

Annona muricata callus extracted particles (AMCEP) as a potential anticancer agent against Human Colorectal Cancer cell line HCT-116 by inducing apoptosis

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Research Article

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Abstract

Although the advances in chemotherapy, radiotherapy and surgery, the mortality and morbidity rate of cancer cases is still high. The use of natural plant derived medicinal product has an emerging modern therapy in order to improve human health and reducing the risk of side effects from synthetic drugs. The aim of this study to evaluate the anticancer potential of *Annona muricata* callus extracted particles against human colorectal cancer cell line HCT-116. *A. muricata* callus extracted particles were screened for the cytotoxic activity by MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide) assay against HCT-116 & WI-38 cell line. Further, Annexin V apoptosis assay were done by the flow cytometry analysis. The particles from ethanolic extract of *A. muricata* callus showed cytotoxic effect against HCT-116 cell line with IC_{50} 60.64 μ g/ml while it showed mild toxicity only at highest concentration against normal WI-38 cell line. Further, Annexin V apoptosis assay reveals the sample treated cell population of HCT-116 followed the process of early apoptosis as compared to the control cells (untreated). It has been concluded that *A. muricata* callus extracted particles have potential anticancer agent against human colorectal cancer cell line HCT-116.

Introduction

Annona muricata L. is also known as Graviola or Soursop (English), Lakshmanphala (Kannada) and it belongs to the family Annonaceae. *A. muricata* is native to the Central America but is now cultivated in tropical and subtropical regions of the world. This plant extract contains many phyto constituents that are highly effective against various cancerous cells. Plant contains acetogenin in the leaf, stem, root and fruits. Acetogenins are the derivatives of fatty acids and has been identified to have an antitumor activity [1, 2, 3]. Natural products from the plants have been used for a considerable period by many cultures and civilizations to help humankind, maintain health and treat various diseases. In a pharmaceutical area, plants used in ethnomedicine are the most abundant source of active phytochemical that afford health benefits against different ailments and diseases [4]. Any uncontrolled growth of a group of cells is commonly known as cancer which is a second major cause of death after heart diseases. According to the worldwide cancer data 2020 total estimated cancer cases were 18.1 million amongst those of 9.3 million cases from men and 8.8 million from women respectively. The three major types of cancers were detected are lung cancer contributing (12.2%), breast cancer (12.5%) and colorectal cancer (10.7%). Therefore, scientific studies of callus extract of *A. muricata* plant has been undertaken in order to establish its potential use as an alternative medicine in the prevention and treatment of cancer.

Materials and Methods

Plant Material Collection and Identification:

Fresh leaves and plantlet of *Annona muricata* were obtained from Mahatma Phule Krishi Vidyapeeth, Rahuri, Ahmednagar (MS) in the month of February to April 2022. The herbarium specimen of plant were

deposited, identified and authenticated from Botanical Survey of India, Western Regional Centre, Pune. (Authentication Letter No: BSI/WRC/Iden.Cer./2022/3005220027363)

Callus culture of *A. muricata*:

Apical tip and third leaf nodal portion of axillary branch of *A. muricata* plant were used as an explant for the initiation of callus culture. Surface sterilization of excised plant material was done with 4-5 drops of Tween 20 followed by 0.1% HgCl₂ solution and washes thrice each times with sterilized distilled water. About 1-1.5cm of explants were cut gently with a sterilized scalpel blade and aseptically inoculated in culture bottles with autoclaved modified Basal Murashige and Skoog medium supplemented with plant growth regulators 1.5 mg/L BAP (6 Benzylaminopurine) and 2.0 mg/L 2,4-D (2,4- Dichlorophenoxy acetic acid). All the culture bottles were maintained at 25± 2⁰C and illuminated for 16 hr. light followed by 8 hr. dark condition. [5]

Sample preparation and extraction:

The callus tissues of *A. muricata* were dried and powdered. 5 gm of powder were suspended in 100 ml of ethanol. Extraction was done using Soxhlet apparatus for 5 hr at specific temperature not exceeding the boiling point. The extract was concentrated using rotary evaporator and extracted sample were preserved in refrigerator for further experiments. [6]

FTIR Analysis of AMCEP:

The ethanol extract fraction of *A. muricata* callus were taken for FT-IR analysis. Based on the peak value in the region of infrared radiation the functional groups of active components were detected. All spectra were obtained with the aid of Perkin Elmer Spectrum RX1, Range 4400 to 450 cm⁻¹; Resolution: 1 cm⁻¹ in the region including IR spectra of solids (KBr pellets) on FTIR spectrophotometer and computerized the absorbance data for analyses. [7]

GC-HRMS analysis of *A. muricata* callus extract:

Ethanol fraction of *A. muricata* callus extract was taken for GC-HRMS analysis (Gas-chromatograph coupled with High Resolution Mass Spectrometer). It is a combined analyzer used for qualitative and quantitative analysis of organic compounds. The analysis was carried out on a GC Agilent 7890 system having FID detector with head space injector and combipal autosampler. The gas chromatograph interfaced to a mass spectrometer (Jeol, AccuTOF GCV, EI / CI Source, Time of Flight Analyser, Mass range -35 - 800 amu and Mass resolution – 5000). 99.99% Helium gas was used as carrier gas at constant flow of 1 ml/min and an injection volume of 1 µl was delivered. The relative percentage amount of each component was calculated by comparing its average peak area to the total areas.

