4

RESULTS AND DISCUSSION

Cancer is one of the most frequent and fatal diseases, and it is still one of the major causes of mortality around the world. Because of the high mortality and occurrence, it is a significant public health and economic issue that necessitates comprehensive prevention. The most prevalent methods for treating cancer are radiotherapy, immunotherapy, and chemotherapy, however these approaches have a negative impact on healthy cells. As a result, the restriction of harmful behaviour to healthy tissues with regularly used medications pushes researchers to look for new cancer treatments that are both safe and effective. Chemoprevention is a revolutionary strategy to cancer control that involves using certain natural or synthetic medicines to suppress, prevent, or reverse cancer before it becomes aggressive. Natural products, such as medicinal plants, herbs, and their phytocompounds, such as flavonoids, alkaloids, and carotenoids, have been shown to have anti-cancer properties (Roy *et al*., 2018).

Plants like *Annona muricata* and others have been demonstrated to have promising phytocompounds that can be used to cure cancer. A. *muricata* plant extracts, which are native to tropical and subtropical regions of the world, contain chemicals that are highly eminent against cancerous cells (Yajid *et al*., 2018). *Annona muricata* has been found to contain 212 bioactive chemicals, according to the published reports. Acetogenins are the most abundant chemicals, followed by alkaloids, phenols, and other substances (Tellez *et al*., 2018).

Acetogenins ability to inhibit NADH oxidase has also been proven to be significant for their anti-tumour activity and also been demonstrated to inhibit ATP generation in mitochondrion. This type of activity has been demonstrated to be successful against cancerous cells that produce more ATP than normal cells, therefore restricting cancer cell's ability to grow. Acetogenin toxicity was only seen in cancer cells, with only a minor unfavourable influence on normal cells (Yajid *et al*., 2018).

Annona muricata extracts proved to have therapeutic potential. On a variety of cancer cell lines, studies have connected that *Annona muricata* derived chemicals possess cytotoxic action, apoptosis induction, eventual cell death, and suppression of proliferation. Furthermore, plant parts are employed as natural medicines, including the bark, fruit, leaves, root, and seeds.

The current study aims at a comprehensive investigation of the anticancer activity of *Annona muricata* against Acute lymphoblastic leukemia cell line (Molt-3) and its normal counterpart Peripheral Blood Lymphocytes (PBL). In this context, the present study focuses to isolate the flavonoid and acetogenin enriched fraction from the *Annona muricata* leaves, followed by the assessment of antioxidant and anticancer activity.

Phase I

4.1. Characterization of Flavonoid (FEF) and Acetogenin (AEF) enriched fraction from *Annona muricata* leaves

4.1.1. UV-Visible absorption spectral analysis

4.1.2. Fourier Transform Infrared spectroscopy analysis

4.1.3. High Performance Thin Layer Chromatographic analysis

4.1.4. High Performance Liquid Chromatographic analysis

4.1.5. Gas Chromatography-Mass Spectrometry analysis

Phase II

4.2. Assessment of Antioxidant potential of Ethanolic crude extract, Flavonoid (FEF), Acetogenin (AEF) enriched fraction and combination of FEF and AEF of *Annona muricata* leaves

4.2.1. Assessment of radical scavenging activity

4.2.1.1. DPPH radical scavenging assay

4.2.1.2. ABTS radical scavenging assay

4.2.1.3. Hydrogen peroxide radical scavenging assay

4.2.1.4. Hydroxyl radical scavenging assay

4.2.2. Assessment of radical generation inhibition assay

4.2.2.2. Inhibition of nitric oxide generation assay

4.2.2.3. Inhibition of superoxide generation assay

4.2.3. Assessment of Chelating activity

4.2.4. Assessment of Reducing power ability

Phase III

4.3. Synergistic effect and assessment of anticancer activity of Ethanolic crude extract, Flavonoid (FEF), Acetogenin (AEF) enriched fraction and combination of FEF and AEF of *Annona muricata* leaves against MOLT -3 and PBL cells

4.3.1. Cytotoxic activity

4.3.1.1. MTT dye reduction assay

4.3.1.2. Sulphorhodamine B assay

4.3.2. Analysis of synergistic effect using combenefit software

4.3.3. Measurement of apoptosis

4.3.3.1. Annexin V/FITC staining

4.3.3.2. Jc-1 staining

4.3.4. Antiproliferative activity

4.3.4.1. Cell cycle analysis

4.3.5. Assessment of ROS level

Phase IV

4.4. *In silico* docking of apoptotic and leukemia targets with Flavonoid (FEF) and Acetogenin enriched fraction (AEF) phytoconstituents

4.4.1. ADME analysis

4.4.2. Molecular docking

Phase I

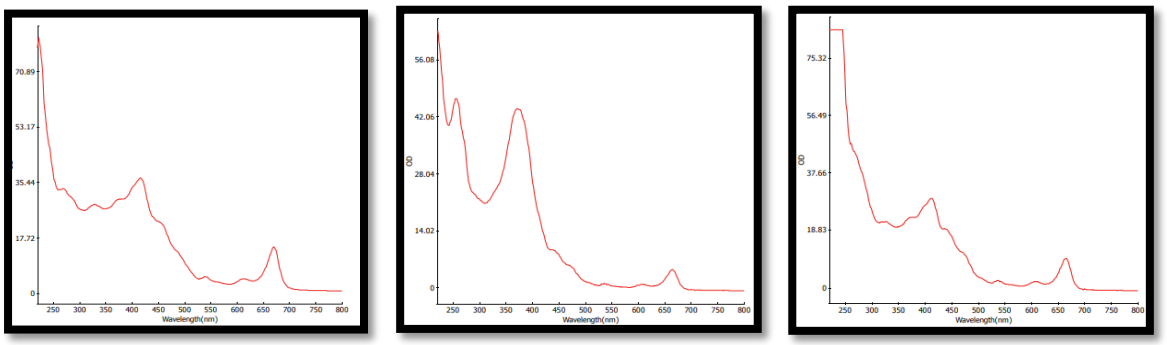
4.1. Characterization of Flavonoid (FEF) and Acetogenin (AEF) enriched fraction from *Annona muricata* leaves

The objective of this first phase of the study was to obtain a simple and practical separation technique for flavonoids and acetogenins, from *Annona muricata* leaves. In order to isolate flavonoids and acetogenins, various solvents were used for the bioassay guided fractionation and various spectral studies were employed to determine the presence of flavonoids and acetogenins in the obtained fractions from the bioassay guided fractionation method.

4.1.1. UV-Visible absorption spectral analysis

The existence of chromophoric groups and aromatic rings in plant samples was determined using UV-Vis spectrophotometry. The unique absorption spectrum found in the UV-Vis peak area indicated the presence of alkaloids, flavonoids, phenolic acids, and tannins in plant extracts.

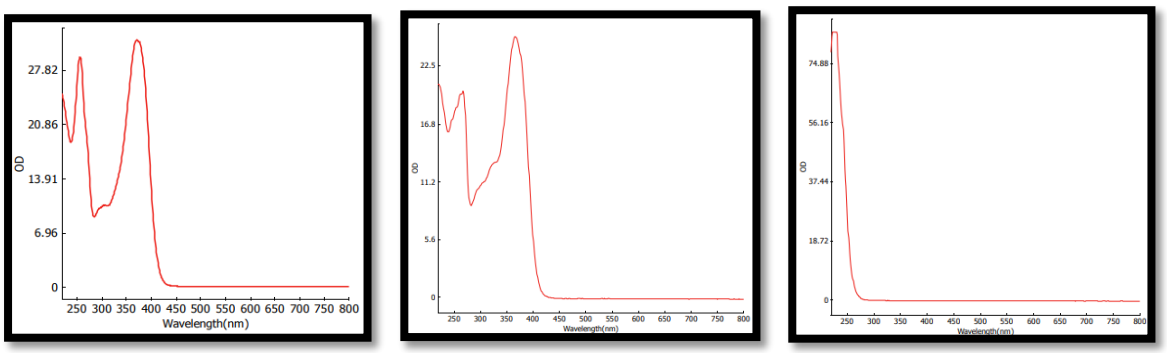
Figure 14: UV-Visible absorption spectra of Ethanolic crude extract, Flavonoid (FEF) and Acetogenin (AEF) Enriched fraction of *Annona muricata* leaves



Ethanolic crude extract **FEF** **AEF**

The presence of various phytochemicals present in the extract was identified using UV-Visible spectroscopy at a wavelength between 250 to 800nm. Distinct peaks were observed in Ethanolic crude extract at 420nm and 670nm, Flavonoid Enriched Fraction showed peaks at 257nm, 370nm, 670nm, Acetogenin Enriched Fraction showed peaks at 250nm, 410nm, 660nm respectively. The absorption spectrums are presented in figure 14.

Figure15: UV-Visible absorption spectra of Quercetin, Kaempferol and Andrographolide



Quercetin **Kaempferol** **Andrographolide**

Quercetin showed peaks at 260nm and 370nm, Kaempferol showed peaks at 265nm, 365nm and Andrographolide showed peaks at 250nm respectively, absorption spectrums are presented in figure 15.

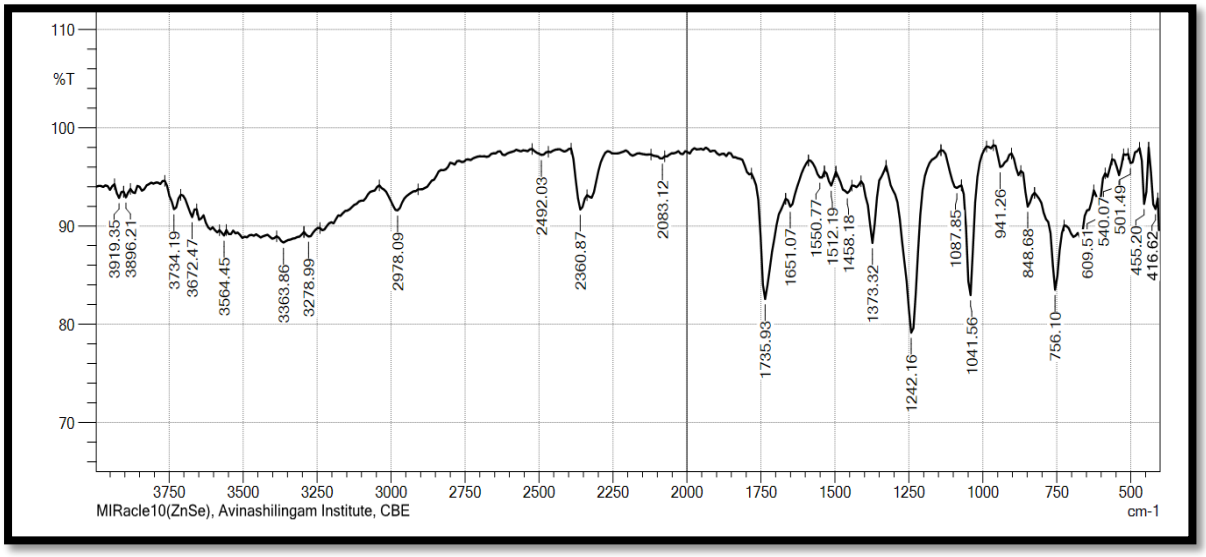
Patle *et al*. (2020) found absorption spectra in the range of 234-676nm in hexane, chloroform, and ethanol extracts of *Dillenia pentagyna* leaves and fruits, showing the presence of polyphenolic compounds, flavonoids, tetraterpenoids, tannins and isoquinoline alkaloids. Then peak occurrence at 234-676nm exposes the existence of polyphenolic and isoquinoline alkaloid compounds in the methanolic extract of *Sargassum wightii* (Rajeswari and Jeyaprakash, 2019). The UV spectrum of the ACCA-1 (Isolated flavonoid) compound shows peak occurrence in the range 250-383nm and indicating the presence of flavones and flavonols (Arora and Itankar, 2018). UV-Vis absorbance at wavelengths of 210-234nm correlate with acetogenins, and the absorbance at 210 nm, which is expected to more precisely indicate acetogenins (Gopal *et al*., 2019). UV absorption spectrum of acetogenins-(Muricin B, Cis-annonacin-10-one, Mosinone and Cis-uvariamicin IV) showed peak maximum UV absorption at range of 200-250nm (Saldana *et al*., 2017).

These reports are supportive of our observations further distinct peaks at different wavelength of ethanolic crude extract, Flavonoid Enriched Fraction and Acetogenin Enriched Fraction corresponds with the standards Quercetin, Kaempferol and Andrographolide reveals the phytoconstituent observed in the *Annona muricata* ethanolic crude extract and fractions were found to be phenolics or flavonoids and acetogenins in nature.

4.1.2. Fourier Transform Infrared spectroscopy analysis

Chemical characterization and identification of functional groups present in isolated phytoconstituents has shown to be a beneficial technique using FTIR (Rengarajan *et al*., 2020). The molecular vibrations of the chemical compounds contained in a sample are featured in FTIR, which provides a unique signature of the chemicals present. As a result, the FTIR spectrum represents the sample's molecular fingerprint (Lucarini *et al*., 2019). The FTIR analysis of *Clitoria ternatea*, *Theobroma cacao* and *Annona muricata* extracts reveals the presence of phenolic molecules (Indrianingsih *et al*., 2021).

