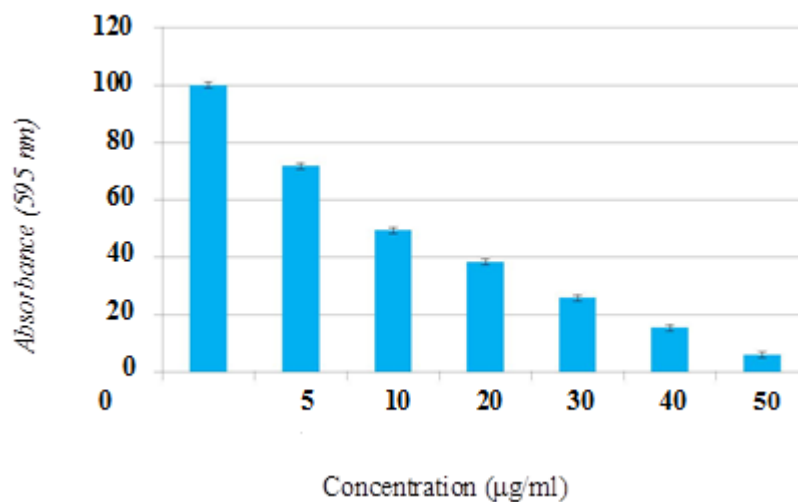


Chart 2. Percentage of cell viability in peel extract treated cells.

spectrophotometer. Each experiment was performed in triplicate. The percentages of viable cells were calculated using the equation:

$$IC_{50} = \frac{\text{Control OD} - \text{Test OD}}{\text{Control OD}} \times 100$$

The viability of the cells was expressed as a percentage of absorbance in cells with treatment to that in cells without treatment. The inhibitory concentration (IC_{50}) was calculated using the formula (Chart 1 to 3).

RESULTS

The percentage of cell viability was found to be gradually decreased from lower concentration to higher

concentration. The inhibitory concentration (IC_{50}) of the bark extract was found to be around 20 µg/ml (Table 1).

The percentage of cell viability was found to be gradually decreased from lower concentration to higher concentration. The inhibitory concentration (IC_{50}) of the peel extract was found to be around 30 µg/ml (Table 2).

The percentage of cell viability gradually decreased from lower concentration to higher concentration. The inhibitory concentration (IC_{50}) of the seel extract was found to be around 10 µg/ml (Table 3).

The plant parts were collected, and the extracts were taken with organic solvents using Soxhlet apparatus. The ethanolic extract of the bark, peel and seed extracts were analyzed for their cytotoxic effects. MTT assay was performed to find a percentage of cell viability and inhibitory concentration value (IC_{50}) of the ethanolic

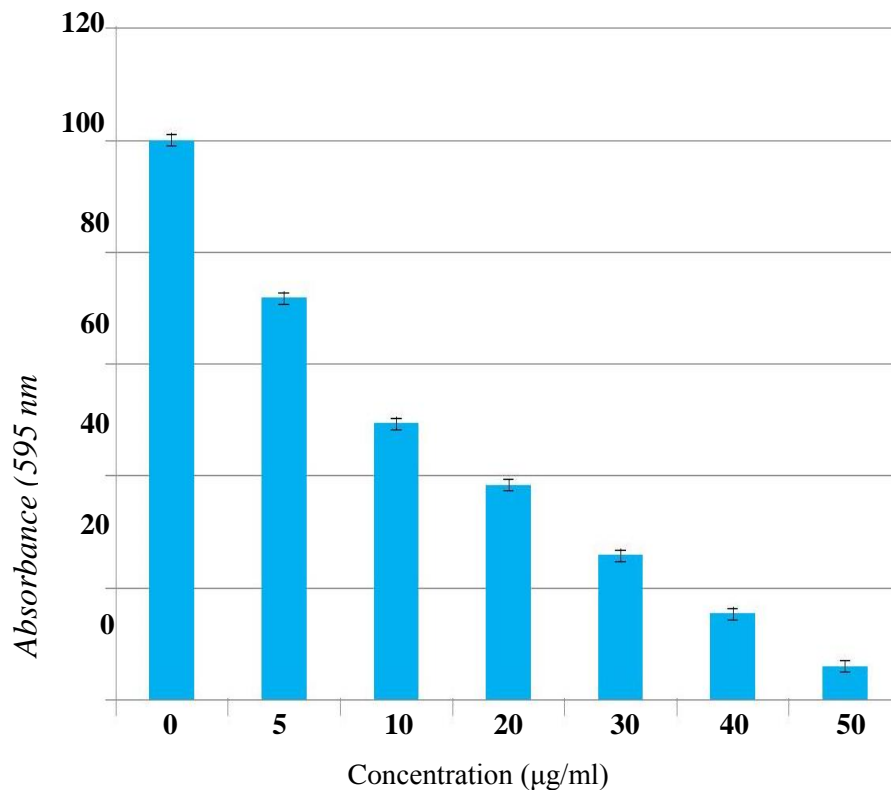


Chart 3. Percentage of cell viability in seed extract treated cells.

