Iraqi journal of Bioscience and Biomedical
Published Online First: 31/ December /2023.
p-ISSN: 3007-5491 / e-ISSN:3007-5505

Cytotoxic Effect of *Annona muricata* leaf extracts on tumor cell lines *in vitro*

Shahlaa M. Salih*[1], Amina Ahmed, Yasir W.issa[3]

1,2 Department of Molecular and Medical Biotechnology, College of Biotechnology, Al-Nahrain University, Baghdad, Iraq.
3 Department of Anesthetic Techniques, Madenat Alelem University College, Baghdad, Iraq.
*Corresponding Author Email. shahlaa.mahdi@nahrsinuniv.edu.iq
Orcid No: 0000-0001-5427-0602
Co-author Email: yasirw.issa@mauc.edu.iq
ORCID No: 0009-0003-5109-5951
Received 02/10/2023,
Accepted 28/11/2023,
Published 31/12/2023.

This work © 2024 by College of biotechnology/ Al-Nahrain university

This is an Open Access article distributed under the terms of the CC BY 4.0. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/ which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Abstract

Cancer is a major cause of mortality and morbidity globally and overall survival rate is still low despite advances in surgery, radiotherapy, and chemotherapy. Plants have played a significant role in the treatment of cancer and infectious diseases for a very long time. *Annona muricata* has been widely used in the treatment of cancer and diseases in many countries. This study was designed to evaluate the cytotoxic effects of *Annona muricata* ethanolic extracts on (MAD_MB_231) human breast cancer cell lines and lung cancer cell line (A549). Various concentrations of *ethanolic* extracts (50, 100,150 and 200 µg/mL) were prepared with ethanolic solvents and used to treat cell lines after 24 h. exposure by MTT assay. The results showed the effect of extract after 24- hour treatment is a dose dependent and has the most effective anticancer activity on the lung cells line with IC50 Values of The IC_{50} was 134.6 mg/ml for breast cancer cell lines and The IC_{50} was (124.6) mg/ml for lung cancer cell line. the current study showed the highly effective action of the ethanolic crude leaves extract of *Annona muricata* and can be used in the management and treatment of cancer.

Keywords: Annona muricata; Cytotoxic effects; Ethanolic extracts; Anticancer activity; MTT assay.

Introduction

Cancer remains a significant health threat and leading cause of death globally, with the International Agency for Research on Cancer reporting 14.1 and 8.2 million new cases and deaths in 2012 and expecting 21.7 and 13 million[1] In the USA alone, 1735350 new cases are expected, with 609640 deaths[2]. Early tumor prognosis has improved, but therapies' limited efficacy and toxicity

Iraqi journal of Bioscience and Biomedical
Published Online First: 31/ December /2023.
p-ISSN: 3007-5491 / e-ISSN:3007-5505

cause significant co-morbidities in advanced patients. Synthetic compounds for molecular targeted therapies have been developed, but resistance has limited their utility[3]. Over the past several decades, identification of plant-based drugs has significantly contributed to anticancer drug discoveries and has resulted in one-third of anticancer drugs approved by the United States Food and Drug Administration (USFDA)[4] historically, e.g., paclitaxel, camptothecin, vincristine and their analogs. Given this history, it is critical to continue to explore and identify new medicinal plants and to determine their potential as a source of new drugs[5]. Annona muricata (Graviola) has extensive traditional use, and considerable evidence has been developed that it may be useful therapeutic agents in the battle against certain cancers[6]. Graviola belongs to the Annonacin family, Annona genus and muricata species. The Annonacin family includes about 130 genera and 2300 species. Graviola is a fruit tree with many uses in traditional and Biological and chemical characterization studies indicate that amentaceous acerogenins are the main ingredients of Graviola[7]. Nowadays more than 100 amentaceous acetogenins that are generally characterized as a family of natural products with antitumor activities, from roots, leaves, barks, fruits, and seeds of Graviola have been widely used in alternative medicine for many purposes[8]. Other reports have demonstrated that Graviola has several biological activities such as antifungal, anti-bacterial, anti-malarial and antioxidant. Furthermore, it has been shown to have anti-cancer properties on multi-drug resistant cancer cell lines[9]. Studies reported that Graviola has been found to selectively inhibit growth against various cancer cells, including lung carcinoma, breast solid tumor lines, prostate adenocarcinoma, pancreatic carcinoma, colon adenocarcinoma cell lines, liver cancer cell lines, human lymphoma cell lines, and multi-drug resistant human breast adenocarcinoma[10]. Due to the activity of phytochemicals including flavonoids, isoquinoline alkaloids, and annonaceous acetogenins (ACGs). These compounds have been tested against various cancer cell lines and have antioxidant activities[11]. The fruit pulp contains 37 volatile chemicals and 80 essential oils, with the analysis of these volatiles promising due to their bioactivity[12]. Acetogenins (AGEs) found in the A.muricata plant have radical scavenging activity against DPPH and ABTS radicals. Anti-inflammatory activity is an emerging field of research for intervention targets in various diseases, including asthma, arthritis, Crohn's disease, Alzheimer's disease, cardiovascular disease, diabetes, high blood pressure, and cancers[13,14]. Natural compounds in dietary supplements and herbal remedies have been used to minimize pain and inflammation, with some working mechanistically by inhibiting inflammatory signaling pathways. For reason mentined above this study was desinged to explore the cytotoxic effect of graviolla ethanolic extract against human breast cancer cell line and lung cancer cell line.