Figure 16: FTIR profile of *Annona muricata* Ethanolic Crude Extract



The FTIR profile of ethanolic crude extract reveals the presence of various secondary metabolites identified based on the functional groups (Figure 16, Table 1). The bands observed at 416.62cm^{-1} and 455.20cm^{-1} showed the presence of alkyl halides. The halo compounds observed in the range of 501.49cm^{-1}, 540.07cm^{-1}, 609.51cm^{-1}, 756.10cm^{-1} and 848.68cm^{-1}. Absorption band at 941.26cm^{-1}, 1041.56cm^{-1}, 1087.85cm^{-1}, 1242.16cm^{-1}, 1373.32cm^{-1} and 1458.18cm^{-1} showed the presence of alkenes, anhydrides, secondary alcohols, amines, phenols and alkanes. The bands observed at 1512.19cm^{-1}, and 1550.77cm^{-1} showed the presence of nitro compound. The presence of bands at 1651.07cm^{-1}, 1735.93cm^{-1}, 2083.12cm^{-1} indicates the presence of alkene, aldehyde and isothiocyanate, at the positions 2360.87cm^{-1}, 2492.03cm^{-1} indicates the presence of alkynes. Bands at 2978.09cm^{-1}, 3278.99cm^{-1} and 3363.86cm^{-1} showed the presence of alkane, alkyne and aliphatic primary amine. The bands observed at 3564.45cm^{-1}, 3672.47cm^{-1} and 3896.21cm^{-1} corresponds to alcohol and 3734.19cm^{-1}, 3919.35cm^{-1} corresponds to phenol.

Figure 17: FTIR profile of Flavonoid Enriched Fraction (FEF) of *Annona muricata* leaf extract

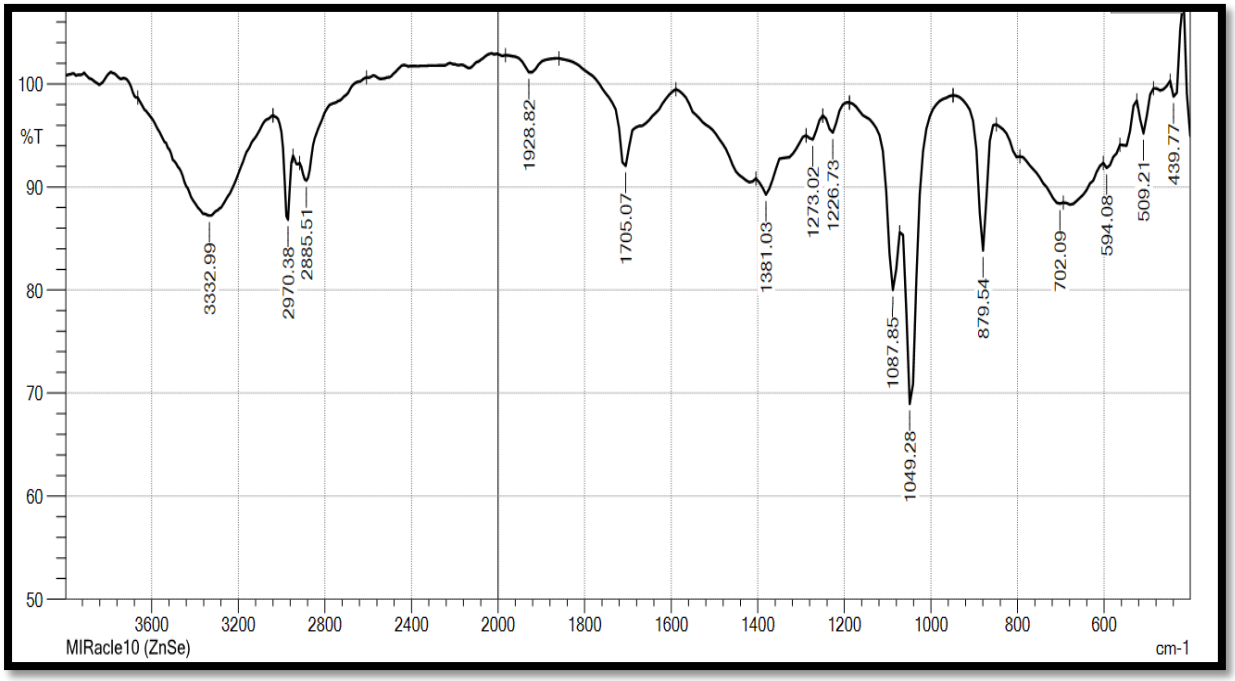
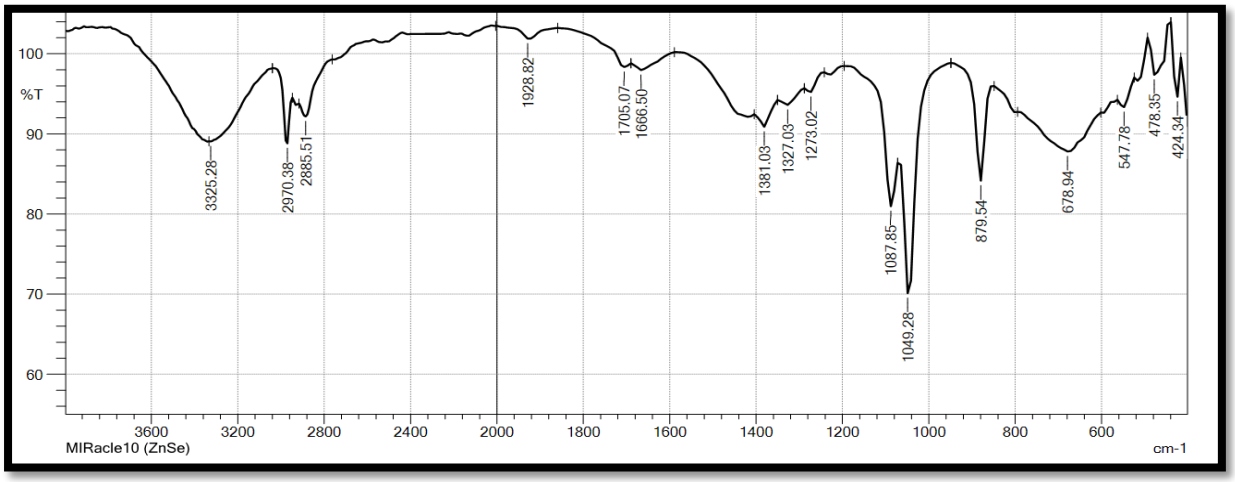


Figure 18: FTIR profile of Acetogenin Enriched Fraction (AEF) of *Annona muricata* leaf extract



The FTIR profile of Flavonoid Enriched Fraction (FEF) (Figure 17, Table 1) reveals the presence of alkyl halides at 439.77cm^{-1}, halo compounds at 509.21cm^{-1}, 594.08cm^{-1} and 879.54cm^{-1}. Absorption band at 702.09cm^{-1}, 1049.28cm^{-1}, 1087.85cm^{-1}, 1226.73cm^{-1} and 1273.02cm^{-1}, 1381.03cm^{-1}, 1705.07cm^{-1}, and 1928.82cm^{-1} reveals the presence of alkene,

anhydride, secondary alcohol, alkyl aryl ether, phenol, conjugated aldehyde, and aromatic compound. The alkane observed at 2885.51cm^{-1}, 2970.38cm^{-1} and alkyne observed at 3332.99cm^{-1} respectively.

The FTIR profile of Acetogenin Enriched Fraction (AEF) (Figure 18, Table 1) reveals the presence of alkyl halides at 424.34cm^{-1} and 478.35cm^{-1}, halo compounds at 547.78cm^{-1}, 678.94cm^{-1}, alkene at 879.54cm^{-1}, 1666.50cm^{-1}, alkane at 2885.51cm^{-1} and 2970.38cm^{-1}. Absorption band at 1049.28cm^{-1}, 1087.85cm^{-1}, 1273.02cm^{-1}, 1327.03cm^{-1}, 1381.03cm^{-1}, 1705.07cm^{-1}, 1928.82cm^{-1} and 3325.28cm^{-1} reveals the presence of anhydride, secondary alcohol, alkyl aryl ether, aromatic amine, phenol, conjugated aldehyde, aromatic compound and alkyne.

The existence of conjugated group C=O (stretching) in *Annona muricata* peel and leaf extract is revealed by FTIR analysis, indicating the existence of a "α, β-unsaturated γ-lactone" which distinguishes the great mass of acetogenins. Similarly, the acetogenin molecule has a vibrational mode that corresponds to the C=C groups (stretching) (Pedroza *et al*., 2021).

Table 1: FTIR functional group analysis of Ethanolic Crude Extract, Flavonoid and Acetogenin enriched fractions of *Annona muricata* leaves

Ethanolic crude extract		**Flavonoid Enriched Fraction (FEF)**		**Acetogenin Enriched Fraction (AEF)**	
Frequency (cm^{-1})	**Functional group**	**Frequency (cm^{-1})**	**Functional group**	**Frequency (cm^{-1})**	**Functional group**
416.62	Alkyl halides	439.77	Alkyl halides	424.34	Alkyl halides
455.20	Alkyl halides	509.21	Halo compounds	478.35	Alkyl halides
501.49	Halo compounds	594.08	Halo compounds	547.78	Halo compounds
540.07	Halo compounds	702.09	Alkene	678.94	Halo compounds
609.51	Halo compounds	879.54	Halo compounds	879.54	Alkene
756.10	Halo compounds	1049.28	Anhydride	1049.28	Anhydride
848.68	Halo compounds	1087.85	Secondary alcohol	1087.85	Secondary alcohol
941.26	Alkenes	1226.73	Alkyl aryl ether	1273.02	Alkyl aryl ether
1041.56	Anhydrides	1273.02	Alkyl aryl ether	1327.03	Aromatic amine
1087.85	Secondary	1381.03	Phenol	1381.03	Phenol

	alcohols				
1242.16	Amines	1705.07	Conjugated aldehyde	1666.50	Alkene
1373.32	Phenols	1928.82	Aromatic compound	1705.07	Conjugated aldehyde
1458.18	Alkanes	2885.51	Alkane	1928.82	Aromatic compound
1512.19	Nitro compound	2970.38	Alkane	2885.51	Alkane
1550.77	Nitro compound	3332.99	Alkyne	2970.38	Alkane
1651.07	Alkene			3325.28	Alkyne
1735.93	Aldehyde				
2083.12	Isothiocyanate				
2360.87	Alkynes				
2492.03	Alkynes				
2978.09	Alkane				
3278.99	Alkyne				
3363.86	Aliphatic primary amine				
3564.45	Alcohol				
3672.47	Alcohol				
3734.19	Phenol				
3896.21	Alcohol				
3919.35	Phenol				

Based on the structure of annonaceous acetogenins, FT-IR analysis revealed the presence of "alkanes, alkenes, ester, aromatic ring, and hydroxyl groups" in the *Annona muricata* ethyl acetate extract (Growther, 2018).

Annona muricata aqueous fruit extract revealed the presence of amines, alkyl halides, carboxylic acids, phenols and alcohols (Hemalatha *et al*., 2020). Aromatic compounds, esters, ethers, alkanes, alkenes, alkynes, carboxylic acids, and hydroxyl groups can all be found in methanolic extracts of A. *muricata* leaves. The broad alcohol/phenol O–H stretching anticipated the high phenolic content in A. *muricata* leaves (Ibrahim *et al*., 2022).

FTIR analysis of ethanol extract of *Annona muricata* seeds reveals the presence of alkyl halide, halogen compound, alkanes, aromaticcompounds, alcohols, carboxylic acids, esters, ethers, nitro compound, and aliphatic amine (Addai *et al*., 2020).

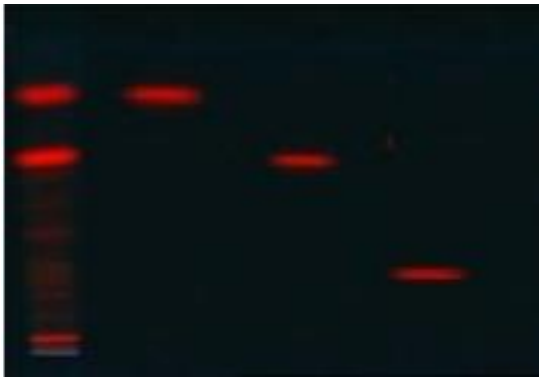
The above-mentioned reports support our study, and the FTIR profile revealed that alkyl halides, halo compounds, alkenes, anhydrides, secondary alcohols, amines, phenols, alkanes, nitro compound, aldehyde and alkyne groups are commonly present in the ethanolic crude extract, flavonoid and acetogenin enriched fractions. Functional groups like amines,

nitro compounds, Isothiocyanate, aliphatic primary amine and alcohols are present in the ethanolic crude extract but not in the flavonoid and acetogenin enriched fractions and this may due to the bioassay guided fractionation of *Annona muricata* leaves. The presence of conjugated group, alkanes, alkenes, and aromatic ring in acetogenin enriched fraction (AEF) confirms the presence of acetogenins and aromatic compounds, while the presence of alkane, alkene, alkyne, and phenol groups confirms the presence of flavonoids in flavonoid enriched fraction (FEF).