Table 1. Various concentrations of bark extract added to the cells.

| Concentrations (µg/ml) | Control | 5 | 10 | 20 | 30 | 40 | 50 | 100 |
|------------------------|---------|-------|-------|--------|-------|-------|-------|-------|
| Row - 1 | 4.78 | 3.09 | 2.89 | 1.0984 | 1.243 | 1.294 | 0.49 | 0.602 |
| Row - 2 | 3.293 | 3.765 | 1.081 | 2.894 | 1.78 | 0.454 | 1.176 | 0.086 |
| Average | 3.958 | 3.515 | 2.401 | 1.800 | 1.304 | 1.06 | 0.791 | 0.332 |

Table 2. Various concentrations of peel extract added to the cells.

| Concentrations (µg/ml) | Control | 5 | 10 | 20 | 30 | 40 | 50 | 100 | 200 |
|------------------------|---------|-------|-------|-------|------|-------|-------|-------|-------|
| Row - 1 | 3.8 | 3.78 | 3.102 | 2.9 | 1.78 | 1.363 | 1.094 | 0.49 | 0.002 |
| Row - 2 | 3.789 | 2.72 | 2.809 | 1.231 | 1.44 | 1.538 | 1.454 | 0.176 | 0.086 |
| Row - 3 | 3.003 | 3.093 | 2.59 | 2.8 | 2.19 | 1.45 | 0.432 | 0.708 | 0.308 |
| Average | 3.530 | 3.197 | 2.833 | 2.310 | 1.80 | 1.45 | 0.993 | 0.458 | 0.132 |

Table 3. Various concentrations of seed extract added to the cells.

| Concentrations (µg/ml) | Control | 5 | 10 | 20 | 30 | 40 | 50 |
|------------------------|---------|-------|-------|-------|-------|-------|-------|
| Row - 1 | 4.76 | 3.902 | 1.99 | 1.79 | 1.003 | 1.094 | 0.034 |
| Row - 2 | 4.789 | 2.869 | 1.781 | 1.989 | 1.28 | 0.094 | 0.28 |
| Row - 3 | 4.003 | 2.949 | 2.9 | 1.409 | 1.205 | 0.89 | 0.489 |
| Average | 4.51 | 3.24 | 2.223 | 1.729 | 1.162 | 0.692 | 0.267 |

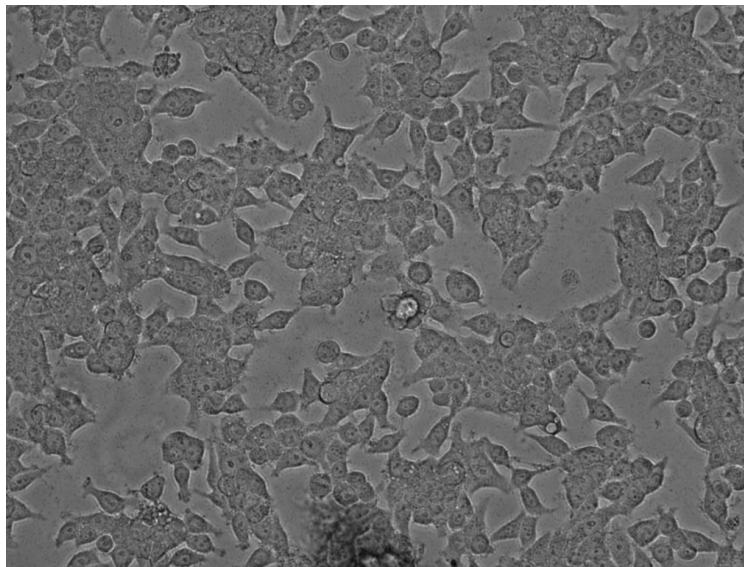


Figure 1. Cells in the control well.