Anticancer Activity: Anticancer activity of AMCEP was performed on human colorectal cancer cell line HCT-116 by the following cytotoxicity and annexin V apoptosis assay.

Cytotoxicity by MTT Assay:

Cytotoxicity assay was performed on both HCT-116 Human Colorectal Cancer cell line & WI-38 Human lung fibroblast cell line procured from NCCS Pune. Briefly, to each well of the 96 well microtitre plate, 200µl of the cell suspension was added and the plate was incubated at 37°C and 5% CO₂ atmosphere for 24 hr. 200µl of different test concentrations of drugs were added to the respective wells and incubated at 37°C and 5% CO₂ atmosphere for 24 hr. Thereafter, the cells were treated with 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltriazolium bromide (MTT) (MP Biomedicals, Germany). The entire medium including MTT solution (5 mg/mL) was aspirated from the wells. The remaining formazan crystals were dissolved in DMSO and the absorbance was measured using a microplate reader at a wavelength of 570 nm and also at 630 nm. The percentage growth inhibition was calculated, after subtracting the background and the blank, and concentration of test drug needed to inhibit cell growth by 50% (IC₅₀) was generated from the dose-response curve for the cell line. [8]

Annexin V Apoptosis Assay by Flow Cytometry:

HCT-116 Human colorectal cancer cell line was used for Annexin V apoptosis assay. Culture cells in a 6-well plate at a density of 3×10^5 cells/2 ml and incubate in a CO₂ incubator overnight at 37°C for 24 hr. Aspirate the spent medium and wash with 1ml 1X PBS. Treat the cells with required concentration of test compound in 2 ml of culture medium and incubate the cells for 24 hr. and leave one of the well as untreated to be used as negative control. At the end of the treatment, remove the medium from all the flasks into 15ml centrifuge tubes and wash with 1ml PBS. Remove the PBS and add 0.5mL of trypsin-EDTA solution and incubate at 37°C for 3-4 minutes. Pour the culture medium back into their respective wells and harvest the cells directly into the centrifuge tubes. Centrifuge the tubes for five minutes at 300 x g at 25°C. Carefully decant the supernatant. Wash with PBS twice. Decant the PBS completely and resuspend cells in 1X Binding Buffer at a concentration of 1×10^6 cells/ml. Transfer 100 µl of the solution (1×10^5 cells) to a 5 ml culture tube. Add 5 µl of AbFlour 488 Annexin V. Gently vortex the cells and incubate for 15 min at RT (25°C) in the dark. Add 2 µl of PI and 400 µl of 1X Binding Buffer to each tube and vortex gently. Analyze by flow cytometry immediately after addition of PI. [9, 10]

Results and discussion

Callus culture of *A. muricata*:

The combination of MS+ BAP 1.5 mg/L and 2,4-D 2.0 mg/L induced an excellent growth of callus from leaf base explant of *Annona muricata* and the morphology of callus was yellowish green and friable in its nature.

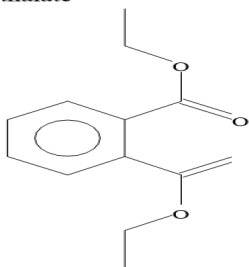
FTIR Analysis of AMCEP:

The IR spectrum revealed the presence of broad and strong adsorption of hydroxyl functional group at 3435.74 cm⁻¹, weak symmetric C-H (s) of aromatic nucleus and asymmetric C-H (s) vibrations CH₃ and CH₂ at 2924.52 cm⁻¹, C=C or N-H vibrations at 1631.74 cm⁻¹, Nitro group at 1381.90 cm⁻¹ and medium vibration of aliphatic C-O-H at 1069.48 cm⁻¹. The presence of alkanes, alkenes, ester, aromatic ring and hydroxyl groups in the extract confirms the presence of annonaceous acetogenins from *A. muricata*.

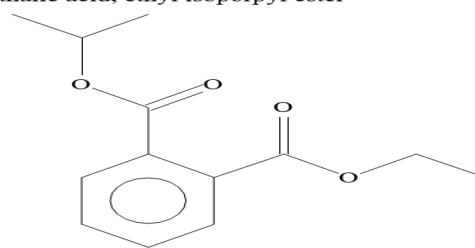
GC-HRMS analysis of *A. muricata* callus extract:

GCHRMS analysis of *A. muricata* callus extract reveals the following phytoconstituents;