4.1.3. High Performance Thin Layer Chromatographic analysis (HPTLC)

HPTLC is a cost-effective and reliable chromatographic technology for detecting and characterising impurities, compounds, and active biomolecules. Because of HPTLC's unique characteristics, such as its ease of use, repeatability, good precision, and ability to record data in comparison to standards. HPTLC has the necessary capabilities for detecting and identifying diverse biomolecules in extracts used in herbal medicine research (Rouhani, 2019). The most advanced form of simple sample preparation and application is HPTLC, which is routinely used to separate and identify complicated mixtures in solution. The use of HPTLC has a long history in quality monitoring of medicinal plants, plant extracts, and natural products (Kustrin and Morton, 2020).

Plate 1: HPTLC profile of Ethanolic crude extract



Lane 1 2 3 4

Lane 1: Ethanolic crude extract (ECE)

Lane 2: Quercetin

Lane 3: Kaempferol

Lane 4: Andrographolide

Figure 19: Densitogram of *Annona muricata* Ethanolic Crude Extract

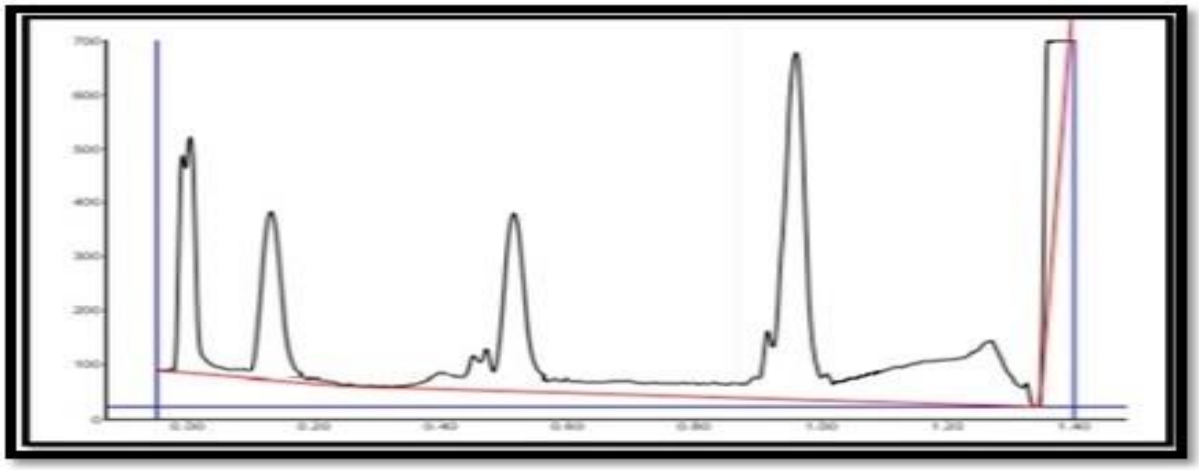


Table 2: Rf values of *Annona muricata* Ethanolic Crude Extract

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area
1	0.02	1.2	0.03	485.6	57.57	0.07	26.0	5942.8
2	**0.13**	**0.2**	**0.15**	**511.3**	**68.79**	**0.17**	**27.8**	**6341.8**
3	0.42	10.4	0.44	18.8	2.23	0.45	9.2	341.5
4	0.45	3.1	0.47	51.6	6.11	0.48	3.4	366.2
5	0.52	97.1	0.54	145.0	13.08	0.56	45.6	5724.8
6	**0.72**	**0.3**	**0.74**	**477.7**	**69.32**	**0.75**	**10.1**	**160.5**
7	**0.96**	**0.9**	**0.98**	**477.0**	**43.06**	**0.99**	**34.1**	**5501.9**
8	1.36	97.1	1.38	145.0	13.08	1.04	45.6	5724.8

Figure 20: Densitogram of Quercetin

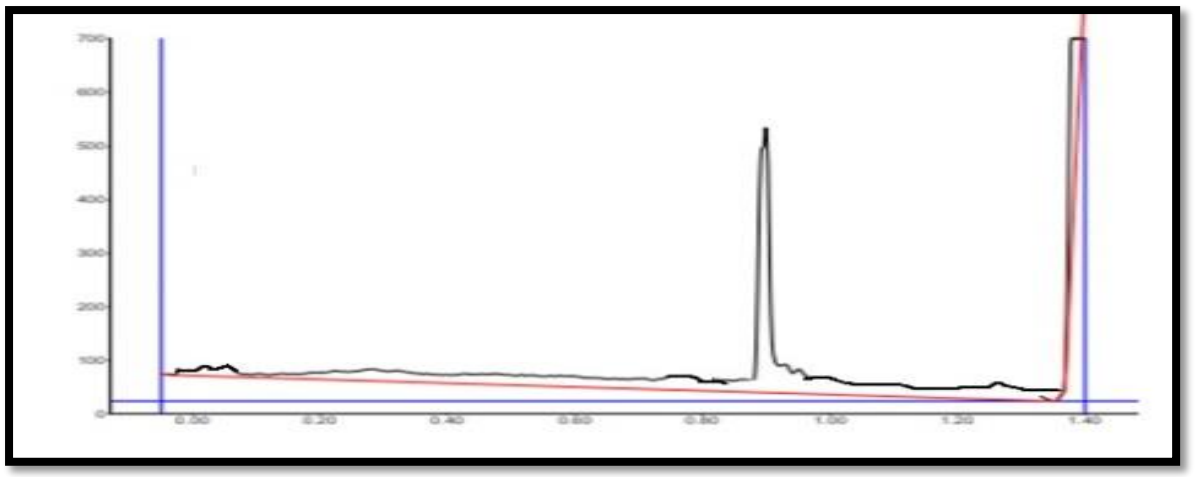


Table 3: Rf value of Quercetin in ECE

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area
1	0.97	0.4	0.96	350.7	60.50	0.97	9.5	5315.2

Figure 21: Densitogram of Kaempferol

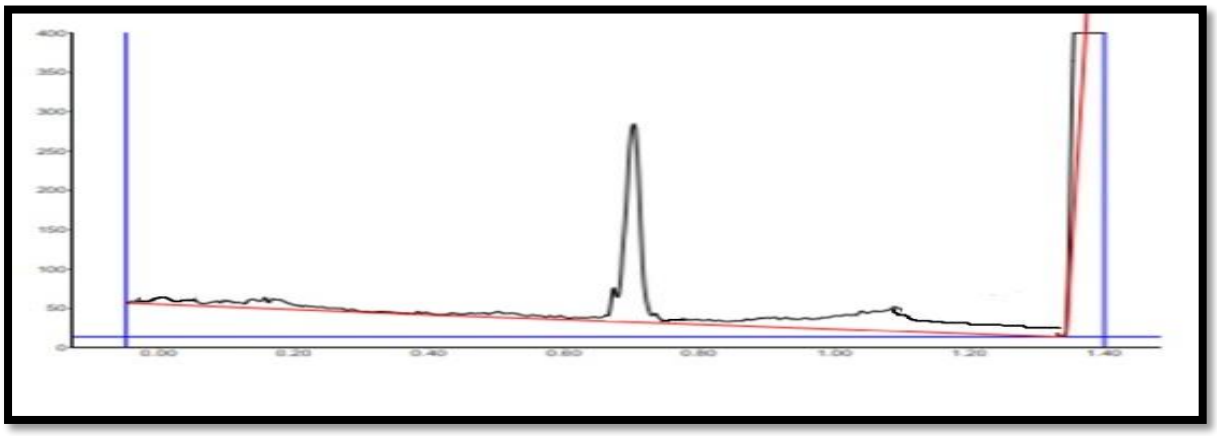


Table 4: Rf value of Kaempferol in ECE

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area
1	0.71	6.5	0.72	252.0	57.95	0.74	2.8	4233.1

Figure 22: Densitogram of Andrographolide

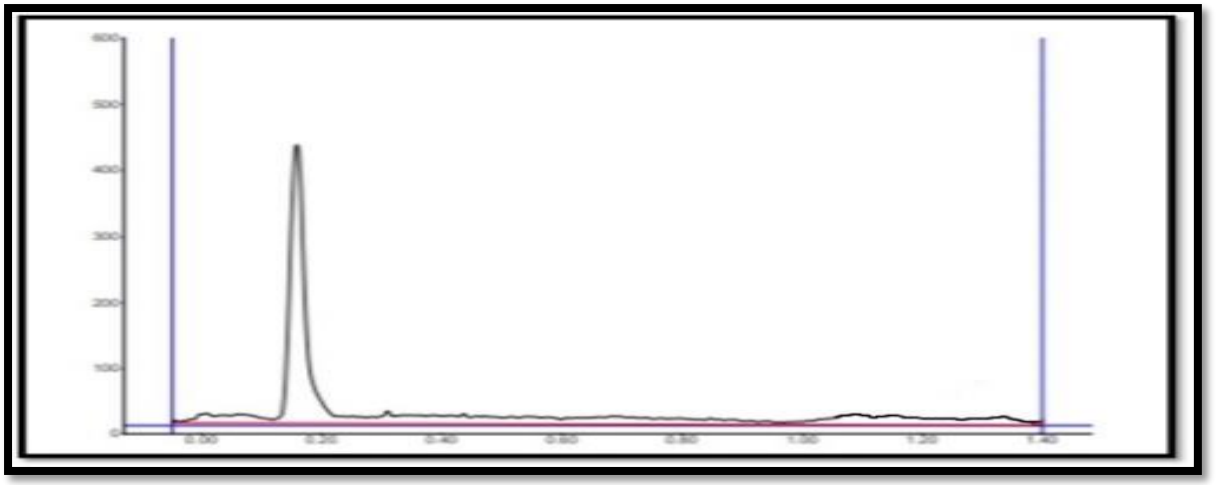
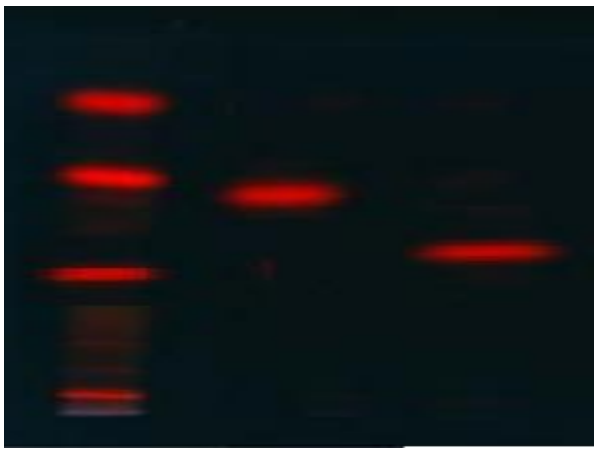


Table 5: Rf value of Andrographolide in ECE

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area
1	0.12	0.3	0.14	477.7	69.32	0.15	20.0	5906.3

HPTLC profile obtained at 366nm for the ethanolic crude extract, Quercetin, Kaempferol and Andrographolide is shown in Plate 1. The results reveal that each of the standards showed a band in lanes 2, 3 and 4. The ECE sample of *Annona muricata* loaded in lane 1 of this HPTLC profile, confirms the presence of Quercetin, Kaempferol and andrographolide like compounds as the RF values of these standards are comparable.

Plate 2: HPTLC profile of Flavonoid Enriched Fraction



Lane 1 2 3

Lane 1: Flavonoid Enriched Fraction (FEF)

Lane 2: Quercetin

Lane 3: Kaempferol

Figure 23: Densitogram of Flavonoid Enriched Fraction

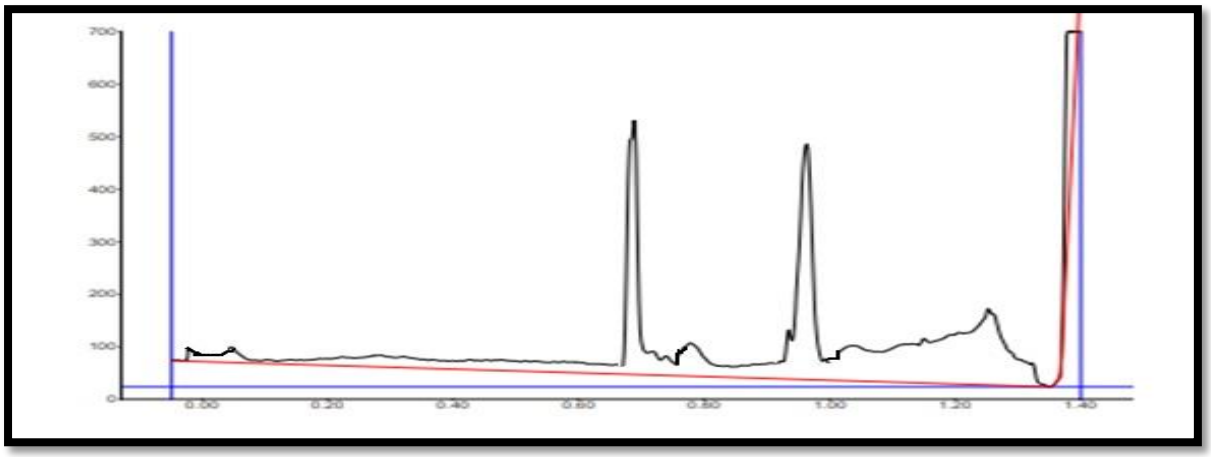


Table 6: Rf value of Flavonoid Enriched Fraction

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area
1	0.02	5.4	0.04	19.7	2.05	0.06	12.6	481.0
2	0.03	17.5	0.05	32.0	3.32	0.08	21.4	239.6
3	**0.70**	**5.1**	**0.72**	**14.2**	**2.06**	**0.74**	**10.1**	**160.5**
4	0.84	26.4	0.86	32.1	2.90	0.88	30.5	1162.9
5	**0.98**	**0.4**	**0.99**	**350.7**	**60.50**	**1.02**	**9.5**	**5315.2**
6	1.09	64.0	1.10	96.3	16.62	1.10	32.5	4447.8
7	1.11	77.9	1.12	98.1	8.86	1.14	96.8	3023.3
8	1.14	34.3	1.16	37.5	3.39	1.18	21.4	442.1
9	1.22	33.3	1.24	40.1	6.92	1.26	0.3	373.4