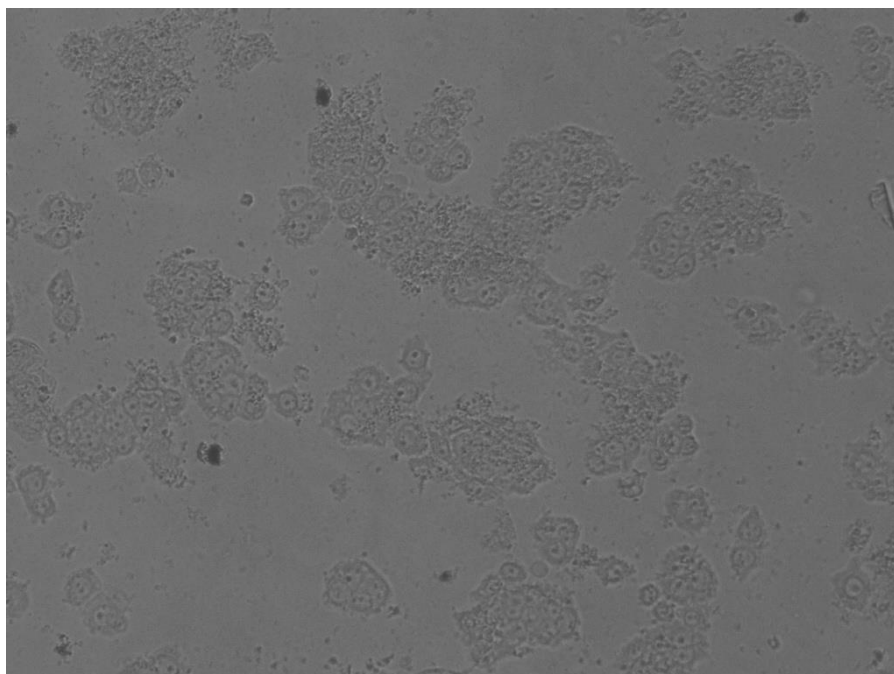


Figure 2. Cells in the bark extract treated well (20 µg).

extracts of bark, peel and seed. The results obtained from MTT assay showed that the inhibitory concentration values of bark, peel and seed were found to be approximately 20, 30 and 10 µg/ml (Figures 1 to 6) respectively. It was observed that the ethanol extracts of the seed had high anticancer activity than bark and peel extract on MCF-7 cell line as they had low IC₅₀ values of 10 µg/ml compared to the latter.

DISCUSSION

Annonaceae species are rich in acetogenin compounds (Craig et al., 1998) exhibit cytotoxic and anti-carcinogenic antioxidant, anti-psoriatic, larvicidal and anthelmintic activities (Martino-Roaro et al., 2008; Jerry, 2008; Feras et al., 1998; Lima et al., 2010; Saelee et al., 2011; Saelee et al., 2011; Kamaraj et al., 2011a,b). Exploration of

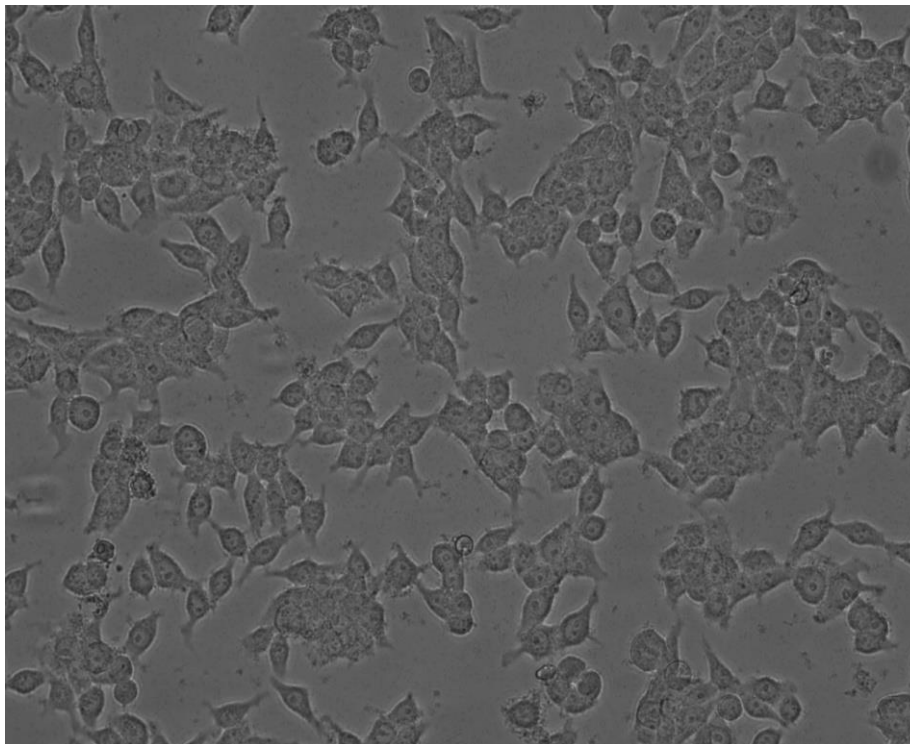


Figure 3. Cells in the control well.

