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Characterization and Antileukemic Potential of *Annona muricata* Leaf Fractions on "MOLT-3" T-Cell Acute Lymphoblastic Leukemia Cells"

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Abstract

Results of this research demonstrate that leaf fractions of *Annona muricata* can cause the T-ALL cell line, molt-3, to undergo apoptosis. To find out what phytochemicals are in the *Annona muricata* leaf fraction (F4C), we used FTIR and GCMS in this investigation. Different functional groups were detected by FTIR findings, and different phytochemicals were detected by GCMS analysis in the F4C fraction. The cytotoxic efficacy of the F4C fraction against molt-3 cell lines were confirmed byMTT and SRB assays.The F4C treated cells terminated at the G0-G1 phase of the cell cycle, according to cell cycle analysis. It was determined that the substantial drop in MMP staining by JC-1 labelling corresponded to the loss of MMP. Anticancer efficacy against molt-3 cell line is thus demonstrated by *Annona muricata* leaf fractions.

Keywords - *Annona muricata*,T-ALL, G0-G1 arrest, MMP, JC-1 staining.

1. Introduction

The aggressive hematologic malignancy known asT-ALLis typified by the abnormal proliferation of immature thymocytes. Even though paediatric T-ALL patients have an 80% overall survival rate, 20% of them pass away from relapsed or refractory disease. Novel remedies are therefore desperately needed(Cordo *et al*., 2021).

Monotherapy or a mixture of radiotherapy and monotherapy is a typical component of conventional cancer treatments. On the other hand, the adverse effects of these methods have been well documented, which has led to the hunt for novel therapeutic medications.In light of this, the scientific community began searching unconventional natural sources, such as conventional plants, for anticancer chemicals. Many investigations have currently assessed the anticancer potential of naturally occurring plant-derived chemicals, both *in vitro* and *in vivo*(Oliveira *et al*., 2021).

Annona muricata L. planthas been used traditionally. Around the world, traditional medicine makes use of this tropical tree. This tropical tree's rich flavonoid, alkaloid, acetogenin, phenolic, and lipophilic chemical content is the basis for its traditional medicinal applications (Carmona *et al*., 2020).

Annonaceae is the family to which A. *muricata* belongs. Plants' leaves, stems, fruits, and roots contain acetogenin. Acetogenins are derivatives of fatty acids with 32–34 carbon chains that ends in 2-propanol, which at the conclusion of the structure generates methyl gamma lactones. Along the hydrocarbon chain, there are also one to three tetrahydrofuran (THF) rings with a range of hydroxyl, acetyl, and/or ketoxyl groups. The cytotoxic potential of acetogenin has been established, and *in vivo* investigations have shown the compound's effectiveness against a variety of pathogens, tumor, pesticides, helminths, viruses, and microbes. To achieve the greatest possible therapeutic effects, A. *muricata*'s active ingredient, acetogenin, also contains flavonoids. The anticancer effects of flavonoids are also well-established. Many plants that contain polyphenol compounds, like quercetin and flavone flavonoids, are regarded to have chemopreventive properties since they reduce the incidence of certain types of cancer.

Another kind of treatment is to utilize herbs to cause tumor cells to undergo apoptosis. In view of the finding that *Annona muricata* demonstrates anticancer potential, we looked into the FTIR and GCMS profiles of the plant's fractions. Our goal is to determine the identity and features of the bioactive compounds within the plant that give rise to the plant's

anticancer properties. To find out if the *Annona muricata* fractions can cause apoptosis in molt-3 cell line, we have also performed MTT, SRB,JC-1 and Propidium Iodide staining.

2. Materials and Methods

2.1 Preparation of sample

Fresh *Annona muricata*L. leaves were dried, and then ground into a fine powder using an appropriate mechanical blender. Fifty grams of the powder was then macerated in ninety-five percent ethanol for five days. A rotary evaporator was used to evaporate the ethanol, and the resulting sediment was then redissolved in acetone. The solution was filtered through silica gel 60 on filter paper using a Buchner funnel. To get the F1, F2, and F3 fractions, the crude extract was leached with H_2O, H_2O - EtOH (7:3 v/v), and H_2O - EtOH (1:1 v/v). Then, using a rotary evaporator, EtOH, EtOH -EtOAc (1:1 v/v), and EtOAc were added one after the other until fraction F4 was obtained (Ilango *et al*., 2022).