Figure 24: Densitogram of Quercetin

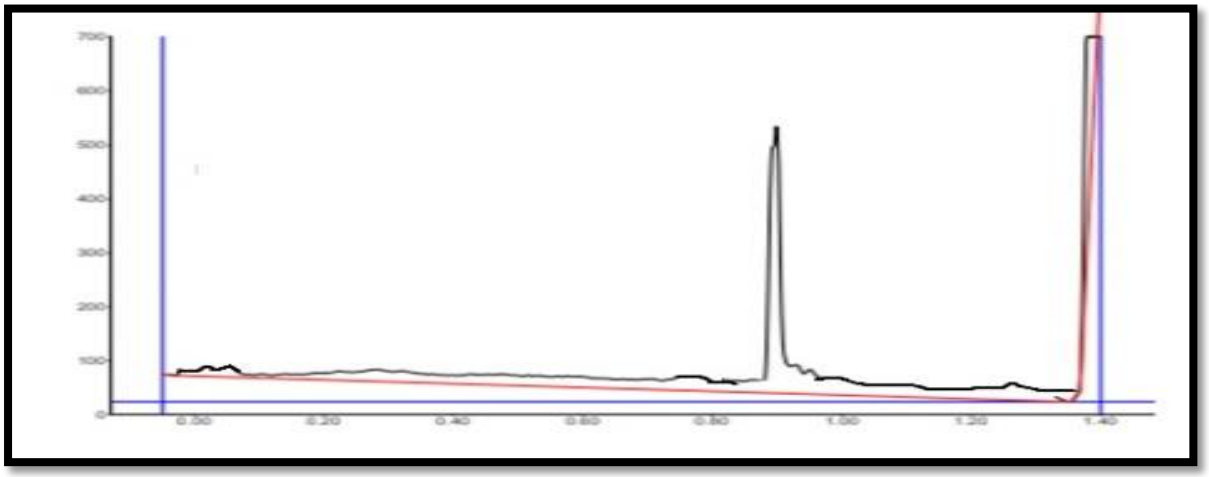


Table 7: Rf value of Quercetin in FEF

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area
1	0.97	0.4	0.96	350.7	60.50	0.97	9.5	5315.2

Figure 25: Densitogram of Kaempferol

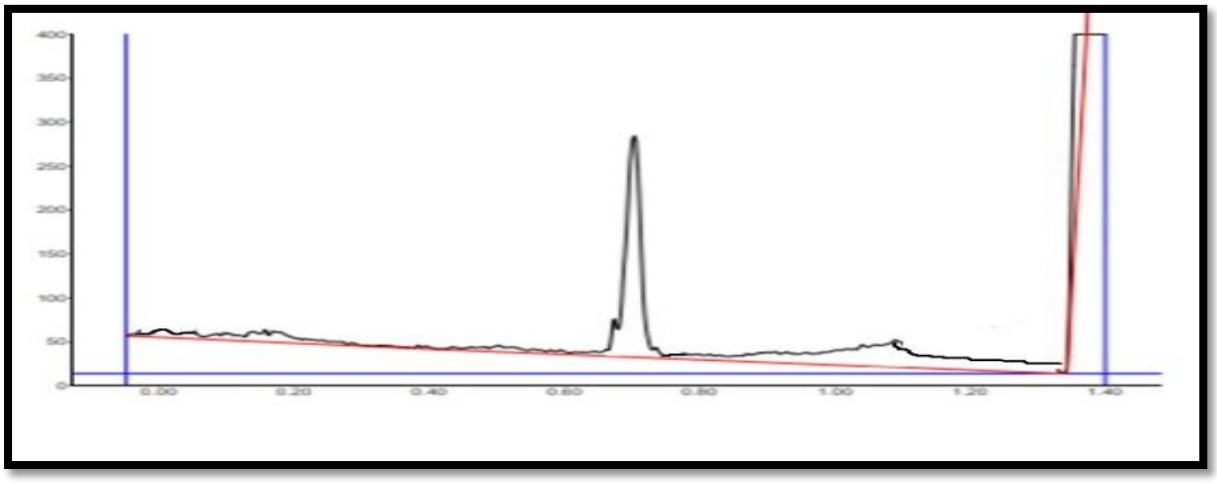
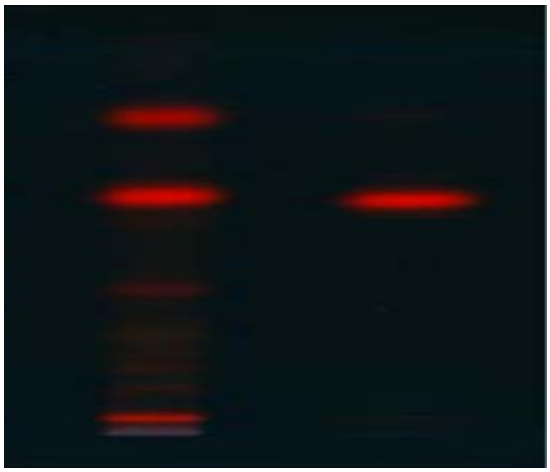


Table 8: Rf value of Kaempferol in FEF

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area
1	0.71	6.5	0.72	252.0	57.95	0.74	2.8	4233.1

HPTLC profile as obtained at 366nm in the Flavonoid Enriched Fraction, Quercetin and Kaempferol. Figure 23 shows the presence of flavonoid related compounds (Quercetin and Kaempferol) in the Flavonoid enriched fraction. The bands obtained (Plate 2) in the Flavonoid Enriched Fraction were compared with the reference compounds Quercetin and Kaempferol. This indicates that the compounds identified from the Flavonoid Enriched Fraction were similar to the reference standard and the Retention factor of the samples are also comparable with that of Quercetin and Kaempferol reiterates the similarity (Table 6).

Plate 3: HPTLC profile of Acetogenin Enriched Fraction



Lane 1 2

Lane 1: Acetogenin Enriched Fraction (AEF)

Lane 2: Andrographolide

Figure 26: Densitogram of Acetogenin Enriched Fraction

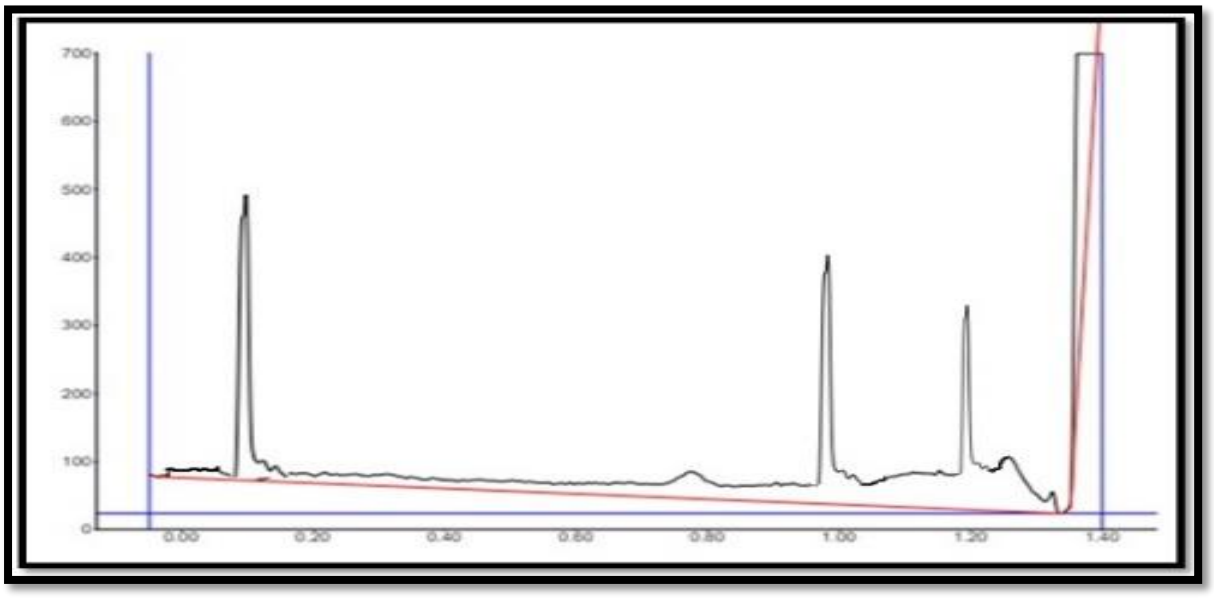


Table 9: Rf values of Acetogenin Enriched Fraction

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area
1	0.04	4.5	0.06	12.3	2.92	0.08	7.4	215.3
2	**0.13**	**1.2**	**0.14**	**351.5**	**70.85**	**0.15**	**6.0**	**45.37**
3	0.73	44.9	0.75	131.2	18.37	0.76	39.8	10405.9
4	1.00	3.2	1.02	437.7	65.61	1.05	14.9	7577.4
5	1.21	1.2	1.23	485.6	57.57	1.25	26.0	5942.8
6	1.32	17.6	1.34	41.5	4.92	0.96	31.8	1838.8

Figure 27: Densitogram of Andrographolide

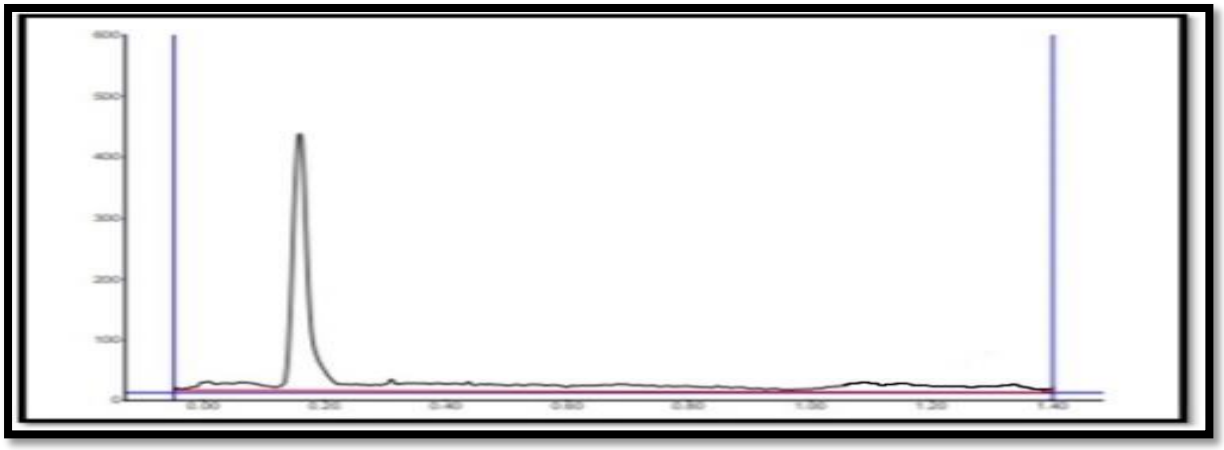


Table 10: Rf value of Andrographolide in AEF

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area
1	0.12	0.3	0.14	477.7	69.32	0.15	20.0	5906.3

Chromatograms were obtained at 366nm in the Acetogenin Enriched Fraction and Andrographolide. Figure 26 shows the presence of acetogenin related compounds in the Acetogenin enriched fraction. The bands obtained (Plate 3) in the Acetogenin Enriched Fraction were compared with the reference compound Andrographolide. This indicates that the compounds identified from the Acetogenin Enriched Fraction were similar to the reference standard and the Retention factor of the samples are also comparable with that of Andrographolide reiterates the similarity (Table 9).

HPTLC chromatogram of *Phlogacanthus thyrsiflorus* methanolic flower extract (MFE) along with some reference compounds (Naringenin, Ascorbic acid, Quercetin, Tannic acid and Gallic acid) revealed the presence of ascorbic acid and naringenin in the MFE sample (Bora *et al*., 2019). Vitamin c, pyrogallol, and caffeine, were found in HPTLC examination of *Kaempferia galanga* rhizome ethanolic extract along with standard reference chemicals (Nonglang *et al*., 2022). HPTLC finger print analysis of *Abrus precatorius* extract, reveals the presence of flavonoids (Saraf and Saraf, 2020). HPTLC analysis of White Mulberry leaves confirms the presence of phenol and flavonoid compounds (Ali *et al*., 2020). The presence of phenolic acids and flavonoid in the A. *reticulata* methanolic leaf extract was confirmed using HPTLC. (Pathak *et al*., 2022). The presence of alkaloid and flavonoid phytoconstituents in G. *wightii* methanolic extract was determined using HPTLC (Palani *et al*., 2020).