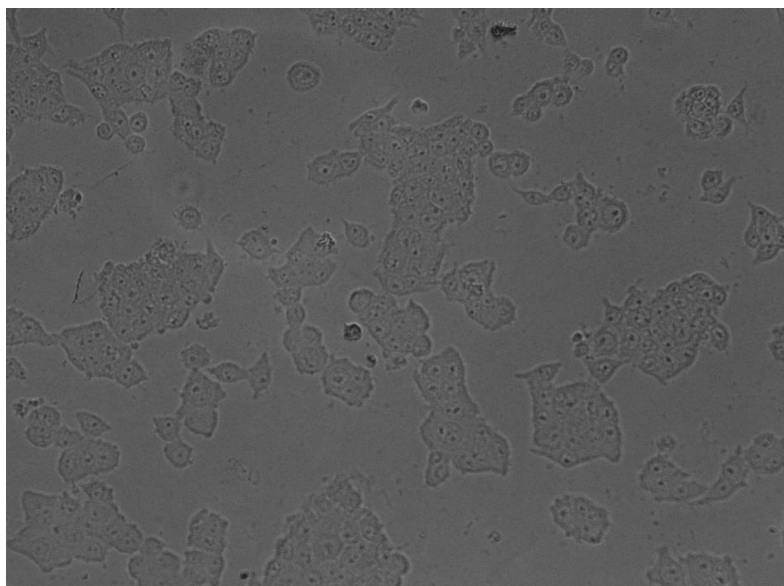


Figure 4. Cells in the peel extract treated well (30 µg).

botanical extract for novel anti-tumor drugs is a significant concern for mankind, as they might prove to be easy and competent approach. Annonaceous acetogenins is the major bioactive compounds from *A. squamosa* seeds that has been identified as well-known inhibitor of multiple

drug resistant cancer cells based on phytochemical and pharmacological studies (Jerry, 2008). Previous studies reported identification and quantification of 2 main compounds of annonaceous acetogenins by HPLC analysis: 12, 15-cis-squamostatin-A and bullatacin from

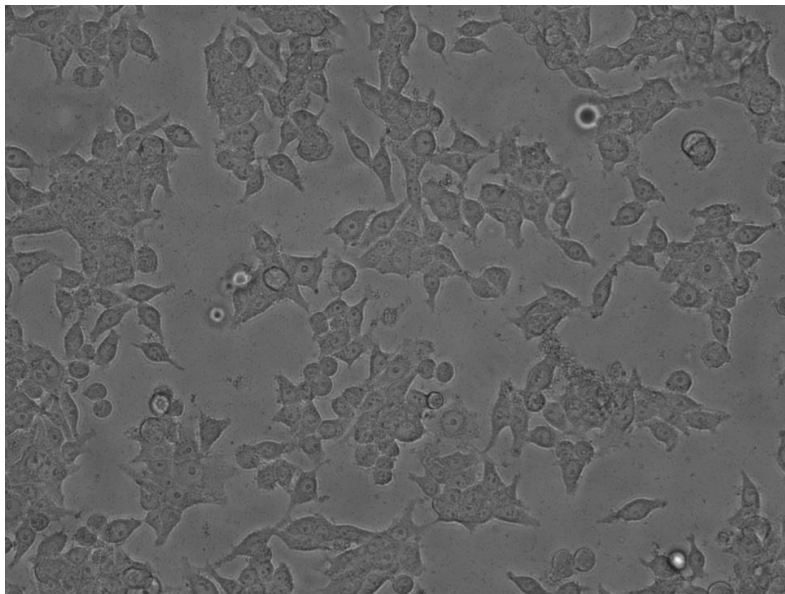


Figure 5. Cells in the control well.