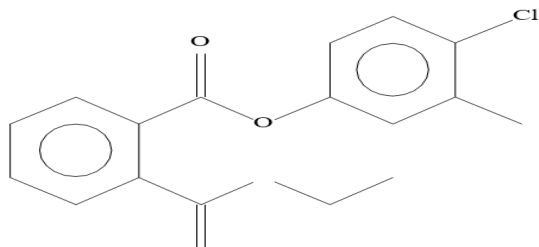
Diethyl Phthalate



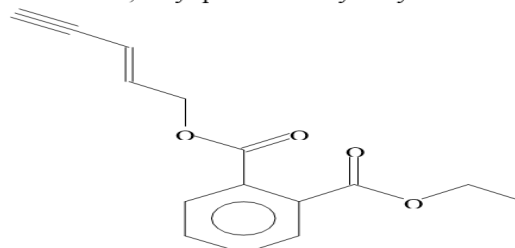
Phthalic acid, ethyl isopropyl ester



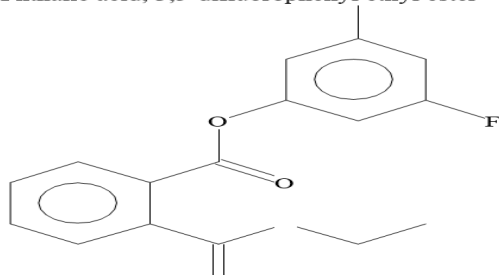
Phthalic acid, 4-chloro-3-methylphenyl ethyl ester



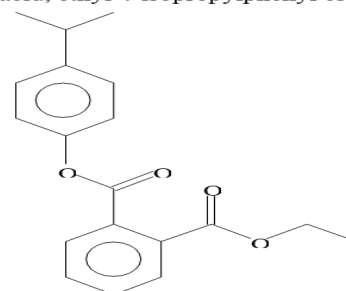
Phthalic acid, ethyl pent-2-en-4-yn-1-yl ester



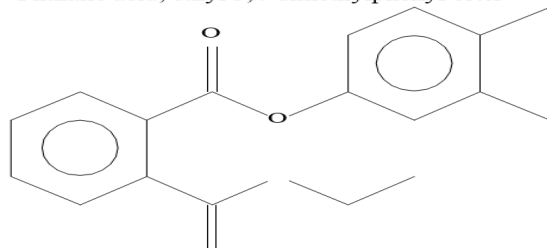
Phthalic acid, 3,5-difluorophenyl ethyl ester



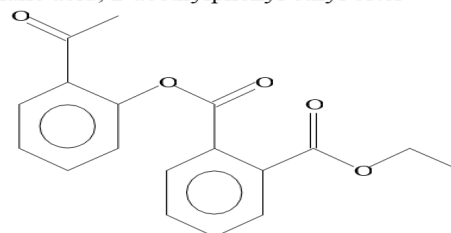
Phthalic acid, ethyl 4-isopropylphenyl ester



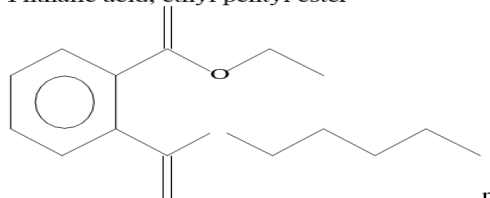
Phthalic acid, ethyl 3,4-dimethylphenyl ester



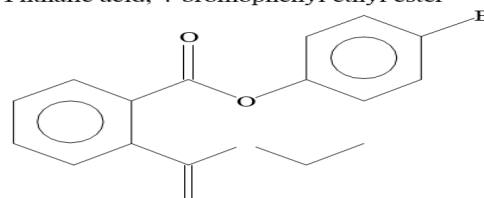
Phthalic acid, 2-acetylphenyl ethyl ester



Phthalic acid, ethyl pentyl ester



Phthalic acid, 4-bromophenyl ethyl ester



Anticancer Activity:

Cytotoxicity by MTT Assay:

Results summarized in Fig.3 & 5 showed the ethanol extract of callus have strong dose dependent inhibition against HCT-116 cell line with IC_{50} 60.64 μ g/ml while it showed mild toxicity only at highest concentration against normal WI-38 cell line (Fig.4). Further, past findings of Yahaya *et al.*, (2014) proved

that ethanolic leaves extract of *A. muricata* showed *in vitro* anticancer activity against EACC, MDA and SKBR3 tumor cell lines with IC₅₀ values of 335.85 µg/ml, 248.77 µg/ml and 202.33 µg/ml respectively.

Annexin V Apoptosis Assay by Flow Cytometry:

Table 2: Annexin V apoptosis assay:

Sr. No	Sample Name	% of Cells				Geometric mean fluorescence intensity (MFI) of AbFlour 488 Annexin V (FL1-A parameter)
		Live	Early Apoptotic	Late Apoptotic	Debris	
1.	Control (Untreated)	91.2	4.17	4.58	0.10	839
2.	Sample EEC - 60.64µg/ml (IC ₅₀)	65.9	20.3	13.6	0.30	2017

Results summarized in table 2 & Fig. 7 showed significant increase in early apoptotic cell population was observed in sample treated cells with increase in mean fluorescence intensity of green fluorescence as compared to the control cells (Untreated). Increase in the AbFlour 488-Annexin V positive and PI negative cells corresponds to the increase in the number of Early apoptotic cells while an increase in the AbFlour 488-Annexin V positive and PI positive cells corresponds to an increase in the Late apoptotic/Necrotic population. Cells with increase in only Propidium fluorescence are considered debris. The Annexin V AbFlour 488 fluorescence was collected in the FL1 detector using a 525nm band pass filter and Propidium Iodide fluorescence was collected in the FL3 detector using a 620nm band pass filter.

