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METHODOLOGY

Herbal plants have traditional origin of both curative and preventive medicinal healing preparations for humans, as well as a source of key bioactive components for extraction. A medicinal plant is made up of numerous key elements that can be used in the medical community and used in the production of various medications. Treatment with the medicinal plant is thought to be quite safe, with no or minor adverse effects. Traditional medications are typically less expensive than modern drugs (Dixit, 2021).

Phytochemicals (from the Greek word "phyto," which means "plant") are functionally active chemical substances found naturally in plants that have health benefits for humans. Phytochemicals are thought to acquire a promising function in the determent and healing of many human diseases because of this feature (Farishta and Sharma, 2021).

The present study aims to isolate and identify the flavonoid and acetogenin rich fractions from *Annona muricata* leaves and to investigate the antioxidant activity with the focus on the anticancer activity of the *Annona muricata* ethanolic leaf extract and its chromatographically separated fractions.

The present research was categorised into four phases. In I phase, an attempt was made to isolate and identify the flavonoid and acetogenin rich fractions from *Annona muricata* leaves using various chromatographic and spectral techniques. The second phase was designed to analyse the antioxidant activity of *Annona muricata* ethanolic leaf extract and its chromatographically separated fractions. In phase III, assessment of synergistic effect of flavonoid and acetogenin enriched fractions of *Annona muricata* leaves and assessment of anticancer activity of *Annona muricata* ethanolic leaf extract and its chromatographically separated fractions in acute lymphoblastic leukemia cell line Molt-3 which was compared with the normal counterpart Peripheral Blood Lymphocytes (PBL). In the final phase, docking studies were carried out to find the interactions between the compounds obtained from the GCMS analysis and apoptotic, cancer, and leukemic targets using Schrodinger software version. The methodology adopted for the study entitled “Synergistic effect and apoptosis induction of flavonoid and acetogenin enriched fractions of *Annona muricata* leaves in Molt-3 cells and Peripheral Blood Lymphocytes” are explained

with the details of the experimental conditions and steps of the procedures employed to study the various parameters are present in this chapter.

Authentication of Plant Material

The plant sample (leaves) of *Annona muricata* was collected from local areas of Coimbatore, Tamil Nadu, India. The leaves were authenticated by the Head, Botanical Survey of India, Coimbatore (BSI/SRC/5/23/2019/Tech/192).

Phase I

Bioassay guided fractionation

Annona muricata leaf powder was macerated in 95% ethanol for 5 days. Using a rotary evaporator, ethanol was evaporated and the sludge was redissolved in acetone for reducing the levels of chlorophyll. The solution was filtered by using a Buchner funnel with silica gel 60 on a filter paper. A total of four fractions F1, F2 and F3 fractions were obtained by using the solvents water, water-ethanol (7:3 v/v), and water-ethanol (1:1 v/v) to leach the solid crude extract. Then ethanol, ethanol-ethyl acetate (1:1 v/v), and ethyl acetate were used consecutively and combined to obtain fraction F4. F4 fraction were tested for flavonoids, and the results revealed the presence of flavonoids, were concentrated at 50ºC and labelled as Flavonoid Enriched Fraction, and used for experimental studies.