HPTLC analysis of hydroalcoholic extract of *Picrorhiza kurroa* (roots), *Andrographis paniculata* (aerial parts) and *Silybum marianum* (seeds) confirms the presence of Picroside-I, andrographolide and silybin (Mehta *et al*., 2021). HPTLC analysis of *Andrographis paniculata* revealed the presence of Andrographolide. The spectra of andrographolide showed maximum absorption at 223nm (Pandey *et al*., 2019).

In correspondence with the above findings, HPTLC analysis performed in the current research revealed that in the *Annona muricata* ethanolic crude extract (ECE), 4 sharp peaks were obtained at the Rf value 0.02, 0.13, 0.52, 0.72 and 1.36, which is comparable with the standards Quercetin, Kaempferol and Andrographolide and confirms the presence of flavonoid and acetogenin related compounds in the *Annona muricata* ethanolic crude extract. Flavonoid enriched fraction showed 2 sharp peaks at Rf value 0.70 and 0.98, which is comparable with the standards Quercetin and Kaempferol which confirms the flavonoids in the flavonoid enriched fraction, while in the Acetogenin enriched fraction, 3 sharp peaks were obtained at the Rf value 0.13, 1.00 and 1.21, which is comparable with the standard Andrographolide, suggesting that acetogenins are present in the Acetogenin enriched fraction.

4.1.4. High Performance Liquid Chromatographic analysis (HPLC)

HPLC is a chromatographic method used to detect, characterize, and isolate the phytocompounds in phytochemical and analytical chemistry. HPLC is a versatile, effective, and frequently used technology for the isolation of natural substances. This methodology is

currently gaining attraction as the recommended way for fingerprinting investigations for herbal plant quality control, according to various analytical techniques. To describe the features of a crude extract, natural materials are extensively extracted in a biological experiment. The resolving capacity of HPLC is suitable for the analytical and preparative processing of multi-component mixtures. Several publications have documented the use of HPLC for identification and quantitation of bioactive molecules in medicinal plants, primarily phenolic compounds, flavonoids, isoquinoline alkaloids and steroids (Thirumal and Laavu, 2017).

Figure 28: HPLC profile of Ethanolic Crude Extract

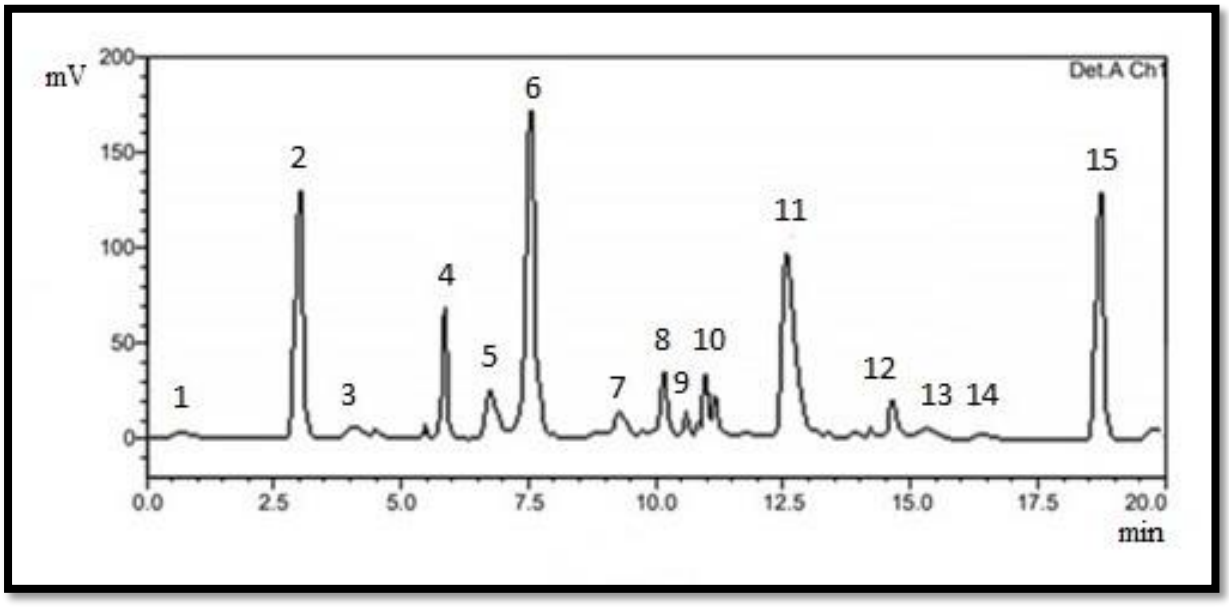


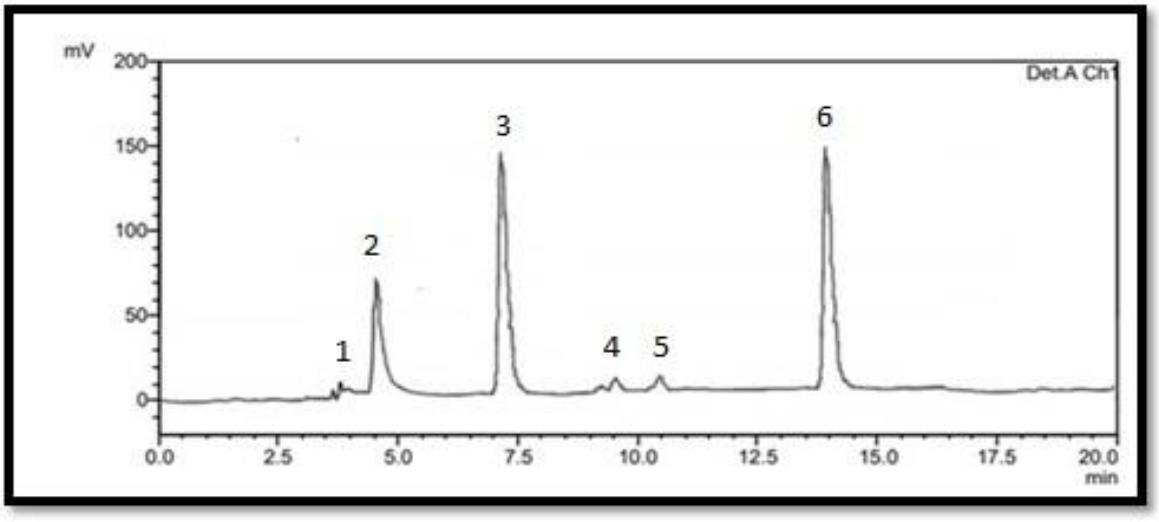
Table 11: Retention time of Ethanolic Crude Extract HPPL Profile

Ethanolic crude extract			
Peak	**Retention Time**	**Area**	**Area %**
1	0.7	352200	1.368
2	3.0	3023087	16.381
3	4.1	3323469	1.3256
4	5.5	919939	10.404
5	6.7	394681	2.139
6	7.5	7197118	38.999

7	9.3	234602	1.2346
8	10.2	758654	4.111
9	10.6	223457	1.6258
10	11.1	467988	1.268
11	12.6	3109845	16.851
12	14.6	357570	1.938
13	15.3	236729	1.432
14	16.4	126581	1.2367
15	18.7	1225618	6.641

The HPLC fingerprint analysis shows the presence of multiple major and minor peaks representing the phytochemical constituents. The Ethanolic crude extract shows the presence of 4 major peaks and 11 minor peaks (Figure 28) and their Retention Time were found to be 0.7, 3.0, 4.1, 5.5, 6.7, 7.5, 9.3, 10.2, 10.6,11.1, 12.6, 14.6, 15.3, 16.4 and 18.7 respectively (Table 11).

Figure 29: HPLC profile of Flavonoid Enriched Fraction



The Flavonoid Enriched Fraction shows the presence of 3 major peaks and 3 minor peaks (Figure 29) and their Retention Time were found to be 3.9, 4.6, 7.3, 9.5, 10.5 and 14 respectively (Table 12). The peaks of the Flavonoid Enriched Fraction were compared with the standard Quercetin (Figure 30) and Kaempferol (Figure 31). The Retention time of

Quercetin and Kaempferol were found to be 8 and 14 which was comparable with the Flavonoid Enriched Fraction, thereby confirming the presence of Flavonoids in FEF (Table 12).

Figure 30: HPLC profile of Quercetin

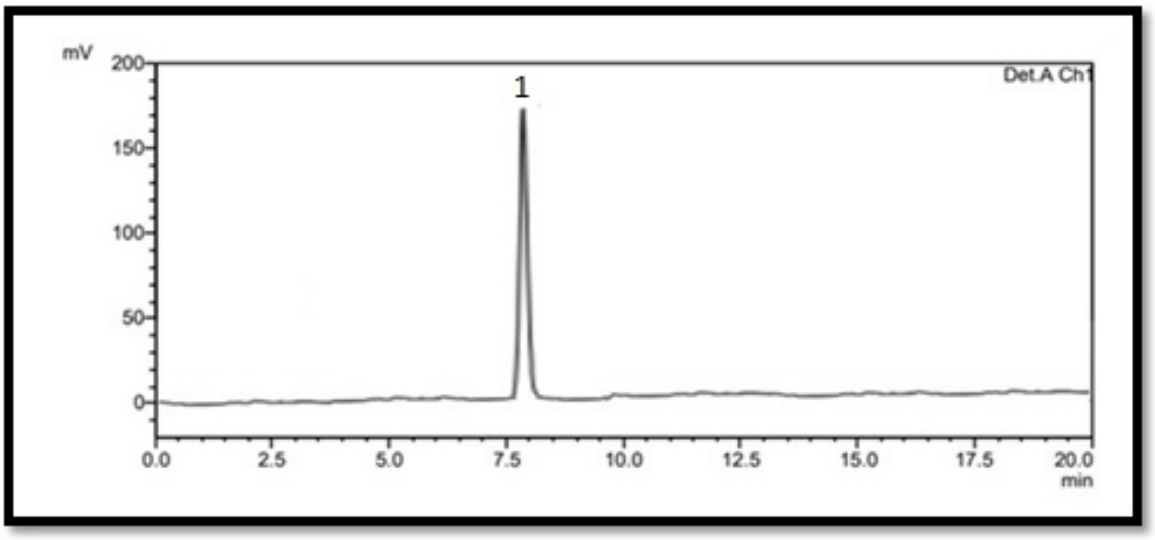


Figure 31: HPLC profile of Kaempferol

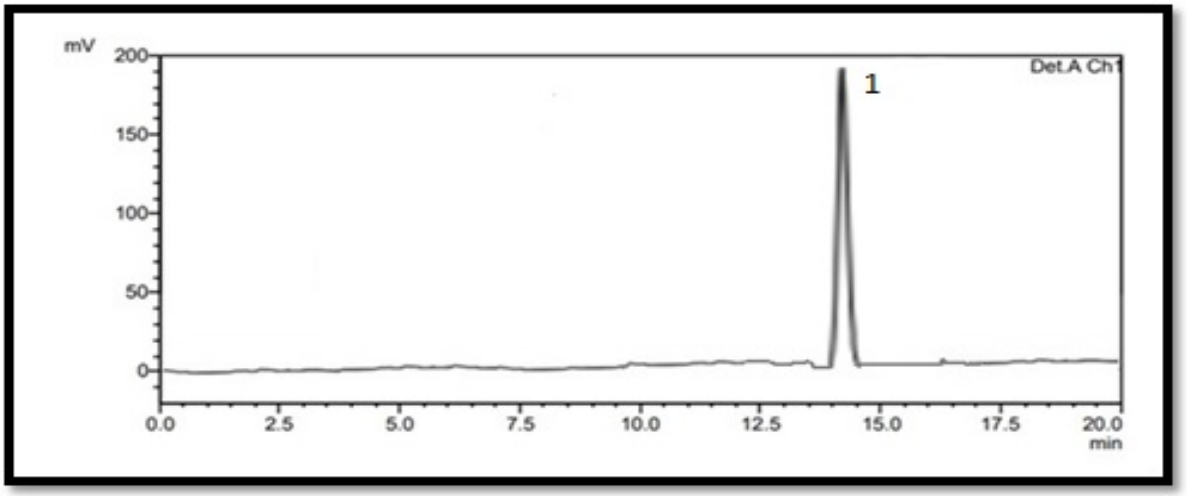


Table 12: Retention time of Flavonoid Enriched Fraction and Standards (Quercetin and Kaempferol) HPLC Profile

Flavonoid enriched fraction				**Standards**							
				Quercetin				**Kaempferol**			
Peak	**RT**	**Area**	**Area %**	**Peak**	**RT**	**Area**	**Area %**	**Peak**	**RT**	**Area**	**Area %**
1	3.9	4184	3.3191	1	**8**	548432	100	1	**14**	2532389	100
2	4.6	24982	4.2421								
3	**7.3**	25797	20.4650								
4	9.5	34386	19.8187								
5	10.5	4168	3.3069								
6	**14**	5350	27.2787								

The Acetogenin Enriched Fraction shows the presence of 4 major peaks and 3 minor peaks (Figure 32) and their Retention Time were found to be 3.5, 6.0, 6.6, 10.1, 10.6, 13.2 and 15.9 respectively (Table 13), The peaks of the Acetogenin Enriched Fraction were compared with the standard Andrographolide (Figure 33). The Retention time of Andrographolide were found to be 3.5 which was comparable with the Acetogenin Enriched Fraction (Table 13), thereby confirming the presence of Acetogenins in AEF.