Conclusion

Ethanolic extract of callus IC50 60.64 µg/ml dosage has significant cytotoxic activity against HCT-116 without harming normal cell line WI-38. Further sample treated HCT-116 cell population followed the early apoptotic pathway as compared to the untreated control cells. Therefore, *A. muricata* callus extracted particles induced apoptosis in HCT-116 cell line and have a potential anticancer agent.

Abbreviations

AMCEP- *Annona muricata* callus extracted particles, PI-Propidium iodide

Declarations

-Ethical Approval: NA

-Conflict of Interest statement: We declare that we have no conflict of interest.

-Consent to Participate: All authors agree to be accountable for all work ensuring the integrity and accuracy.

-Author's contribution:

Experimental performance: Harshad Sanjay Tambe under the guidance of Dr. A. M. Bhosale and Dr. R. D. Borse

All authors read and approved the final manuscript.

Data availability: The MTT and Apoptotic assay data sets are available from Cellkraft Biotech Pvt. Ltd. Bangaluru while FTIR and GCHRMS analyzed from CIF, Pune and SAIF IIT Bombay upon reasonable request.

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Figures

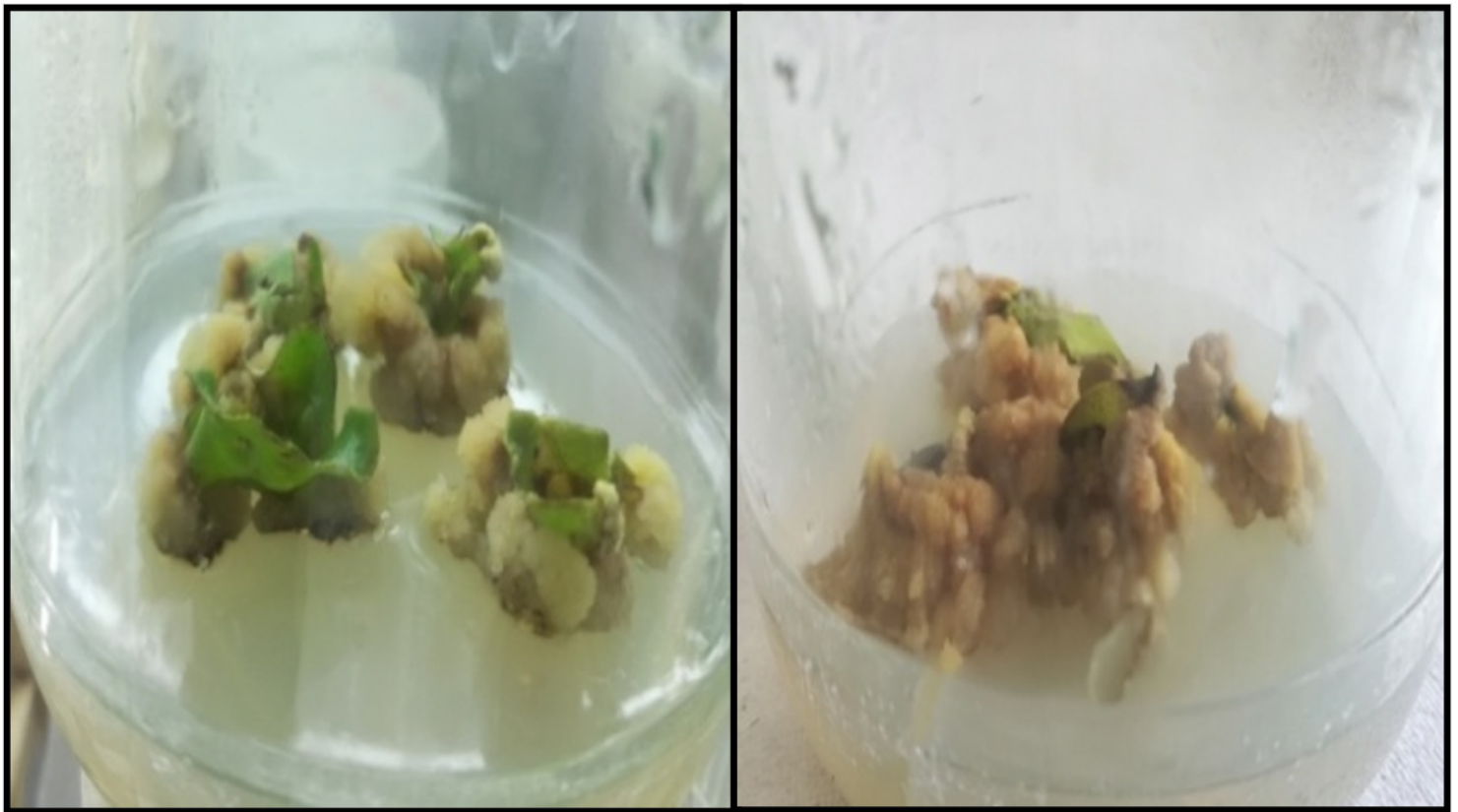


Figure 1

Annona muricata callus tissue

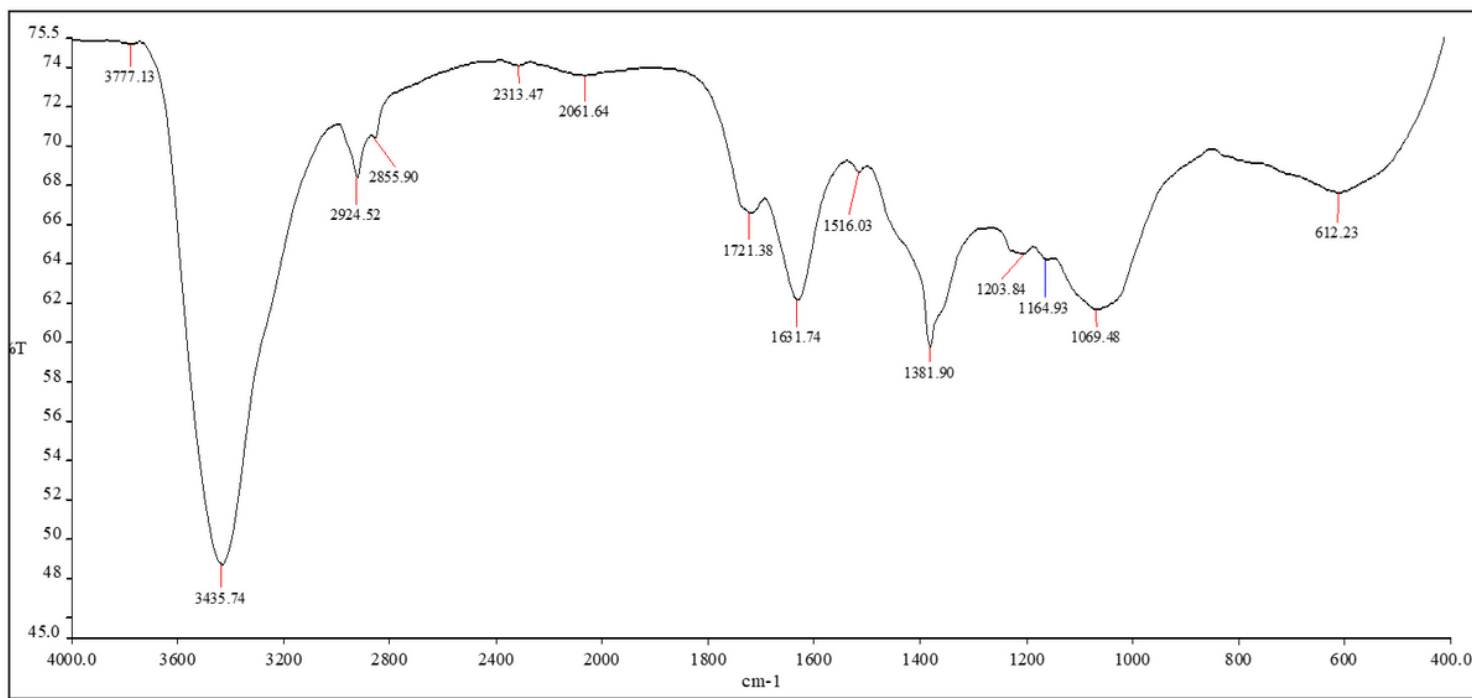


Figure 2

FTIR analysis of AMCEP

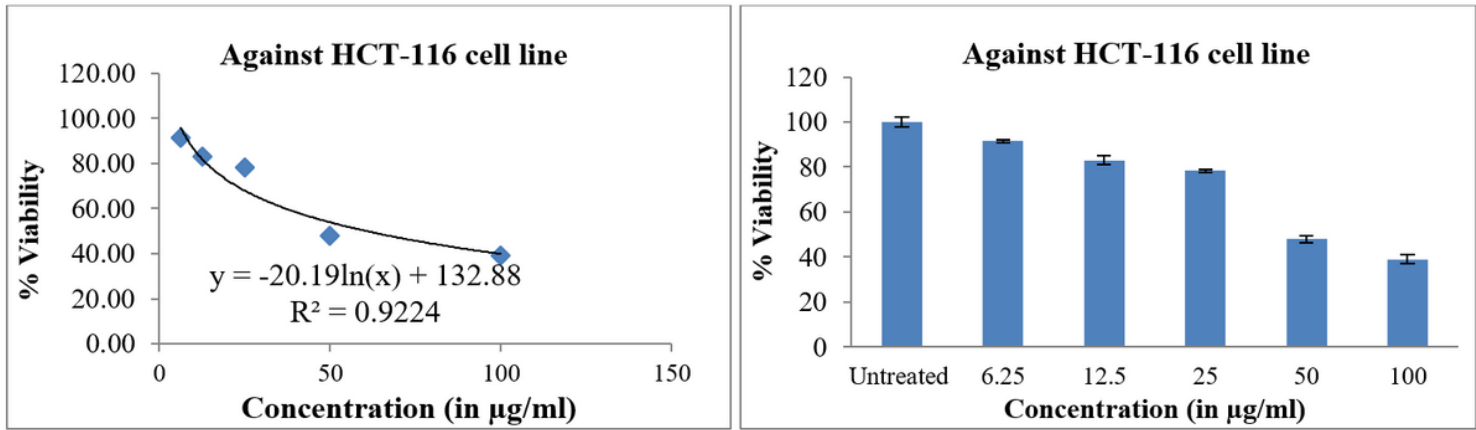


Figure 3

MTT Data Analysis against HCT-116 cell line

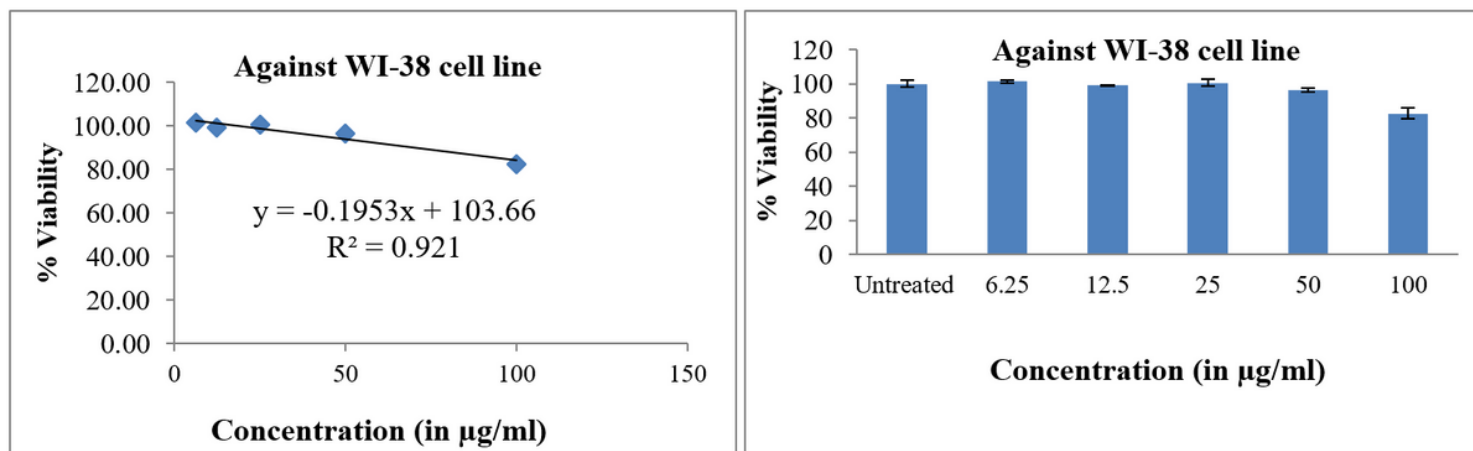


Figure 4

MTT Data Analysis against WI-38 cell line

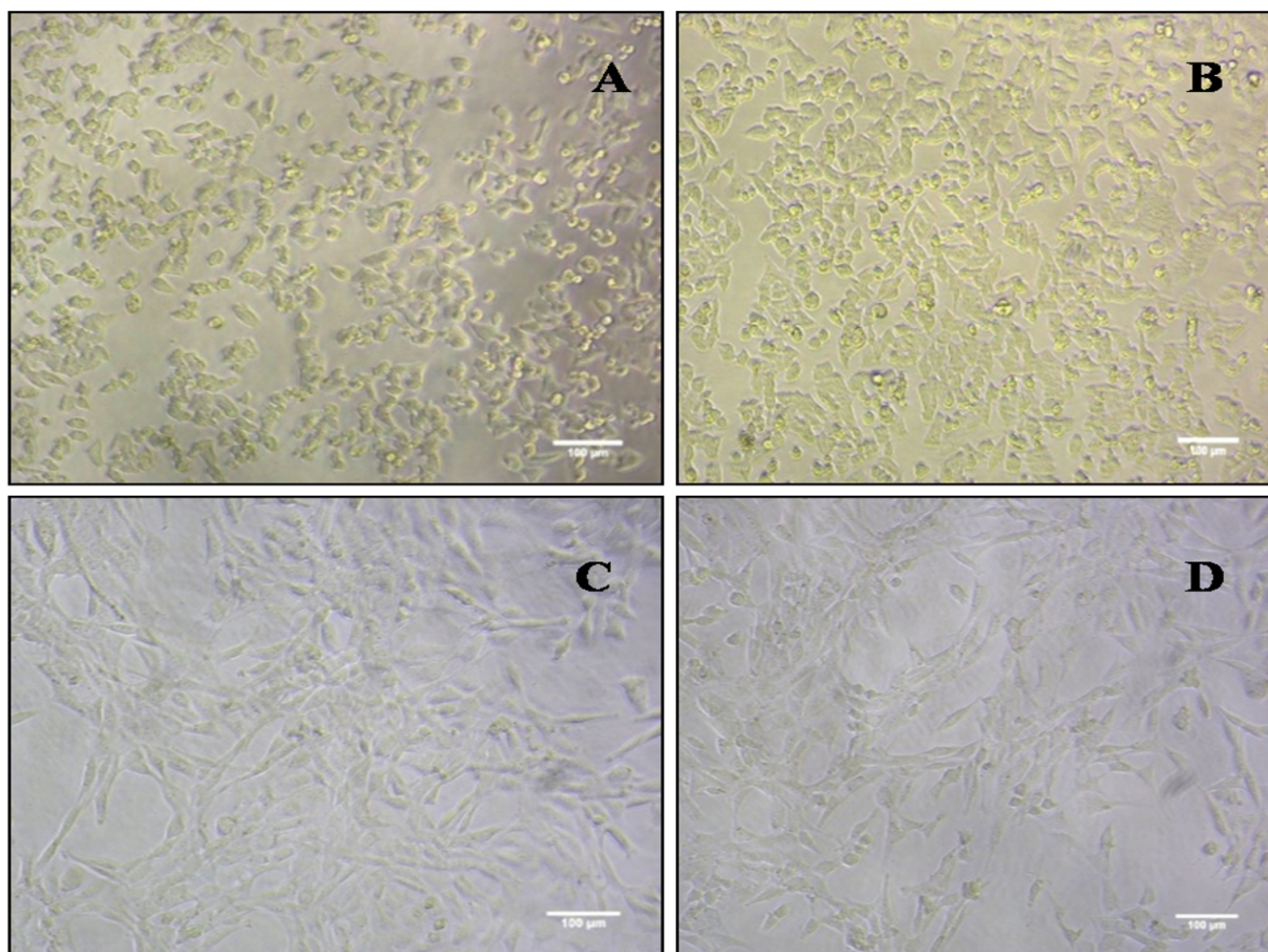


Figure 5

A: Treated HCT-116, B: Untreated HCT-116, C: Treated WI-38 and D: Untreated WI-38 Cell line