2.2 Fractionation using open column chromatography

A 30-cm column was used to fractionate the sample. After the F4 fraction was placed onto a silica gel 60 column chromatography, the fractions F1C, F2C, F3C, and F4C were obtained.Figure 1 listed the mobile phase solvents that were utilized as eluents, with silica gel 60 serving as the stationary phase. Samples that had been fractionated were lyophilized and kept at room temperature. After that, the samples were employed for the studies after being dissolved in DMSO (1 mg/ml)(Ilango *et al*., 2022).

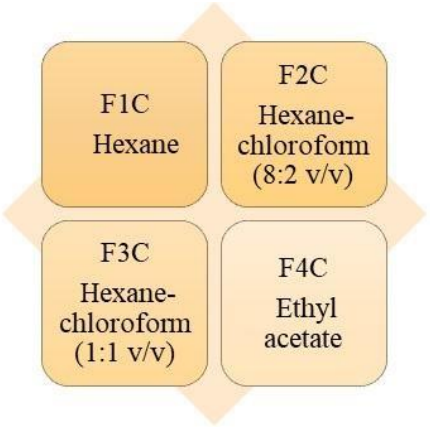


Figure 1: Eluents for obtaining fractions

2.3 Fourier transforms infrared spectroscopy (FTIR)

A Shimadzu (Miracle 10) FTIR instrument was used to analyse the fractions that were produced above, and data on infrared absorbance was gathered over a wave number range of 3750 cm-1 to 500 cm-1.

2.4 Gas chromatography mass spectrometry (GCMS)

A Schimadzu CH-GCMSMS-02 (GC-MS) equipment, was used to analyse the compounds present in plant extract. The compounds were identified using the GC-MS peaks and the corresponding chemicals' library data (NISP Search).

2.5 Culturing of molt-3 cell lines

The Molt-3 T-ALL cell line was provided by NCCS, Pune, India. It was cultured at 37°C in RPMI1640 media with 10% FBS. After determining the cell count and viability using a haemocytometer, cell viability assays and staining analysis were performed, respectively.

2.6 MTT assay

The MTT assay was carried out in accordance with Miyazawa and Igarashi (2001).

2.7 SRB assay

The SRB assay was carried out in accordance with Suganya Ilango *et al*., 2022.

2.8 JC-1 staining

The JC-1 staining was carried out in accordance with Kim *et al*., 2020.

2.9 Cell cycle analysis

The Cell cycle was carried out in accordance with Kim *et al*., 2020.

3. Results and Discussion

3.1 FTIR

The most widely utilized technique for determining chemical components and clarifying the structures of phytocompounds is FTIR. Because of its fingerprint properties, it plays a significant role in pharmaceutical research [6-11]. The results of FT-IR analysis confirmed the presence of C-Br, N–H, C–H, CO-O-CO, S=O, O-H, C=C, N=C=S, O–H, C-H, N-H and C-I functional groups (Figure 2 and Table 1). *Annona muricata* leaf extract was found to include = OH groups, = CH_2, CH alkenes groups, = CH_3 alkane groups, and = COC ester group(Daud *et al*., 2016).