Figure 32: HPLC profile of Acetogenin Enriched Fraction

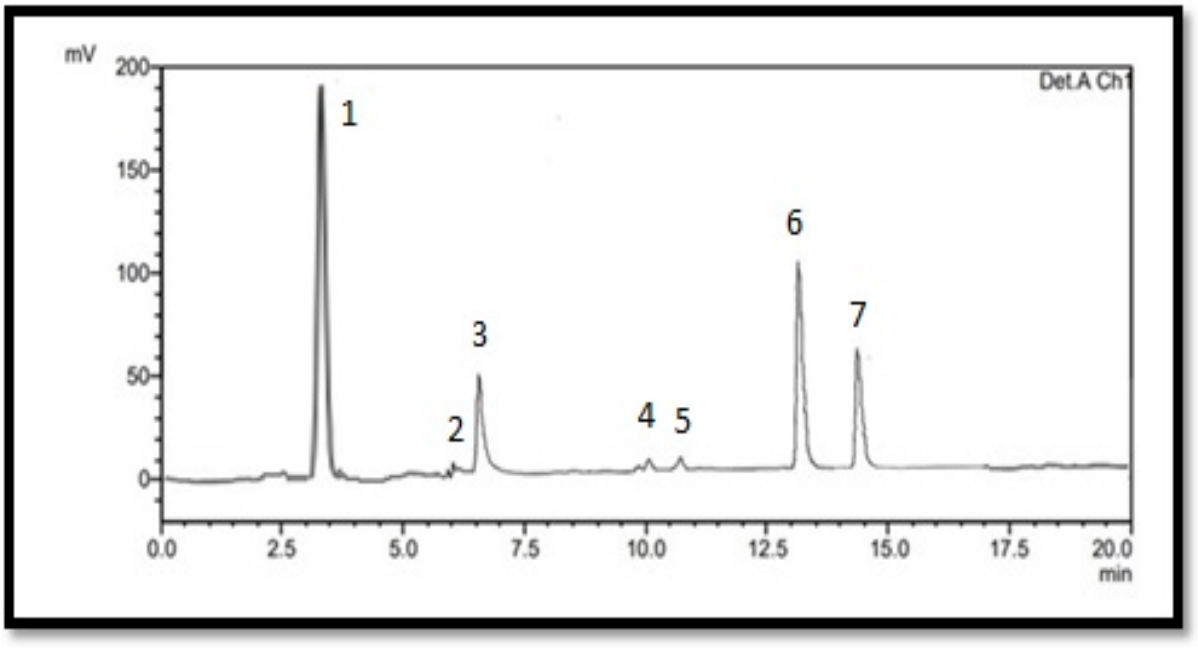


Figure 33: HPLC profile of Andrographolide

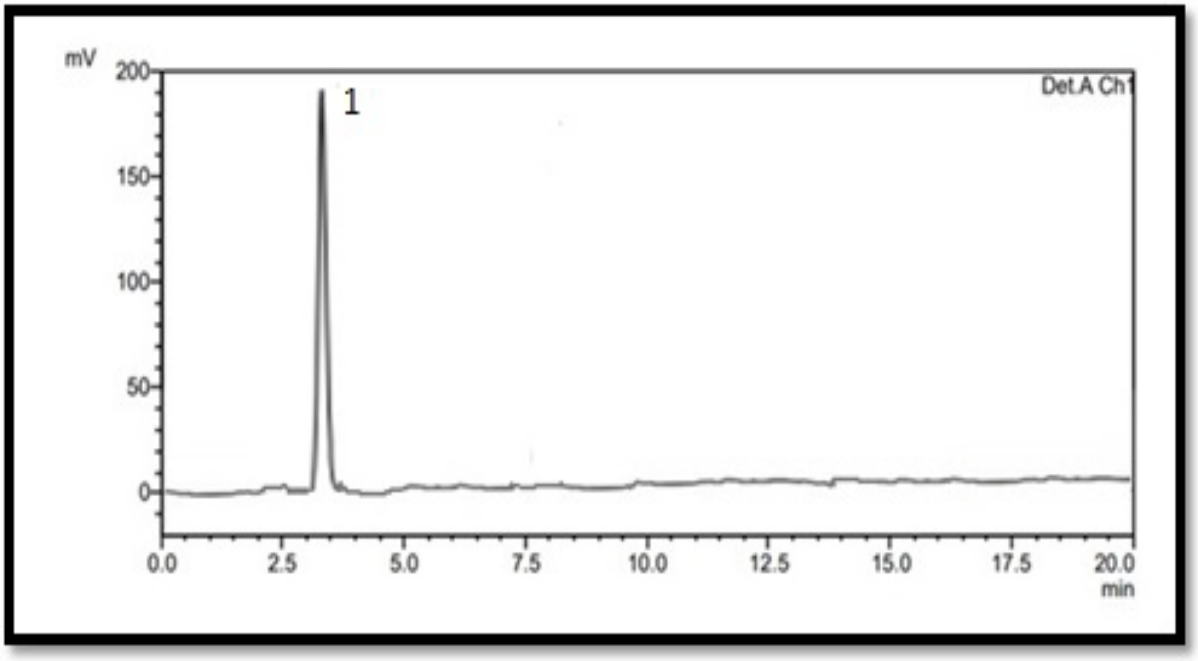


Table 13: Corresponding Rf value of Acetogenin Enriched Fraction

Acetogenin enriched fraction				**Standard (Andrographolide)**			
Peak	**Retention Time**	**Area**	**Area %**	**Peak**	**Retention Time**	**Area**	**Area %**
1	**3.5**	7193218	38.999	1	**3.5**	3416802	100
2	6.0	4248	3.3596				
3	6.6	26972	4.2341				
4	10.1	223457	1.6258				
5	10.6	1232618	6.543				
6	13.2	25386	20.4650				
7	15.9	343797	19.8187				

The HPLC chromatographic profile of *Annona muricata* aqueous leaves extract at 280 nm shows the presence of five major peaks, which were identified as quercetin 3-glucoside, rutin, chlorogenic acid, catechin and gallic acid (Cercato *et al*., 2021).

HPLC analysis of *Annona muricata* aqueous extract shows the presence of various polyphenolic compounds such as "p-coumaric acid, 5-Ocaffeoylquinic acid, quercetin-3-

Oglucoside, 5-Ocaffeoylquinic acid, quercetin-3-O-galactoside, kaempferol-3-O-rutinoside, quercetin-3-O-rutinoside, and catechin" (Mancini *et al*., 2018). The existence of quercetin and kaempferol-related substances was identified in flavonoids isolated from mulberry leaves and analysed using HPLC (Ju *et al*., 2018). The HPLC chromatograms of *Pteris cretica* L. extracts shows the presence of flavonoids, including luteolin-7-*O*-glucoside, rutin, quercetin, luteolin, and apigenin (Hou *et al*., 2019).

HPLC was used to assess the phenolic and flavonoid content of *Coccoloba uvifera* L. leaf extracts. "Benzoic, ellagic, gallic, and o-coumaric acids" were the major phenolic acid components found in ethanol, acetone, and aqueous extracts. Flavonoids such as "rutin, myricetin, and quercetin" were discovered in aqueous, acetone, and ethanol extracts (Ashmawy *et al*., 2020).

HPLC analysis of *Annona muricata* leaf fractions revealed the presence of "Squamostattin-A, 12, 15-Cis-squamostatin-A, Squamostatin-D, Bullatacin, Isodesacetyl uvaraicin, Asiminecin and Squamocin" (Mulia *et al*., 2013). Similarly, acetogenins compound "12, 15-cis-squamostatin-A, Squamostatin-A, Bullatacin, Squamocin, Isodesacetyluvaricin and Desacetyluvaricin" were detected in *Annona muricata* leaf extract (Daud *et al*., 2016).

The above-mentioned reports support our study and the results revealed that Ethanolic crude extract contain more phyto constituents, and the bio-assay guided fractionation could help to reduce the phytocompounds showing minor peaks in the chromatograms. However compounds were more similar to flavonoids in FEF and acetogenins in AEF. To further understand the properties of secondary metabolites, additional research is required.

4.1.5. Gas Chromatography-Mass Spectrometry analysis (GC-MS)

Researchers use a combination analytical technique that combines gas chromatography and mass spectroscopy to detect and characterize chemicals constituents. In phytochemical studies and chemotaxonomic research of medicinal plants containing biologically active components, GC-MS plays a significant role (Olivia *et al*., 2021).

Figure 34: GCMS profile of Ethanolic Crude Extract

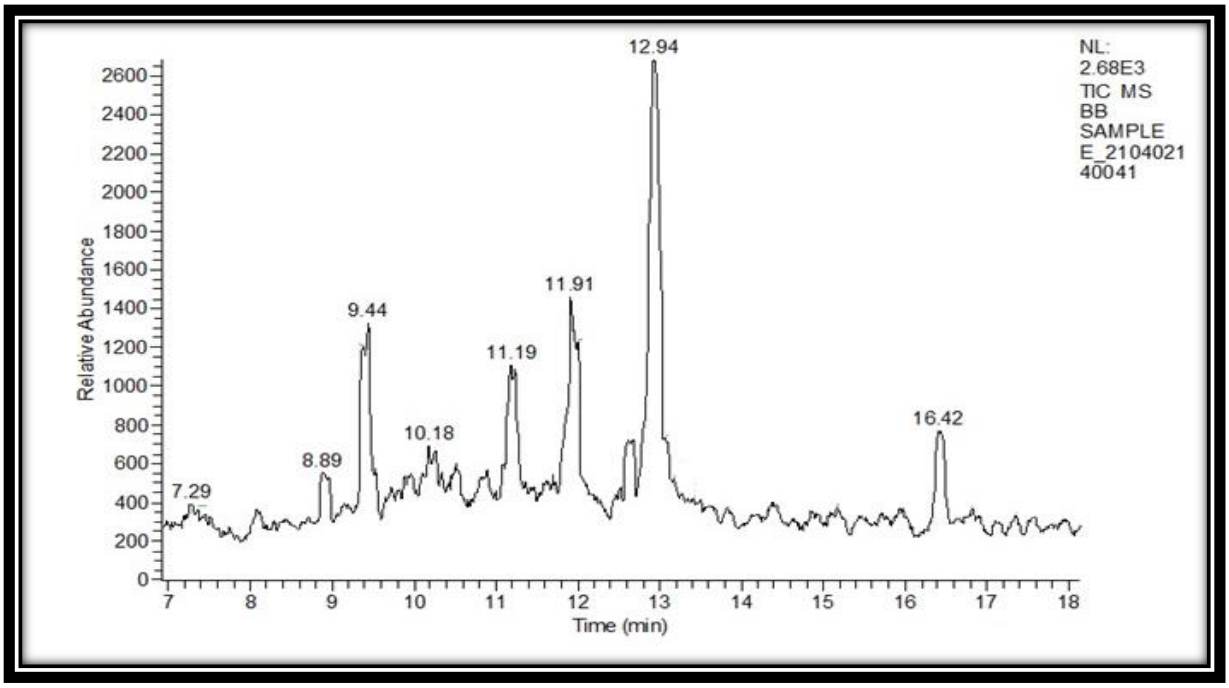


Table 14: Bioactive constituents identified in Ethanolic Crude Extract of *Annona muricata* leaves

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 15: Biological properties of Ethanolic Crude Extract phytoconstituents

Compound Name	Biological activity
10-Oxodecanoic acid, methyl ester	Antioxidant, anti-inflammatory activity
Pentadecanoic acid	Cytotoxic activity
α-Gurjunene	Antiproliferative, Anti-microbial activity
dodecanoic acid	Cytotoxic activity
Phytol	Cancer preventive properties
Pentadecanol	Antibacterial activity
Hexadecatrienoic acid	Antioxidant activity
Oleyl alcohol	Antitumor activity

Figure 34 shows a GC-MS chromatogram of *Annona muricata* leaves ethanolic crude extract. The presence of 10-Oxodecanoic acid, methyl ester, Pentadecanoic acid, α-Gurjunene, Dodecanoic acid, phytol, pentadecanol, Hexadecatrienoic acid, and Oleyl alcohol were identified when mass spectra contents were compared to the NIST library. Table 14 provides comprehensive tabulations of the extract's GC-MS analysis, as well as their biological attributes (Table 15).

The *Annona muricata* ethanolic leaves extract revealed 25 compounds, according to Yahaya *et al*. (2018), and the GC-MS study of *Annona muricata* leaf extracts indicated that the hydromethanolic extract are rich in fatty acids (Atanu *et al*., 2022).