Figure 11: Flavonoid enriched fractions from *Annona muricata* leaves

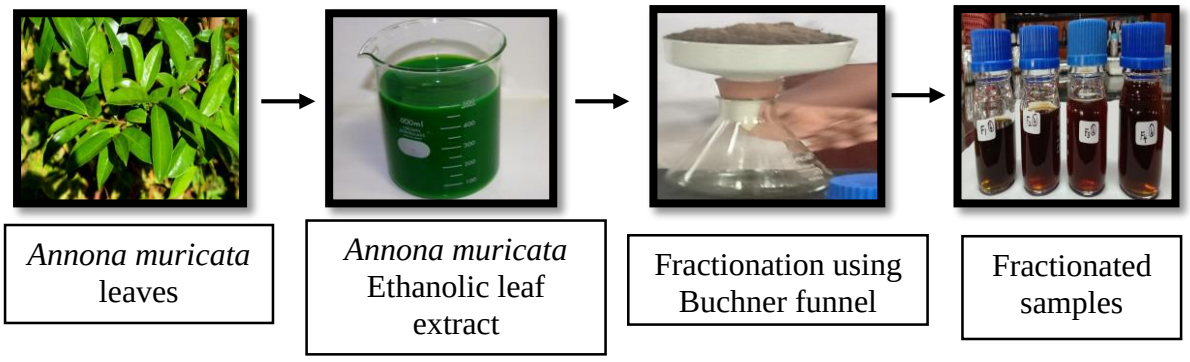
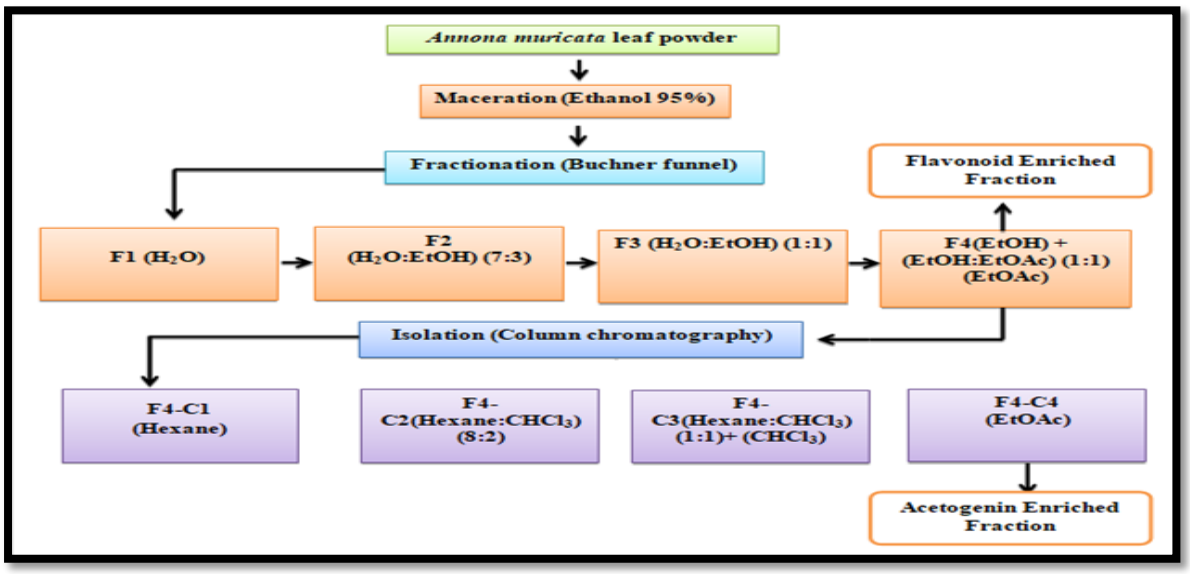
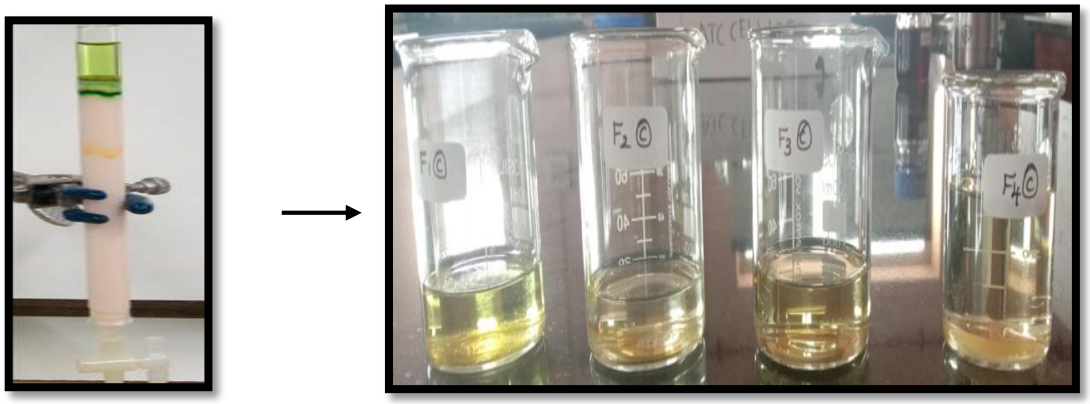


Figure 12: Bioassay guided fractionation of flavonoid and acetogenin rich fractions from *Annona muricata* leaves (Mulia *et al*., 2015)



The sample was fractionated using a 50cm column. Hexane, hexane-chloroform (8:2 v/v), hexane-chloroform (1:1 v/v), and ethyl acetate were employed as eluents in an open silica gel column chromatography of the F4 fraction. Eluted F4C fraction were tested for annonaceous acetogenin, fraction tested positive with kedde's reagents were concentrated at 50ºC and labelled as Acetogenin Enriched Fraction, and used for experimental studies.