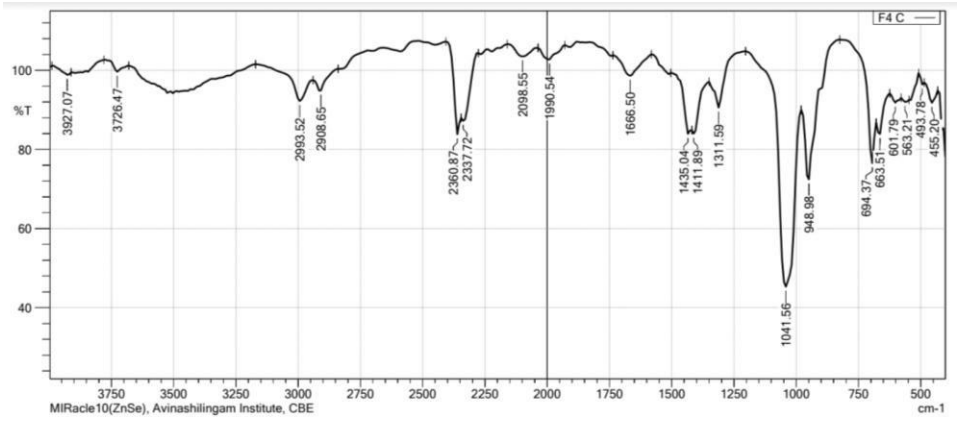


Figure-2: FTIR analysis

S.No.	**Wave numbers (cm^{-1})**	**Functional group**	**Class**
1	455.20	C–Br stretch	alkyl halides
2	493.78	C–Br stretch	alkyl halides
3	563.21	C-Br stretching	halo compound
4	601.79	C-Br stretching	halo compound
5	663.51	C-Br stretching	halo compound
6	694.37	N–H	amines
7	948.98	C–H	aromatics
8	1041.56	CO-O-CO stretching	anhydride
9	1311.59	S=O stretching	sulfone
10	1411.89	S=O stretching	sulfate
11	1435.04	O-H bending	carboxylic acid
12	1666.50	C=C stretching	alkene
13	1990.54	N=C=S stretching	isothiocyanate
14	2098.55	N=C=S stretching	isothiocyanate
15	2337.72	O–H stretch	carboxylic acids

16	2360.87	O–H stretch	carboxylic acids
17	2908.65	C-H stretching	alkane
18	2993.52	N-H stretching	amine salt
19	3726.47	O-H stretching	alcohol
20	3927.07	C-I	Alkyl halides

Table-1: FTIR functional group analysis of Annona muricata fraction – F4C

3.2 Gas chromatography mass spectrometry (GCMS)

GC, which separates the different components of chemical compound mixtures, and MS, which analyses the metabolites that the GC separates, are combined in the GC-MS technique for analytical compound separation. Drug metabolites, thermostable volatile chemicals, and active medicinal components can all be analysed using GC-MS (12 – 14). Table 2 lists the elements of the F4C fraction, and Figure 3 displays the fraction's GC-MS profile.