Figure 35: GCMS profile of Flavonoid Enriched Fraction (FEF) of *Annona muricata* leaves

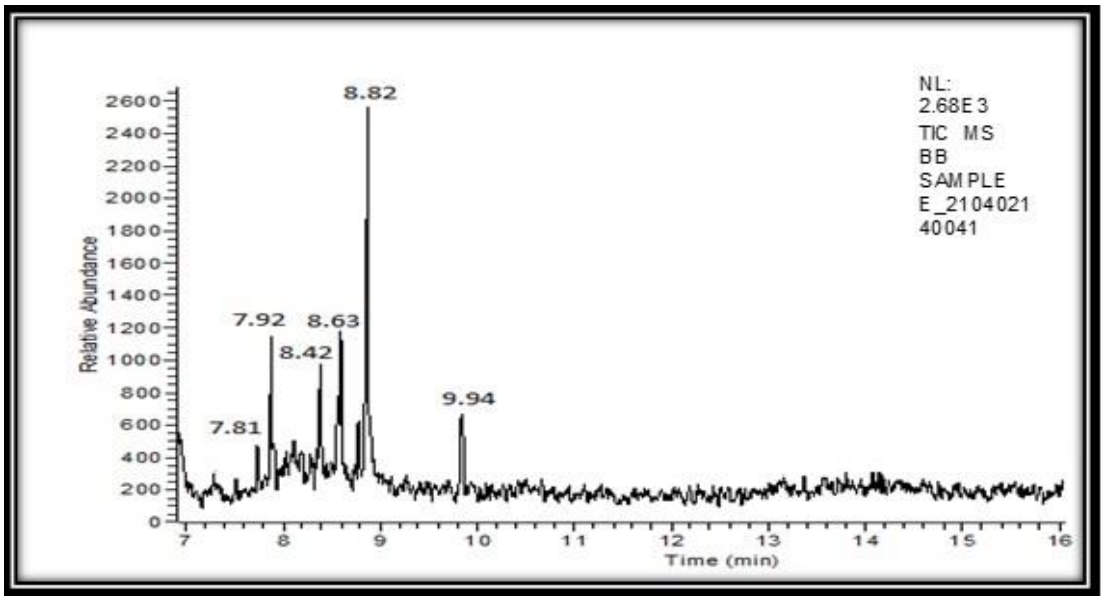


Figure 35 shows a GC-MS chromatogram of the Flavonoid enriched fraction (FEF) of *Annona muricata* leaves. The presence of Isoferulic acid, Pentanedioic acid, Myricetin, Apigenin-6-C-glucoside, Luteolin 3-di-O-glucoside, and Glycitein was identified by comparing the mass spectra components with the NIST library. Table 16 contains comprehensive tabulations of the FEF's GC-MS analysis, as well as their biological attributes (Table 17).

Table 16: Bioactive constituents identified in Flavonoid Enriched Fraction (FEF) of *Annona muricata* leaves

Apex RT	Start RT	End RT	Area	%Area	Height	%Height	Compound
7.81	7.48	7.99	1667.946	2.73	242.389	3.43	Isoferulic acid
7.92	7.72	7.99	7711.012	12.64	966.360	13.67	Pentanedioic acid
8.42	8.24	8.66	5496.529	9.01	655.096	10.27	Myricetin
8.63	8.42	8.81	9509.375	15.59	1026.307	13.92	Apigenin-6-C-glucoside
8.82	8.62	8.91	21415.233	35.10	2216.587	31.36	Luteolin 3´7-di-O-glucoside
9.94	9.64	9.99	3751.875	6.15	483.352	6.84	Glycitein

Table 17: Biological properties of Flavonoid Enriched Fraction phytoconstituents

Compound Name	Biological activity
Isoferulic acid	Cytotoxicity, antioxidant and Antiproliferative activity
Pentanedioic acid	Neuroprotective properties
Myricetin	Antimicrobial, antioxidant and anticancer activity
Apigenin-6-C-glucoside	Antioxidant and antitumor activity
Luteolin 3´7-di-O-glucoside	Antioxidant and cytotoxic activity
Glycitein	Preventive supplement of cancer and osteoporosis

Wahab *et al*. (2018) reported 34 phenolic compounds have been isolated from the leaves of *A. muricata*. The purification of n-butanol leaf extract of *A. muricata* led to the isolation of flavonoid related compounds.

Wahab *et al*. (2018) revealed that 34 polyphenolic compounds were extracted from A. *muricata* leaves. Separation and purification of A. *muricata* n-butanol leaf extract resulted in the isolation of flavonoids.

Figure 36: GCMS profile of Acetogenin Enriched Fraction (AEF) of *Annona muricata* leaves

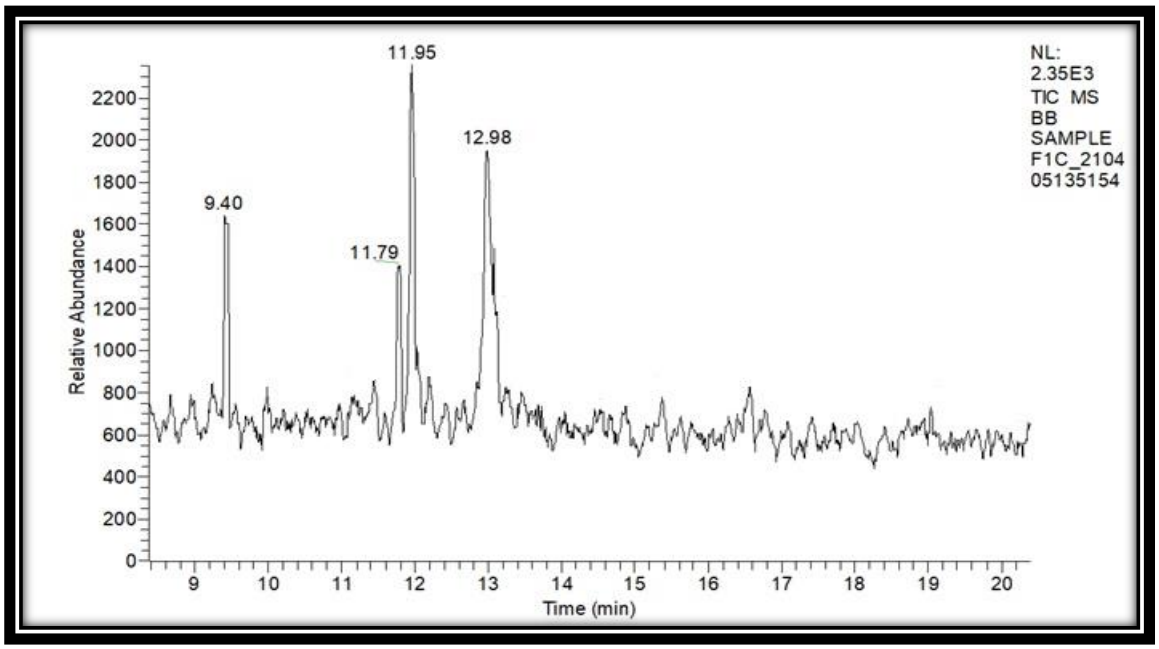


Figure 36 shows a GC-MS chromatogram of the Acetogenin enriched fraction (AEF) of *Annona muricata* leaves. The presence of α-Muurolene, Cis-solamin, Muricatacin, and Germacrene B was identified when the mass spectra constituents were compared to the NIST library. Table 18 provides comprehensive tabulations of the AEF's GC-MS analysis, as well as their biological attributes (Table 19).

Table 18: Bioactive constituents identified in Acetogenin Enriched Fraction (AEF) of *Annona muricata* leaves

Apex RT	Start RT	End RT	Area	%Area	Height	%Height	Compound
9.40	9.37	9.48	3968.079	14.82	961.089	20.82	α-Muurolene
11.79	11.71	11.83	3120.326	11.65	738.019	15.99	Cis-solamin
11.95	11.86	12.09	8600.178	32.12	1685.298	36.51	Muricatacin
mMue	12.82	13.15	11087.702	41.41	1231.612	26.68	Germacrene B

Table 19: Biological properties of acetogenin enriched fraction phytoconstituents

Compound Name	Biological activity
α-Muurolene	Antipathogenic and cytotoxic activity
Cis-solamin	Cytotoxic and haemolytic properties
Muricatacin	Anti-rheumatism, anticancer activity and neuralgia properties
Germacrene B	Anti-microbial and anti-proliferative activity

Wahab *et al*. (2018) reported that 47 acetogenins were isolated from the *Annona muricata* leaves, including Cis-solamin, solamin. *Annona muricata* leaves and fruit revealed the presence of *α*-Pinene, (D)-, Limonene, (D)-, Methyl (9E)-9-octadecen-12-ynoate, 6-Methyloctadecane, *α*-Muurolene, Andrographolide (Gyesi *et al*., 2019). GCMS analysis of *Annona cherimola* leaves revealed the presence of germacrene D, bicyclogermacrene, (E)-caryophyllene, sabinene and β-pinene (Valarezo *et al*., 2022).

The above-mentioned reports support our study and the compounds identified in the *Annona muricata* ethanolic extract ranges from the Retention Time 7.29 - 16.42, Retention

Time of flavonoid (FEF) ranges from 7.81 - 9.94 and the Retention Time of acetogenin enriched fraction (AEF) ranges from 9.40 - 12.98, bio-assay guided fractionation results in separation of flavonoids and acetogenins compounds from the ethanolic crude extract. Thus, the cited literature confirms that isolated compounds are flavonoid and acetogenin related compounds which has diverse biological properties, further research is required for the development of novel drugs and to explore its pharmacological potential.

Phase II

4.3. Assessment of Antioxidant potential of Ethanolic crude extract, Flavonoid (FEF), Acetogenin (AEF) Enriched Fraction and combination of FEF and AEF of *Annona muricata* leaves

Free radicals are atoms or molecules that have one or more unpaired electrons, and they are collectively referred to as ROS. Hydroxyl, superoxide, nitric oxide (NO), and hydrogen peroxide radicals are examples of ROS. Free radicals target macromolecules like lipids, nucleic acids, and proteins, causing cell death and disrupting homeostasis. Excess free radicals are constantly created in the human body as a result of metabolic process and also due to external causes, resulting in oxidative stress. Antioxidants function as scavenging free radicals, protecting living organisms from the detrimental effects of excessive ROS formation. A symbiotic relationship in between antioxidant defense system and radicals can lead to a variety of diseases. Natural antioxidants have played a prominent role in the pharmaceutical markets in recent years due to their nutritional and therapeutic benefits (Nalini *et al*., 2018).

Yang *et al*. (2015) reported that acetogenins and flavonoids are the two important phytochemical groups in the plant *Annona muricata*. Mulia *et al*. (2013) reported that acetogenins from the *Annona muricata* plant are usually extracted using ethanol as the organic solvent.

Previous study done by Ilango *et al*. (2021) in our lab reported the antioxidant activity of *Annona muricata* leaf extracts in solvents with varying polarity, reveals polar solvents possess more antioxidant and anticancer activity. To further analyse and narrow down the importance of secondary metabolites that played a significant role in the antioxidant and anticancer activity, in the present study bioassay guided fractionation procedure was performed using ethanol extract to isolate flavonoid enriched fraction (FEF) and acetogenin

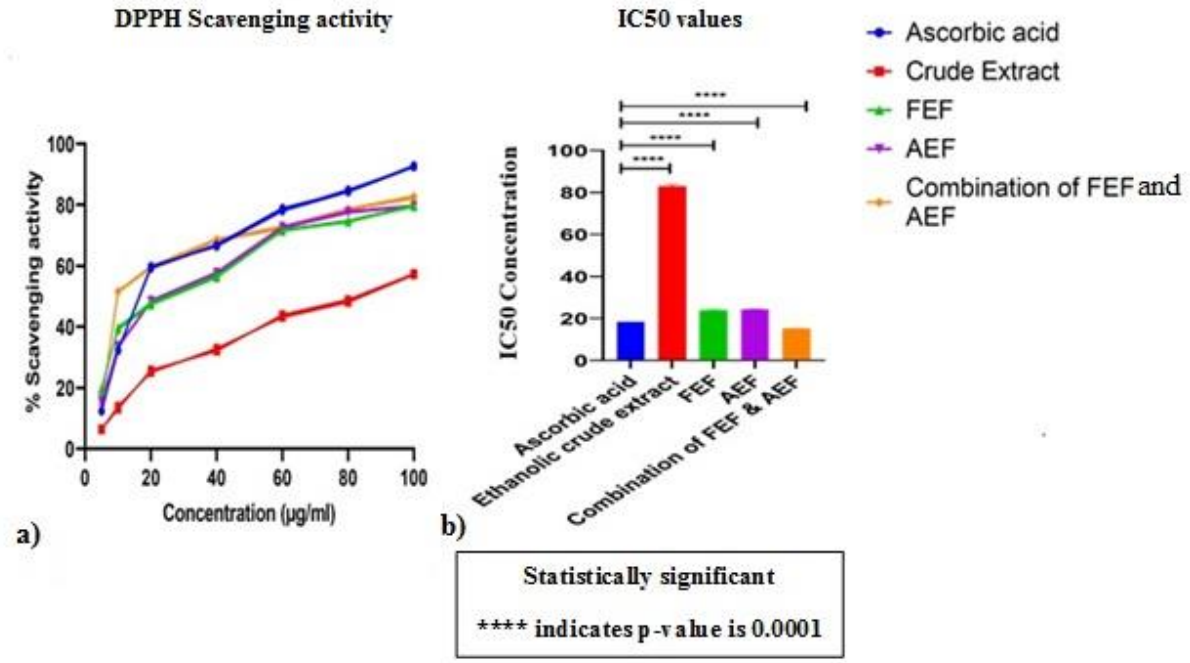
enriched fraction (AEF) from the *Annona muricata* leaves and to determine its antioxidant activity and anticancer potential.