Materials and Methods

Preparation of graviolla extract

The samples were collected from the local market. Briefly, (20g) leaves were extracted with ethanol (100 mL) in a shaking incubator at 25°C for 72 h. The extracts were filtered with Whatman's No. 1 filter paper and re-extracted three times. The filtrate was concentrated under reduced pressure by using Rotary evaporator. After measuring the weight of dry extracts, stock solution of 200 mg /ml prepared. The extracts used for evaluation were sterilized by filtration with 0.20 mm membrane and kept at −80 C in the dark till used for further analysis.

Studying antitumor activity

The anticancer efficacy of methanolic extract from graviolla against MCF7 cell line normal hepatic human cell line WRL 68 was evaluated. The colorimetric cell viability MTT assay was used as described by [14]. At first, 200 µL/well of cells (106 cell/ mL) were cultured in 96-well tissue culture plate. Then, Different concentrations of graviolla extract test solution were prepared to evaluate

Iraqi journal of Bioscience and Biomedical
Published Online First: 31/ December /2023.
p-ISSN: 3007-5491 / e-ISSN:3007-5505

cytotoxic effect against two examined cell line (200,150,100, 50, 25, 12.5 µg/ mL) in water. After that, 100 µL of various concentrations was added to each well and incubated at 37 ºC for 24h, 28h. After the incubation, 10µL of MTT solution (5 mg/ mL) was added to each well and incubated at 37 ºC for 4 h. Finally, 50 µL of DMSO (dimethyl sulfoxide) was added to each well and incubated for 10 min. L20B and MCF7 cells were cultured in complete medium without graviolla extract solution as a control. The absorbance was measured for each well at 620 nm using an ELISA reader. Only viable cells were able to take the stain while the dead cells were not. The live cells, percentage of viability and inhibition ratio were calculated according to the formula GI% of graviolla Extracts[15].

Statistical analysis

The experiments were performed in triplicate and all data are expressed as means. ± standard deviation. The values were analyzed by one-way ANOVA using prism pad graph version 16.0 software and individual comparisons were obtained by Tukey's method. P value ≤ 0.05 was considered statistically significant.

Results and Discussions

Cytotoxic effect of *Graviolla* Ethanoic extract

The present study, breast cancer cell line (MAD_MB_231) was used to determine the cytotoxic activity of graviolla extract at various concentrations (50, 100,150 and 200 µg/mL). The effect was a dose dependent, and the 200 mg/ml showed the highest activity (74.0 ± 1.9) The IC_{50} was 134.6 mg/ml presented in table 1 and fig.1

Table 1: Cytotoxic effect of *Annona muricata* on (MAD_MB_231) cell lines

***Graviolla* extract concentration (µg/ml)**	**MCF7 (Mean ± SEM)**
12.5	**12.0 ± 4.3**
25	**25..0 ± 2.8**
50	**33.0 ± 3,5**
100	**53.0 ± 3.0**
150	**63.0 ± 2.2**
200	**74.0 ± 1.9**

Iraqi journal of Bioscience and Biomedical
Published Online First: 31/ December /2023.
p-ISSN: 3007-5491 / e-ISSN:3007-5505