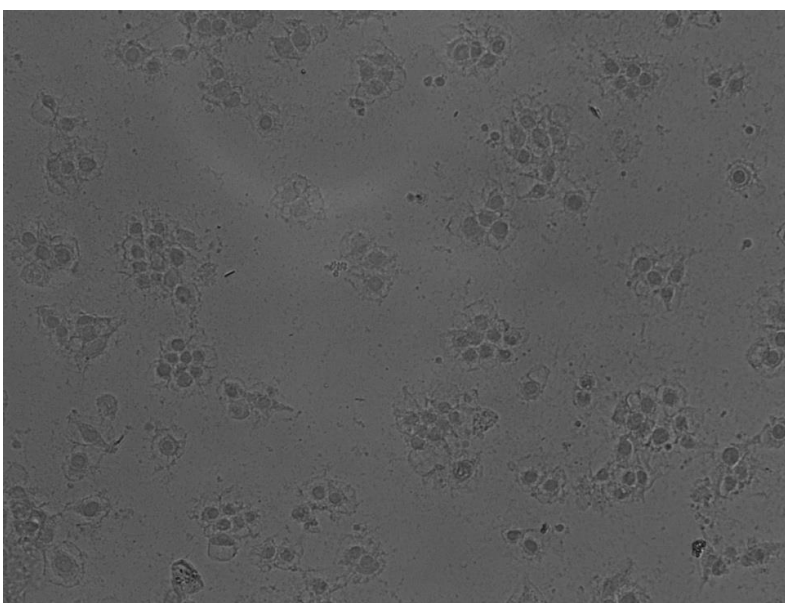


Figure 6. Cells in the seed extract treated well (10 µg).

the extract of *A. squamosa* seeds. Bullatacin, a bistetrahydrofuran annonaceous acetogenin high degree inhibitory action (300 times more effective than taxol) of the mitochondrial respiratory chain complex I, as tested *in vivo* (Jerry, 2008; Liaw et al., 2010). 12, 15-cis-squamostatin-A and bullatacin also showed anti-cancer property against various tumor cell lines (Yang et al., 2009; Chen et al., 2011). Supplementary assays should be performed to find the accurate inhibitory concentration

values. Thus, it can be concluded that the plant parts of *A. squamosa* such as bark, peel and seed were found to have anti-cancer activity against the breast cancer cell lines, which is a reliable indication of the presence of drug molecules in the *A. squamosa* plant that could treat breast cancer. Further studies should be carried out for the isolation of the lead molecules from the parts of the plant to treat the breast cancer. The cell line used has characteristic feature of cancer, hence its cell death

contributes directly to the anti-cancer property.

Conclusion

The ethanolic seed extract of the *A. squamosa* is considered to have a high anticancer potential compared to bark and peel. The outcome of the present study encourages future investigation by isolating a lead molecule responsible for anticancer efficacy, so as to design a particular drug for the cancer. Further studies can be performed in the future to predict the underlying mechanism of the anticancer potential of the plant.

Conflict of Interests

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENT

The authors express our sincere thanks to Dr. A. Antony Joseph Velanganni, Assistant Professor, Department of Biochemistry, Bharathidasan University for helping us by providing necessary equipment used to carry out cell line studies. They also extend their thanks to the Head of the University for providing the necessary instrumental facilities for Soxhlet extraction to complete the project work. The project is self-funded by the authors.