Figure 13: Isolation of Acetogenin enriched fractions from *Annona muricata* leaves



Isolation using open silica gel chromatography

Eluted Fractions

UV-Visible spectral analysis

An absorption spectral analysis was done for the ethanolic crude extract and flavonoid and acetogenin enriched fractions of *Annona muricata* leaves in a biospec-nano (Shimadzu). The equipment was set to mode of operation, and the absorption spectra was collected from 200 to 800nm.

FT-IR Spectral Analysis

Ethanolic crude extract, flavonoid enriched fraction and acetogenin enriched fraction of *Annona muricata* leaves were analysed with a Shimadzu (Miracle 10) FTIR instrument, and infrared absorbance data was collected over a wave number range of 3600 cm^{-1} to 600 cm^{-1}.

HPTLC analysis

HPTLC fingerprinting profiling of ethanolic crude extract, flavonoid enriched fraction and acetogenin enriched fraction of *Annona muricata* leaves was performed by following the procedure as described in Appendix I.

HPLC analysis

HPLC analysis of ethanolic crude extract, flavonoid enriched fraction and acetogenin enriched fraction of *Annona muricata* leaves was performed by following the procedure as described in Appendix II.

GCMS analysis

GCMS analysis of ethanolic crude extract, flavonoid enriched fraction and acetogenin enriched fraction of *Annona muricata* leaves was performed by following the procedure as described in Appendix III.

Phase II

Oxidants are formed as intermediates of cellular redox processes that produce adenosine triphosphate (ATP) to provide energy for cells using oxygen. ROS and RNS are useful chemicals for cellular processes and immunological responses when they are in balance, but when their concentrations are out of balance, they create oxidative stress, which can lead to chronic and degenerative diseases. Plant secondary metabolites, such as phenolic compounds and flavonoids, which are usually known as the major phytochemical molecules

with antioxidant characteristics from plants, have long been reported as a potential hub of natural antioxidant chemicals in medicinal plants (Tungmunnithum *et al*., 2018).

DPPH radical scavenging assay

The method of Mensor *et al*. (2001) was used to measure DPPH radical scavenging activity, which is detailed in Appendix IV.

ABTS radical scavenging assay

The radical scavenging activity of ABTS was determined using the method given in Appendix V by Shirwaikar *et al*. (2006).

Hydrogen peroxide scavenging activity

The hydrogen peroxide scavenging activity was evaluated using the method given in Appendix VI by Ruch *et al*. (1989).

Hydroxyl radical scavenging activity

The hydroxyl radical scavenging activity was determined using the method described in Appendix VII by Klein *et al*. (1991).

Determination of inhibition of nitric oxide generation

The nitric oxide production inhibition assay was performed using the method described in Appendix VIII by Green *et al*. (1982).

Determination of inhibition of Superoxide generation

The method of Winterbourn *et al*. (1975), as detailed in Appendix IX, was used to evaluate the inhibition of superoxide production.

Reducing power ability

The capacity of the reducing power assay was determined using the method given in Appendix X by Yildrim *et al*. (2001).

Chelating activity

Chelating activity was tested by the method of Hegazy and Ibrahium (2012) as given in Appendix XI.

Phase III

Carcinoma is the second major cause of death in the globe, and because of this, the development of new anticancer medications is critical. Plant-based research has made significant progress in the last century, with the invention of numerous chemicals that are now employed in cancer treatment procedures (Majolo *et al*., 2019).

In this research, we looked into the anticancer action of the ethanolic crude extract, flavonoid and acetogenin enriched fractions of *Annona muricata* leaves in Molt-3 leukemic cell line was elucidated.

Culturing of Molt-3 cell line

National Centre for Cell Science (NCCS), Pune, India, provided the Molt-3 (T-cell acute lymphoblastic leukemic cell line). The cells were incubated at 37˚C and cultured in RPMI 1640 media supplemented with 10% FBS, 0.5 % penicillin and streptomycin, and 1mM sodium pyruvate.

Culturing of Peripheral Blood lymphocyte (PBL)

Fresh blood was drawn from healthy individual by vein puncture under aseptic conditions, using a heparinized syringe. The blood was diluted with sterile PBS in the ratio 1:1. The diluted blood was added along the side of the centrifuge tube containing sterile lymphosep in the ratio 1:2. Then, it was carefully spun at 400g for 30 minutes at 18-20°C. After centrifugation, the lymphocytes which has formed the grey coloured layer was carefully separated without disturbing the blood plasma and separation medium. The lymphocyte suspension was washed thrice with PBS. The pellet was resuspended in the RPMI 1640 medium supplemented with 10% FBS, 0.5% penicillin-streptomycin and phytohemagglutinin.