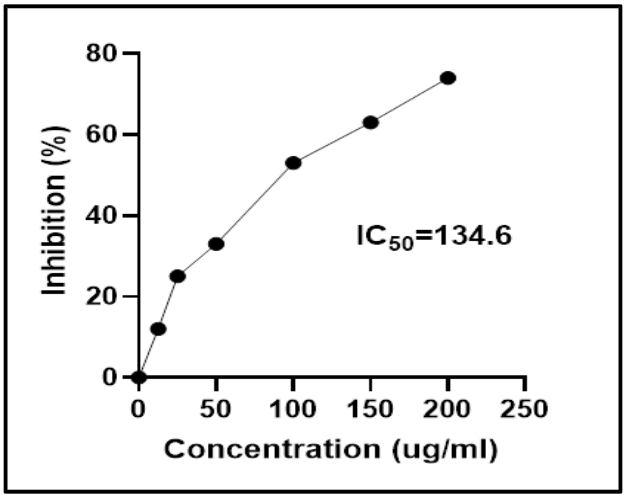

Figure 1: Cytotoxic effect of *Annona muricata* on (MAD_MB_231) cell lines

The cytotoxic activity of Graviola extract on lung cancer cell lines (A549) was investigated and results showed that the effect was a dose dependent, and the highest activity was recorded at (200 mg/ml), The IC_{50} was (124.6) mg/ml as shown in table 2 and fig.2

Table 2. Cytotoxic effect of *Annona muricata* on (A549) cell line

***Graviolla* extract concentration (µg/ml)**	**MCF7 (Mean ± SEM)**
12.5	10.0 ± 2.3
25	22..0 ± 1.8
50	31.0 ± 1.5
100	42.0 ± 3.0
150	53.0 ± 2.0
200	66.0 ± 3.8

Iraqi journal of Bioscience and Biomedical
Published Online First: 31/ December /2023.
p-ISSN: 3007-5491 / e-ISSN:3007-5505

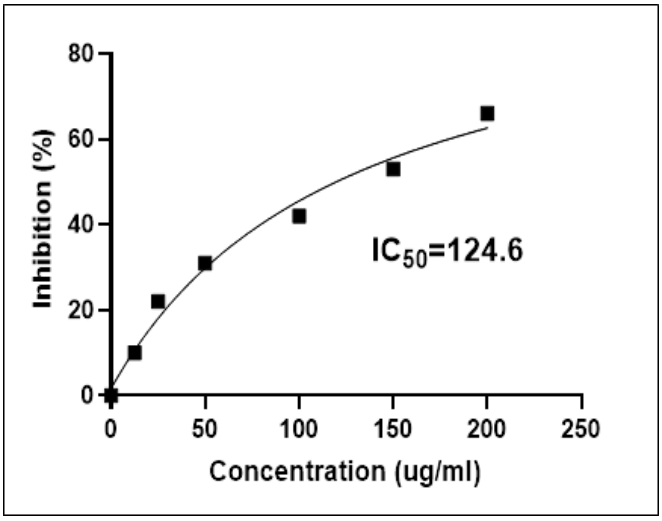

Figure 2. Cytotoxic effect of *Annona muricata* on (A549) cell line

Cytotoxicity test is a qualitative and quantitative test to determine cell death. The MTT assay is a method used to see cytotoxic effects extracts on cancer cells. The principle of the MTT assay is a spectroscopic method and is by determining the absorbance value of formazan[16]. MTT will be absorbed into the cell and entered the system of cell respiration in mitochondria. The action of the enzyme active mitochondria in cells is to metabolize tetrazolium salts, resulting in termination of tetrazolium ring by dehydrogenase enzymes which lead to tetrazolium formazan being transformed into water-insoluble but soluble in SDS 10%. Formazan formed is colored purple and is proportionate to the number of living cells. Cells that die dissolve in water and remain yellow because the mitochondria of cells that die are not respirational, hence tetrazolium ring is disconnected so it cannot reduce MTT reagent to formazan and the color remains yellow[17].