REFERENCES

- Avni GD, Ghulam NQ, Ramesh KG, Mahmoud El-Tamer, Jaswant Singh, Ajit KS, Yashbir SB, Subhash CT, Hari KB (2008). Medicinal Plants and Cancer Chemoprevention. *Curr. Drug Metab.* 9(7):581-591.
- Biba VS, Amily A, Sangeetha S, Remani P (2014). Anticancer, Antioxidant and Antimicrobial activity of Annonaceae family. *World J. Pharm. Pharmaceutical Sci.* 3(6):1595-1604.
- Chen Y, Chen JW, Li X (2011). Cytotoxic bistetrahydrofuran annonaceous acetogenins from the seeds of *Annona squamosa*. *J. Natural Products* 74:2477-2481.
- Craig Hopp D, Feras QA, Zhe-Ming Gu, McLaughlin JL (1998). Mono-THF ring annonaceous acetogenins from *Annona squamosa*. *Int. J. Phytochem.* 47(5):803-809.
- Edmondson JM, Linda SA, Andrew OM (1998). A rapid and simple MTT-based spectrophotometric assay for determining drug sensitivity in monolayer cultures. *J Tissue Cult. Methods* 11:15-17.
- Feras QA, Xiao-Xi Liu, Jerry LM (1998). Annonaceous Acetogenins: Recent Progress. *J. Natural Products* 62:504-540.
- Ferlay J, Shin HR, Bray F, Forman D, Mathers C, Parkin DM (2010). Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *Int. J. Cancer.* 127(12):2893-2917.
- Guangdong Food and Drug Administration (2004). Guangdong Chinese Materia Medica Standards. Guangdong Science and Technology Press, Guangdong. 1:194.
- Jerry LM (2008). Paw Paw and Cancer: Annonaceous Acetogenins from Discovery to Commercial Products. *J. Natural Products* 71(7):1311-1321.
- Kamaraj C, Bagavan A, Elango G, Zahir AA, Rajakumar G, Marimuthu S, Santhoshkumar T, Rahuman AA (2011a). Larvicidal activity of medicinal plant extracts against *Anopheles subpictus* & *Culex tritaeniorhynchus*. *Indian J. Med. Res.* 134:101-106.
- Kamaraj C, Rahuman AA, Elango G, Bagavan A, Zahir AA (2011b). Anthelmintic activity of botanical extracts against sheep gastrointestinal nematodes, *Haemonchus contortus*. *Parasitol. Res.* 109:37-45.
- Liaw CC, Wu TY, Chang FR, Wu YC (2010). Historic perspectives on Annonaceous acetogenins from the chemical bench to preclinical trials. *Planta Medica.* 76:1390-1404.
- Lima LARL, Piment LPS, Boaventura MAD (2010). Acetogenins from *Annona cornifolia* and their antioxidant capacity. *Food Chem.* 122:1129-1138.
- Martino-Roaro Gerardo Zepeda-Vallejo, Eduardo Madrigal-Bujaidar (2008). Anti carcinogenic and genotoxic effects produced by Acetogenins isolated from *Annona muricata*. Abstracts 180:32-246.
- Neha P, Dushyant B (2011). Phytochemical and Pharmacological Review on *Annona squamosa* Linn. *Int. J. Res. Pharmaceutical Biomed. Sci.* 2(4):404-1412.
- Rajsekhar S (2011). Pharmacognosy and pharmacology of *Annona squamosa*: A Review. *Int. J. Pharm. Life Sci.* 2(10):1183-1189.
- Saelee C, Thongrakard V, Tencomnao T (2011). Effects of Thai medicinal herb extracts with anti-psoriatic activity on the expression on NF-kB signaling biomarkers in HaCaT keratinocytes. *Molecules* 16:3908-3932.
- Sumitra C, Krunal N (2013). In vitro and in vivo Methods for Anticancer Activity Evaluation and Some Indian Medicinal Plants Possessing Anticancer Properties: An Overview. *J. Pharmacogn. Phytochem.* 2(1):14-19.
- Suzanne AE, Aboagye EO, Ali S, Anderson AS, Armes J, Berditchevski F, Blaydes JP, Brennan K, Brown NJ, Bryant HE, Bundred NJ, Burchell JM, Campbell AM, Carroll JS, Clarke RB, Coles CE, Cook GJ, Cox A, Curtin NJ, Dekker LV, Silva Idos S, Duffy SW, Easton DF, Eccles DM, Edwards DR, Edwards J, Evans D, Fenlon DF, Flanagan JM, Foster C, Gallagher WM, Garcia-Closas M, Gee JM, Gescher AJ, Goh V, Groves AM, Harvey AJ, Harvie M, Hennessy BT, Hiscox S, Holen I, Howell SJ, Howell A, Hubbard G, Hulbert-Williams N, Hunter MS, Jasani B, Jones LJ, Key TJ, Kirwan CC, Kong A, Kunkler IH, Langdon SP, Leach MO, Mann DJ, Marshall JF, Martin L, Martin SG, Macdougall JE, Miles DW, Miller WR, Morris JR, Moss SM, Mullan P, Natrajan R, O'Connor JP, O'Connor R, Palmieri C, Pharoah PD, Rakha EA, Reed E, Robinson SP, Sahai E, Saxton JM, Schmid P, Smalley MJ, Speirs V, Stein R, Stingl J, Streuli CH, Tutt AN, Velikova G, Walker RA, Watson CJ, Williams KJ, Young LS, Thompson AM (2013). Critical research gaps and translational priorities for the successful prevention and treatment of breast cancer. *Breast Cancer Res.* 15(5):R92.
- Yang HJ, Zhang N, Li X, Chen JW, Cai BC (2009). Structure-activity relationships of diverse annonaceous acetogenins against human tumor cells. *Bioorganic Medicinal Chem. Lett.* 19:2199-2202.