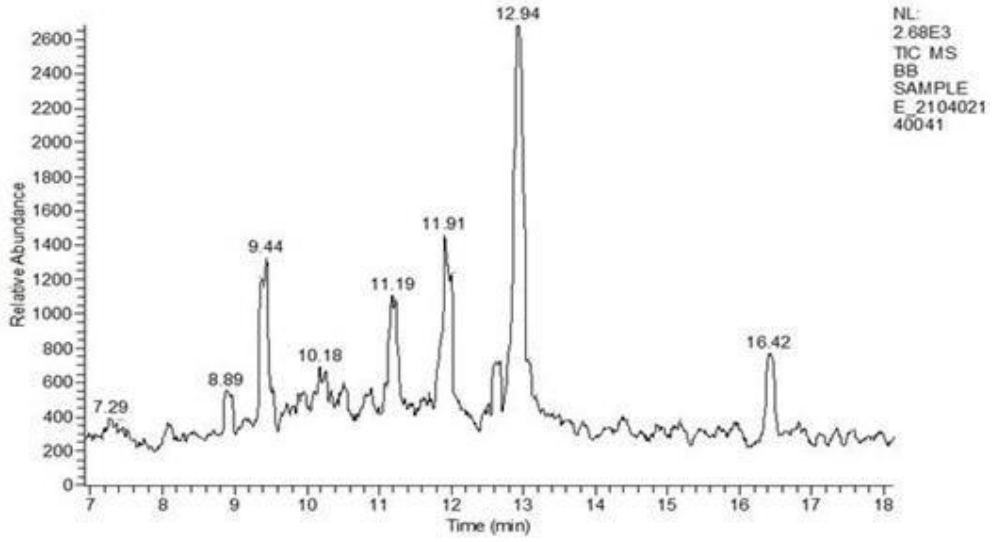


Figure-3: GCMS profile of F4C fraction

Apex RT	Start RT	End RT	Area	%Area	Height	%Height	Compound Name
7.29	7.16	7.48	1198.761	1.96	118.307	1.67	10-Oxodecanoic acid, methyl ester
8.89	8.84	8.99	1667.946	2.73	242.389	3.43	Pentadecanoic acid
9.44	9.30	9.56	7711.012	12.64	966.360	13.67	α-Gurjunene
10.18	10.05	10.30	2850.432	4.67	302.070	4.27	dodecanoic acid
11.19	11.06	11.32	5496.529	9.01	655.096	9.27	Phytol
11.91	11.76	12.21	9509.375	15.59	1026.307	14.52	pentadecanol
12.94	12.74	13.20	21415.233	35.10	2216.587	31.36	Hexadecatrienoic acid
16.42	16.31	16.54	3751.875	6.15	483.352	6.84	Oleyl alcohol

Table -2: Bioactive constituents identified in Ethanolic crude extract of Annona muricata leaves

3.3 MTT dye reduction assay

The findings indicated that the F4C fraction has the potential to kill cells; of the quantities tested, 500µg of the fraction shown the greatest cytotoxicity towards the molt-3 cell line, followed by 300µg, 200µg, 100µg, 50µg, and 25µg (Figure-4).

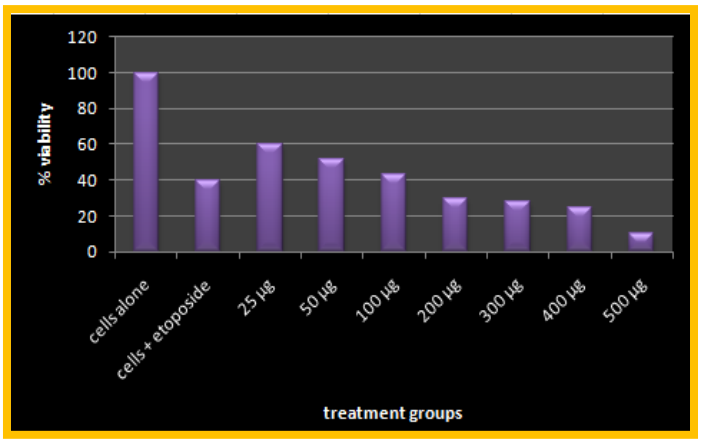


Figure-4: MTT Assay

3.4 Sulphorhodamine B assay

Similar outcomes were seen in the SRB assay, which demonstrated that the F4C fraction could cause cell death. Of the concentrations assessed, the F4C fraction at 500µg exhibited the best cytotoxicity towards the molt-3 cell line, followed by concentrations at 400µg, 300µg, 200µg, 100µg, 50µg, and 25µg (Figure-5).