For 10 minutes, the mixture containing Molt-3 cells and PBL was spun at 1500rpm. The pellet was resuspended in l ml of media after the supernatant was discarded. Cell viability was determined using the trypan blue exclusion test on a haemocytometer, and 10^6 cells were seeded into a 96-well plate for further assays.

The optimal dose was identified by treating Molt-3 cell line with different concentrations of extract and fractions (10 to 150µg) for 24 hours based on the literatures available. Followingly, the anticancer action of the *Annona muricata* ethanolic leaf extracts

and chromatographically separated fractions were evaluated by MTT, SRB assay, and flow cytometric analysis.

Treatment groups

1. Cells alone (Molt-3)
2. Molt-3 Cells + Ethanolic crude extract
3. Molt-3 Cells + Flavonoid Enriched Fraction (FEF)
4. Molt-3 Cells + Acetogenin Enriched Fraction (AEF)
5. Molt-3 Cells + Combination of Flavonoid and Acetogenin Enriched Fraction (FEF + AEF)
6. Cells alone (PBL)
7. PBL + Ethanolic crude extract
8. PBL + Flavonoid Enriched Fraction (FEF)
9. PBL + Acetogenin Enriched Fraction (AEF)
10. PBL + Combination of Flavonoid and Acetogenin Enriched Fraction (FEF + AEF)

Cell viability assays

In order to determine the optimal dose and cytotoxic effect of the ethanolic extract, flavonoid enriched fraction, acetogenin enriched fraction, combination of flavonoid and acetogenin enriched fraction of *Annona muricata* leaves, MTT and SRB assay was performed.

MTT dye reduction assay

The decrease of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was used to assess cell viability, as detailed in Appendix XII by Igarashi and Miyazawa (2001).

Sulphorhodamine B assay

The SRB test, described by Skehan *et al*. (1990) and given in Appendix XIII, was used to investigate the cytotoxic effect of the extracts and fractions on the Molt-3 cell line and PBL.

Analysis of synergistic effect using combenefit software

Synergistic pharmaceutical product interactions could potentially cut dosages and treatment periods for undesirable side effects. In these process, pharmaceutical products are

evaluated using concentrations in defined or nonfixed ratios, the latter of which can be expanded into matrix or factorial arrays. The results of analysing drug combinations taken concurrently or sequentially may generate inconsistent results, but they may reveal the molecular nature of drug interactions. (Perez *et al*., 2018). Synergistic effect of flavonoid and acetogenin enriched fractions was analysed using combenefit software. The procedure is outlined in Appendix XIV.

Cell cycle analysis by flow cytometry

Cell cycle analysis was performed using flow cytometry (BD FACSverse) as per the protocol described by Krishan (1975). The effect of ethanolic crude extract, flavonoid enriched fraction and acetogenin enriched fraction of *Annona muricata* leaves on the cell cycle distribution of Molt-3 cells and PBL were examined using flow cytometer kit method as given in Appendix XV.

Detection of cell death by Annexin V/FITC- PI apoptosis staining by flow cytometery analysis

The magnitude of apoptosis elucidated by ethanolic crude extract, flavonoid enriched fraction and acetogenin enriched fraction of *Annona muricata* leaves was determined using Annexin V/FITC Apoptosis detection kit (BD Biosciences) as given in the Appendix XVI.

Detection of mitochondrial membrane potential (ΔΨm) by – JC-1 staining

The mitochondrial membrane potential of ethanolic crude extract, flavonoid enriched fraction and acetogenin enriched fraction of *Annona muricata* leaves was determined by JC-1 staining kit procedure as given in the Appendix XVII.

Measurement of ROS using flow cytometry

The oxidative damage in intact cells was assessed with the cell permeable dye 2', 7'-dichlorofluorescein diacetate (DCF-DA) using the kit protocol described in Appendix XVIII.

Phase IV

Molecular docking is a approach to anticipate how a molecule will interact with another when they are linked together to create a stable complex. In order to confirm the cytotoxicity results obtained using Molt-3 cell lines under *in vitro*, and also to understand the possible mechanism of action involved in the apoptosis inducing property of *Annona muricata* leaf extracts, molecular docking studies were done. Thus, phase IV was

designed to predict drug likeness properties and to analyse the interactions between the protein and a ligand which was performed using Schrödinger software version 9.0. The procedure is outlined in Appendix XIX.

Data analysis

For phase I and II results, statistical analysis was performed using one-way ANOVA followed by post hoc Tukey's multiple comparison tests using Graph pad prism 8.4.1 (Trial version) to determine the significance of the standard and treatment groups. The $P \leq 0.0001$ was considered statistically significant.