Several studies examining the anticancerous properties in A. muricata extracts have observed the induction of apoptosis. According to the available data, there are about six extract types about solvent extraction of A. muricata parts, including water ethanol, methanol, ethyl acetate, chloroform, and n-hexane extracts. Leaf extracts of A. muricata induce apoptosis in breast MDA-MB-468 cancer cells through caspase-3 activation[18,19]. Studies concluded that apoptosis has been receiving great attention as a major mechanism of cell death in normal as well as tumor cells. However, the programmed cell death might be interrupted due to defective signaling pathway nonetheless in tumor cells with higher rate of mutation. Defective apoptosis has been reflected in the form of cell resistance to apoptotic inducing agents and, consequently, treatment failure. Annonaceous acetogenins, from Annona muricata were found to be promising new anti-tumor and anticancer agents in numerous in vitro studies. These acetogenins are demonstrated to be selective for cancer cells, having potent antiproliferative and toxic outcomes in these cells[20,21]. Specific acetogenins in extracts of Annona muricata have been reported to be selectively toxic in vitro to certain types of tumor cells including lung carcinoma cell lines; human breast solid tumor lines; prostate adenocarcinoma; pancreatic carcinoma cell lines; colon

adenocarcinoma cell lines; mammary adenocarcinoma cell lines; liver cancer cell lines; human lymphoma cell lines; and multi-drug resistant human breast adenocarcinoma[22,23].

Conclusion

The study reveals the cytotoxic potential of Annona muricata ethanolic extracts against human breast and lung cancer cell lines. The extract's dose-dependent anticancer activity suggests it could be a valuable source of anticancer compounds. The extract's induction of apoptosis in cancer cells supports its therapeutic potential. The research supports the use of traditional medicinal plants in modern cancer treatment and could lead to the development of novel anticancer drugs.

Acknowledgment

We express our profound gratitude to all those who contributed to the successful completion of this study. Special thanks to the laboratory staff at Al-Nahrain University research lab, whose expertise and support were invaluable in the execution of our experiments. Our appreciation also extends to the participants for their willingness to contribute to this research. This work was supported by the authors

Authors Declaration

- We hereby confirm that all the Figures and Tables in the manuscript are original and have been created by us.
- The author has signed use of laboratory cell lines in this research.
- We have obtained ethical clearance for our study, which involved human participants, from the local ethical committee at [Al-Nahrain University/College of Biotechnology]. This approval underscores our commitment to ethical research practices and the well-being of our participants.
- Ethical Clearance: The project was approved by the local ethical committee at [Al-Nahrain University/College of Biotechnology], ensuring adherence to ethical standards and the protection of participants' rights and welfare.

Authors Contribution Statement

[First Author's Name]: Contributed to the conception and design of the study, conducted the experiments, data analysis, and drafted the initial manuscript.

[Second Author's Name]: Assisted in the preparation of the Annona muricata ethanolic extracts and performed the MTT assay experiments.

[Third Author's Name]: Played a crucial role in the statistical analysis of the data and interpretation of the results.

References

1. Dai YS, Hogan S, Schmelz EM, Ju YH, Canning C, Zhou K. Selective growth inhibition of human breast cancer cells by graviola fruit extract in vitro and in vivo involving downregulation of EGFR expression. Nutr Cancer. 2011;63(5):795-801.
2. Justino AB, Miranda NC, Franco RR, Martins MM, da Silva NM, Espindola FS. Annona muricata Linn. Leaf as a Source of Antioxidant Compounds with in Vitro Antidiabetic and Inhibitory Potential against α-Amylase, α-Glucosidase, Lipase, Non-Enzymatic Glycation and Lipid Peroxidation. Biomed Pharmacother. 2018;100:83-92.
3. Nguyen MT, Nguyen VT, Minh LV, Trieu LH, Cang MH, Bui LB. Determination of the Phytochemical Screening, Total Polyphenols, Flavonoids Content, and Antioxidant Activity of Soursop Leaves (Annona muricata Linn.). IOP Conf Ser Mater Sci Eng. 2020;736:062011.