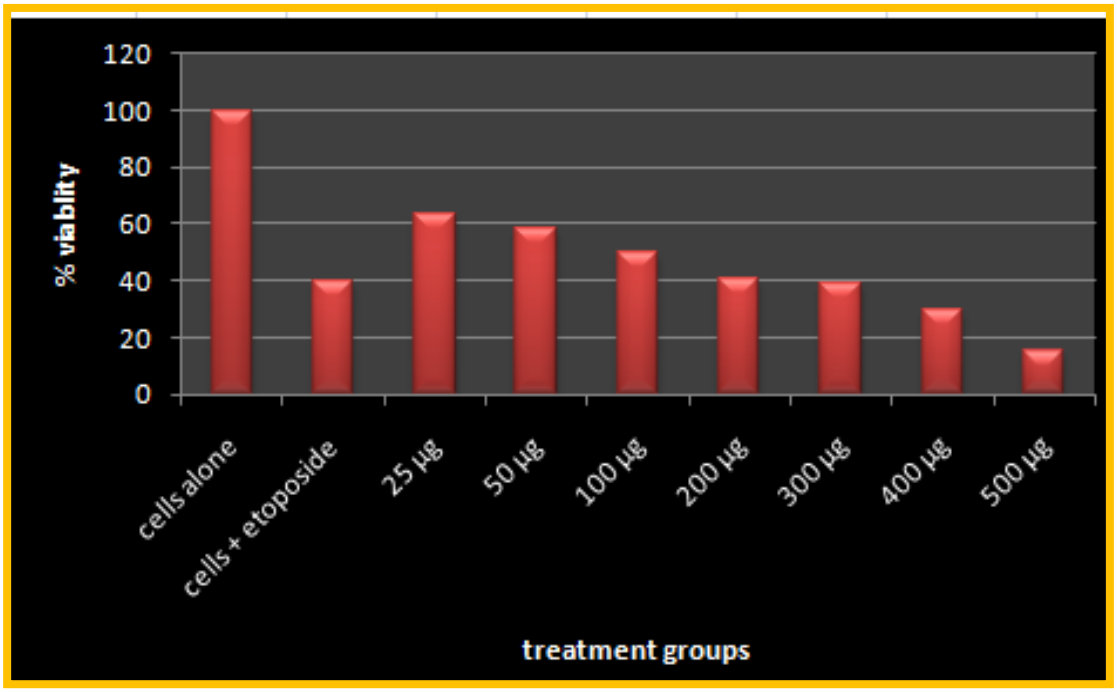


Figure-5: SRB Assay

3.5 JC-1 staining

The synthesis of ATP is mostly dependent on the mitochondria, an essential organelle that is also involved in the apoptotic process. Normal cells will have more J-aggregates; this will vary when the MMP changes. The initial step in the onset of apoptosis is depolarization.There are less J-aggregates and more monomers formed as a result of membrane depolarization. The F4C fraction was shown to be more effective in inducing apoptosis, as evidenced by the higher proportion of monomersin the treated group (Figure-6).

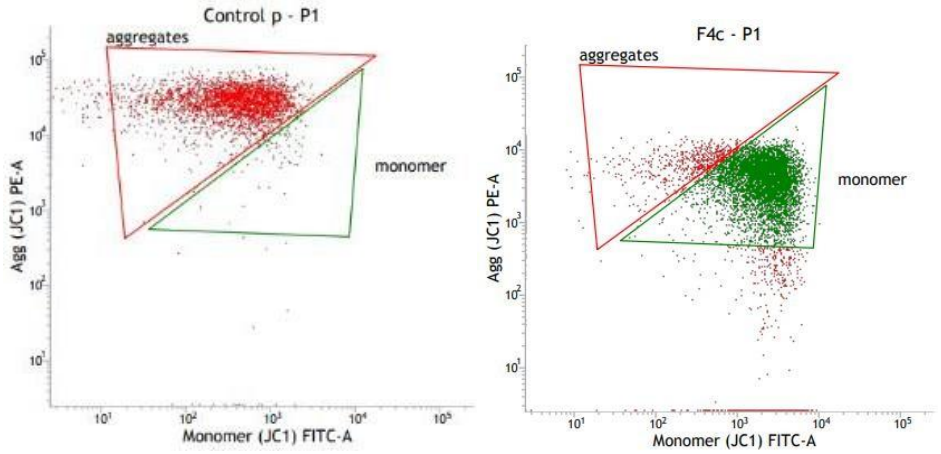
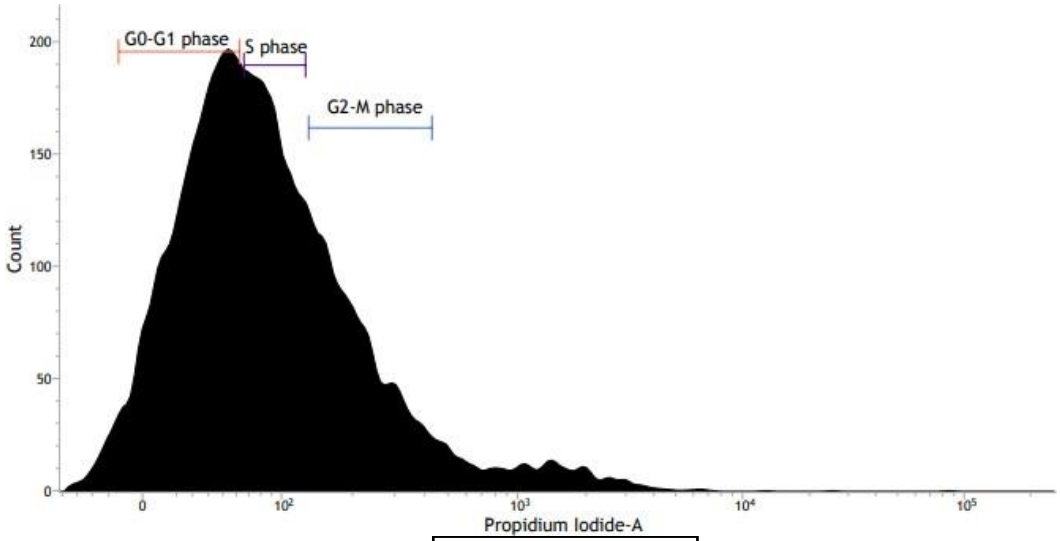