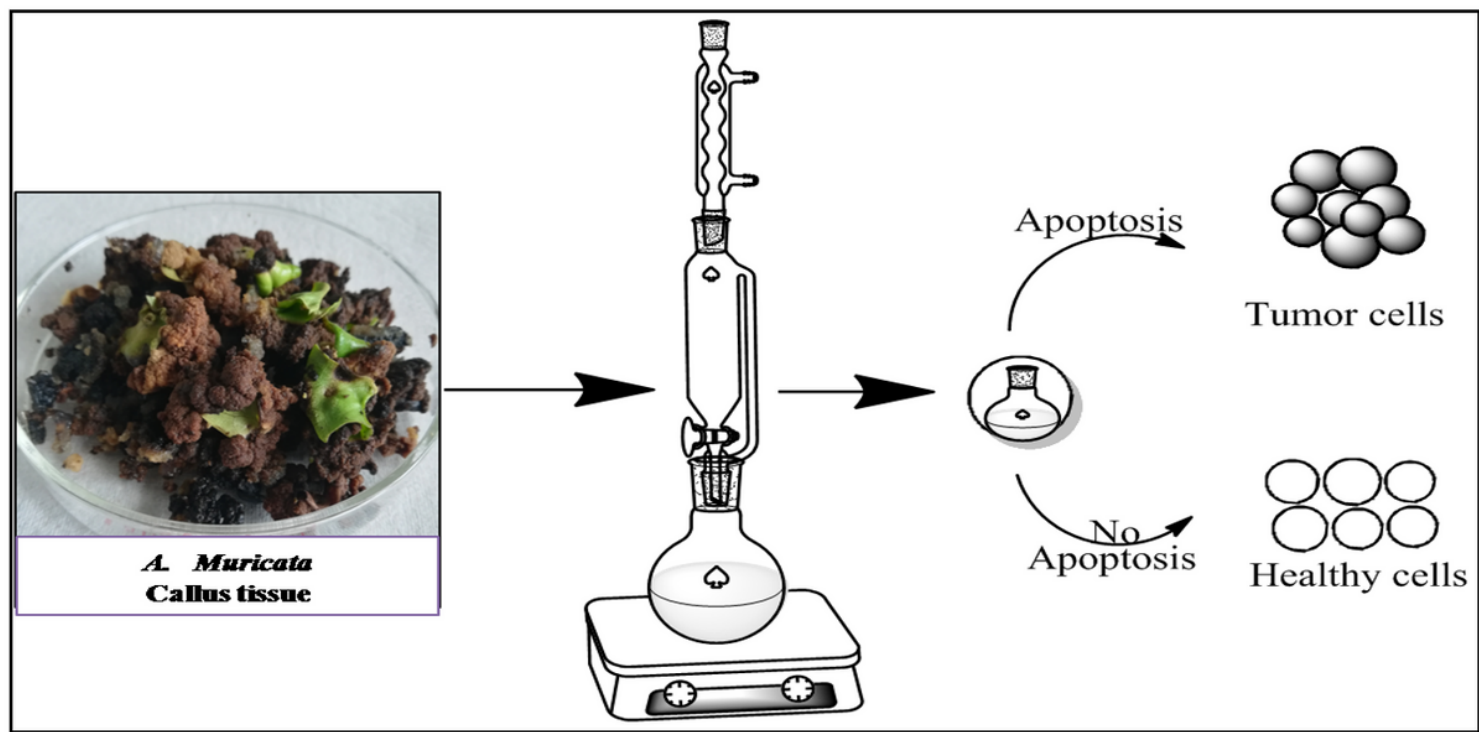


Figure 6

Workflow showing anticancer and