4. Zubair H, et al. Cancer Chemoprevention by Phytochemicals: nature's Healing Touch. Molecules. 2017;22:E395.
5. Nugraha AS, Haritakun R, Lambert JM, Dillon CT, Keller PA. Alkaloids from the Root of Indonesian Annona muricata L. Molecules. 2021; 35:481-489.
6. Errayes AO, Abdussalam-Mohammed W, Darwish MO. Review of Phytochemical and Medical Applications of Annona muricata Fruits. J Phytochem Rev. 2020;2:70-79.
7. Asare GA, Afriyie D, Ngala RA, et al. Antiproliferative activity of aqueous leaf extract of Annona muricata L. on the prostate, BPH-1 cells, and some target genes. Integr Cancer Ther. 2014;14(1):65-74.
8. Cheong K, Tan C, Mirhosseini H, Chin S. Optimization of equilibrium headspace analysis of volatile flavor compounds of Malaysian soursop (Annona muricata): Comprehensive two-dimensional gas chromatography time-of-flight mass spectrometry (GC × GC-TOFMS). J Chromatogr A. 2011;125:1481-1489.
9. Abdullah M, Syam AF, Meilany S, et al. The value of caspase-3 after the application of Annona muricata leaf extract in COLO-205 colorectal cancer cell line. Gastroenterol Res Pract. 2017;2017:4357165.
10. Adewole SO, Caxton-Martins EA. Morphological changes and hypoglycemic effects of Annona muricata Linn. (Annonaceae) leaf aqueous extract on pancreatic B-cells of streptozotocin-treated diabetic rats. Afr J Biomed Res. 2006;9:173-187.
11. Aguilar-Hernández G, Zepeda-Vallejo LG, García-Magaña MDL, Vivar-Vera MDLÁ, Pérez-Larios A, Girón-Pérez MI, Coria-Tellez AV, Rodríguez-Aguayo C, Montalvo-González E. Extraction of Alkaloids Using Ultrasound from Pulp and By-Products of Soursop Fruit (Annona muricata L.). Food Chem. 2020;10:4869.
12. Antony P, Vijayan R. Acetogenins from Annona muricata as Potential Inhibitors of Antiapoptotic Proteins: A Molecular Modeling Study. Molecules. 2016;10:1399.
13. Chih H, et al. The MTT assay method for cytotoxicity evaluation. Appl Microbiol Biotechnol. 2004;68(4):523-530.
14. Dai YS, Hogan S, Schmelz EM, Ju YH, Canning C, Zhou K. Selective growth inhibition of human breast cancer cells by graviola fruit extract in vitro and in vivo involving downregulation of EGFR expression. Nutrition and Cancer. 2011;63(5):795-801.
15. van Meerloo J, Kaspers GJL, Cloos J. Cell sensitivity assays: the MTT assay. Methods Mol Biol [Internet]. 2011 [cited 2024 Mar 8];731:237–45.
16. Dang YJ, et al. In situ absorption in rat intestinal tract of solid dispersion of annonaceous acetogenins. J Pharmacol Exp Ther. 2012;341(3):821-829.
17. de Sousa OV, et al. Antinociceptive and anti-inflammatory activities of the ethanol extract of Annona muricata L. leaves in animal models. Int J Mol Sci. 2010;11(5):2067-2078.
18. De Sousa OV, Vieira GD-V, de Pinho JDJR, Yamamoto CH, Alves MS. Antinociceptive and anti-inflammatory activities of the ethanol extract of Annona muricata L. leaves in animal models. Int J Mol Sci. 2010;11(5):2067-2078.
19. De Souza R, Benassi E, da Silva RR, Afonso S, Scarminio IS. Enhanced extraction yields and mobile phase separations by solvent mixtures for the analysis of metabolites in Annona muricata L. Leaves. J Chromatogr A. 2009;1216(32):4176-4185.
20. Naik AV, Sellappan K. Assessment of Genotoxic Potential of Annonacin and Annona muricata L. Extracts on Human Breast Cancer (MCF-7) Cells. Mutagenesis. 2021;36(2):123-134.
21. Nugraha AS, Damayanti YD, Wangchuk P, Keller PA. Anti-Infective and Anti-Cancer Properties of the Annona Species: Their Ethnomedicinal Uses, Alkaloid Diversity, and Pharmacological Activities. Molecules. 2019;24(22):4419.
22. Yang C, et al. Synergistic interactions among flavonoids and acetogenins in Graviola (Annona muricata) leaves confer protection against prostate cancer. Carcinogenesis. 2015;36(6):656-665.
23. Sun S, et al. Novel annonaceous acetogenins from Graviola (Annona muricata) fruits with strong anti-proliferative activity. Tetrahedron Lett. 2017;58(6):583-585.