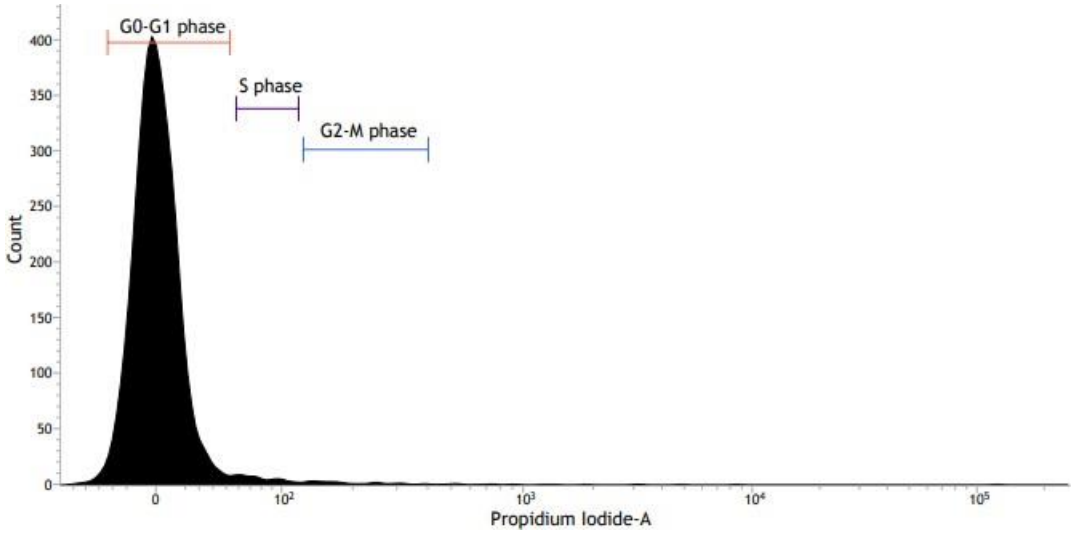
Figure -6: MMP by flow cytometry

3.6 Cell cycle analysis

There had been no cell cycle arrest since the untreated molt-3 cells (Control) were evenly dispersed throughout the cell cycle (Figure 7a). Our results were confirmed by the distribution of cells that underwent apoptosis-mediated cell death upon exposure toF4C fraction. The distribution of cells revealed that they were arrested at G0-G1 phase (Figure 7b).



a) Control



b) F4C

Figure-7: Cell cycle analysis

4. Discussion

The MTT assay results showed unequivocally that the extract concentration of 50 µg/mL for non-polar to polar solvents had significantly reduced the absorbance when compared to the control. This was reflected in the compromised cell viability because of these *Olea ferruginea Roylebark* extracts' cytotoxic potential (Liaquat *et al*., 2021).The results of the SRB experiment demonstrated that the leaf extract of *Clinacanthus nutans* induced substantial antiproliferative properties (Toha *et al*., 2022).Duration of incubation with *Annona muricata* leaf extract increased, flow cytometry analysis revealed a rise in the proportion of Jurkat cells with reduced MMP (Liliana *et al*., 2021).The capacity of *Annona muricata* leaf methanol extracts to cause apoptosis and/or terminate the cell cycle was studied in an attempt to identify a possible tactic for limiting or preventing the development of MCF-7 cells (Mahmood *et al*., 2022).

5. Conclusion

According to the research, *Annona muricata* fractions may be utilized to develop more effective anticancer medications to fight the Molt-3 cell line.

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