apoptotic activity of AMCEP

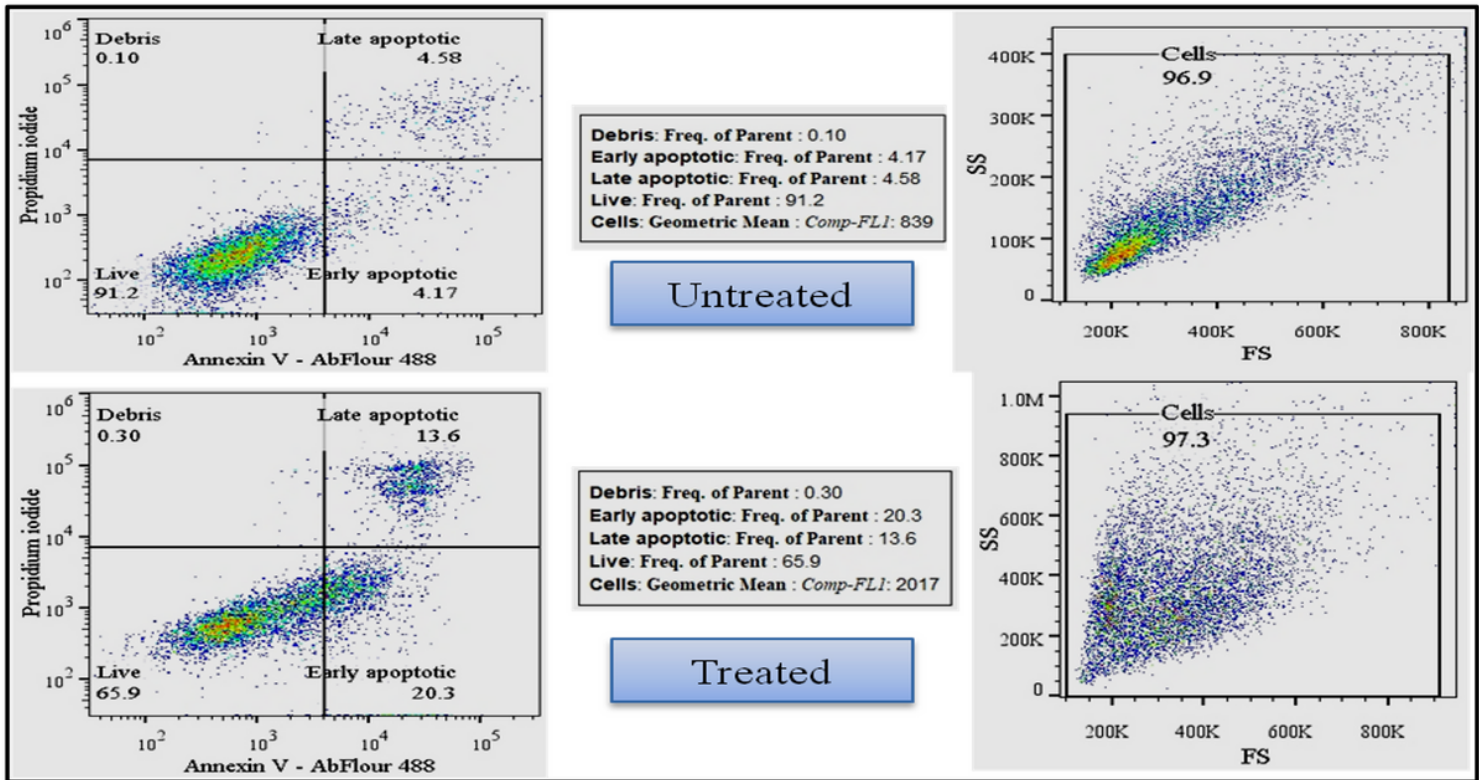


Figure 7