4.3.1. Assessment of radical scavenging activity

4.3.1.1. DPPH radical scavenging assay

Antioxidants could convert the stable free radical DPPH (deep violet colour) to 1, 1-diphenyl-2-picryl hydrazine, resulting in discoloration. When comparing the ethanolic crude extract and all fractions to standard ascorbic acid, there was a significant difference ($p < 0.05$) in DPPH radical scavenging activity.

Figure 37: DPPH radical scavenging activity of *Annona muricata* Ethanolic Crude Extract and fractions



a) % Scavenging activity of ethanolic extract and fractions against DPPH b) IC50 concentration of ethanolic extract and fractions of *Annona muricata*

The DPPH results showed that the ethanolic crude extract and all fractions could scavenge DPPH radicals, with the scavenging activity increasing as the extract and fraction concentrations increased. However, the activity peaks at 100µg, demonstrating that the ethanolic extract and chromatographically derived fractions have dose-dependent activity.

The combination of FEF and AEF group had the highest scavenging activity, followed by AEF, FEF, and ethanolic crude extract (Figure 37a), IC50 values were calculated using graph pad prism software and the IC50 values were found to be 18.21µg/ml for Ascorbic acid, 82.96µg/ml for crude extract, 23.96µg/ml for AEF, 24.24µg/ml for FEF, and 15.21µg/ml for combination of FEF and AEF (Figure 37b), respectively.

The antioxidant action of ethanolic *Annona muricata* leaf extract was shown to be high, which is consistent with our findings. The presence of phenolic and flavonoid components could cause this effect (Nguyen *et al*., 2020). By scavenging DPPH radicals, the antioxidant activity of ethyl acetate, hexane, and methanol extracts of A. *muricata* leaves was demonstrated (Lawal *et al*., 2017).

In a concentration dependent manner, ethyl acetate, butanol. hexane, dichloromethane, and water fractions of *Annona muricata* ethanolic stem bark extract displayed DPPH free radical scavenging activity. The butanol fraction had higher free radical scavenging activity than the ethyl acetate, dichloromethane, and aqueous fractions, which could be attributed to the presence of polyphenols, which are common components of herbal medicines and are known for their antioxidant activity due to their radical scavenging ability (Sanni *et al*., 2020).

The antioxidant activity of *Annona muricata* ethanolic leaf extract was found in the aqueous, methanol, and n-hexane fractions, with fraction of methanol having the greatest scavenging activity among the fractions examined. The methanol fraction has the highest quantity of polyphenolic components in *Annona muricata* leaves, about twice as much as the other fractions. The methanol fraction has the maximum lactone content, preceded by the aqueous and hexane fractions. That suggests that acetogenins, potent phytoconstituents identified in A. *muricata* leaf, are much more soluble in ethyl acetate solvent than in water or n-hexane solvent. The greater antioxidant activity is due to the methanol fraction's increased total polyphenolic content (Krisanti *et al*., 2021).

Similar to the *in vitro* mode of action of ascorbic acid, which contains allylic hydrogens, the antiradical activity of acetogenins against DPPH could be linked to the "α, ß - unsaturated lactone ring moiety", acetogenins also contain allylic hydrogens, it might act similarly. (Gonzalez *et al*., 2017).

Antioxidants are believed to provide health benefits, acting as lowering the risk of a variety of diseases linked to oxidative stress. Meanwhile, phenolic compounds are abundant in plants and have a broad variety of biological impacts, including anti-inflammatory, antibacterial, and antioxidant properties. As a result, leaves rich in polyphenol components can serve as antioxidants (Zhaohong *et al*., 2017).

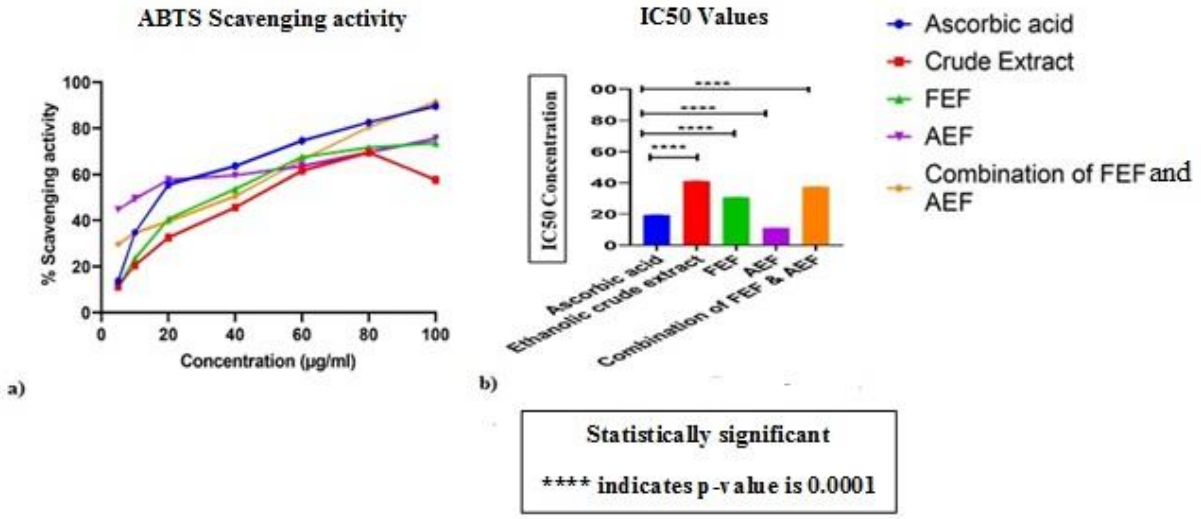
According to these supporting studies, the free radical quenching ability of the *Annona muricata* leaf ethanolic extract and chromatographically derived fractions in the current investigation could be attributed to the presence of various polyphenols in the *Annona muricata* leaves, such as flavonoids and acetogenins. The extract group having the combined fractions exhibited the maximum scavenging potential against DPPH, this could be due to the presence of allylic hydrogen as observed in ascorbic acid scavenging action. Thus, the results are comparable and better with ascorbic acid. The combined fraction could scavenge due to the abundant polyphenolic compounds and acetogenins which could readily be extracted in the more polar solvents like ethanol. The IC50 value of the combined fractions when compared to Ascorbic acid clearly indicates that the fractions in combination could operate synergistically to detoxify the DPPH radicals.

4.3.1.2. ABTS radical scavenging assay

The ABTS results revealed that the ethanolic crude extract and all fractions could scavenge ABTS radicals, with scavenging activity increasing as the ethanolic crude extract and fraction concentrations increased. The activity however, peak at 100µg, demonstrating that the ethanolic crude extract and its chromatographically derived fractions have dose-dependent activity.

Among the groups tested, combination of FEF and AEF treated group exhibited greater scavenging activity (Figure 38a), and the IC50 values were found to be 19.35µg/ml for Ascorbic acid, 41.12µg/ml for crude extract, 30.75µg/ml for FEF, 11.04µg/ml for AEF and 37.41µg/ml for combination of FEF and AEF (Figure 38b) respectively.

Figure 38: ABTS radical scavenging activity of *Annona muricata* Ethanolic Crude Extract and fractions



a) % Scavenging activity of ethanolic extract and fractions against ABTS b) IC50 concentration of ethanolic extract and fractions of *Annona muricata*

Antioxidants' primary job is to scavenge proton radicals. A protonated ABTS radical with a maximum absorption wavelength of 734 nm, is a key factor in deciding antioxidant capability. ABTS's scavenging effect is proportional to its concentration.

A. *muricata* ethanolic leaf extract was found to be prompt and efficient ABTS radical scavengers and the antioxidant activity was linked to total phenolic and flavonoid content (Nguyen *et al*., 2020).

The activity of all fractions; n-hexane, dichloromethane, dichloromethane: methanol (1:1), methanol, and ethanol fractions of *Annona muricata* ethanolic leaf extract were comparable with Vitamin C (standard), using the ABTS radical scavenging method (Nwaehujor *et al*., 2020).

ABTS assay reveals that the acetogenin fraction has antioxidant activity. The presence of flavonoids in the acetogenin fraction is likely responsible for its antioxidant effect. Polyphenols have a variety of radical-scavenging mechanisms, including metal scavengers, electron transfer, and hydrogen ion donation. Radicals are more easily quenched by phenolic substances (Gonzalez *et al*., 2017).

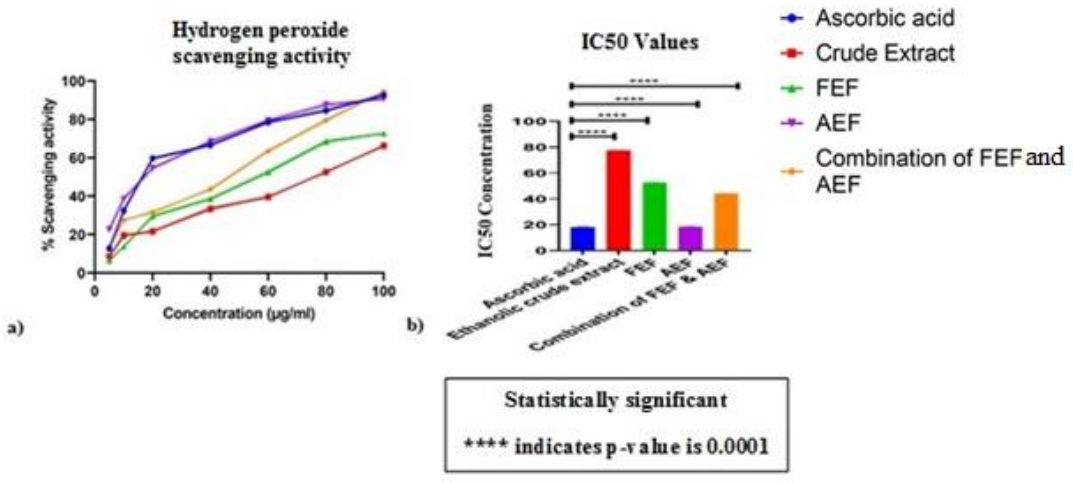
Annona muricata fruit pulp has strong antioxidant properties due to its high amount of phenolics and acetogenins. The antioxidant capacities of ethyl acetate fruit pulp and leaf fractions of *Annona muricata* were higher when compared to the acetogenin, "15–acetyl guanacone", implying a synergic radical scavenging mechanism between the phytoconstituents (Agu *et al*., 2018).

In line with the earlier studies, the current study findings suggest that the flavonoids and acetogenins found in *Annona muricata* may be responsible for the extract and fractions ABTS scavenging ability. The combined fractions with acetogenin and flavonoid could cause the potential scavenging of ABTS radical indicating its antioxidant capacity and the individual fractions could cause better scavenging when compared to the combined fractions indicating the absence of synergism between them.

Hydrogen peroxide radical scavenging assay

The hydrogen peroxide radical scavenging results demonstrated that the ethanolic crude extract of *Annona muricata* and all fractions were capable of scavenging hydrogen peroxide radicals, and the free radical scavenging action raised as the concentration of the extract and fractions increased (Figure 39a). And the maximum scavenging activity was found to be at concentration of 100µg, indicating that the extract and fractions have dose-dependent activity.

Figure 39: Hydrogen peroxide radical scavenging activity of *Annona muricata* Ethanolic Crude Extract and fractions



a) % Scavenging activity of ethanolic extract and fractions against hydrogen peroxide
b) IC50 concentration of ethanolic extract and fractions of *Annona muricata*

Among the groups tested, combination of FEF and AEF treated groups exhibited greater scavenging activity, and the half maximal inhibitory concentration values were determined to be 18.20µg/ml for Ascorbic acid, 77.55µg/ml for crude extract, 52.71µg/ml for Flavonoid enriched fraction, 18.39µg/ml for Acetogenin enriched fraction and 44.13µg/ml for combination of FEF and AEF respectively (Figure 39b).

Despite the fact that hydrogen peroxide does not react favourably with most biologically important substances, it is an internal progenitor of hydroxyl radicals, which are very destructive to cells. As a result, the ability of the plant extract and fractions to scavenge H_2O_2 is a measure of their antioxidant activity (Tolsarwad *et al*., 2020).

At 10 – 100µg/ml, the F4 fraction acquired from the *Annona muricata* ethanolic extract demonstrated the strongest H_2O_2 radical scavenging activity. F4 activity at 50µg/ml was substantially higher ($p<0.05$) than that of other concentrations, including the standard (Vitamin C). The synergetic impact of the chemicals contained in the fraction could be responsible for F4 fraction effectiveness. Plant extracts have been shown to have antioxidant effects owing to phenolic chemicals including flavonoids (Nwaehujor *et al*., 2020).

The *Moringa oleifera* ethyl acetate fraction scavenged hydrogen peroxide in a concentration-dependent manner, which could be associated to the existence of phenols. Flavonoids are principally derivatives of phenolic substances, which are significant phytoconstituents in plants. Phenolics primarily operate as free radical scavengers, allowing plants to withstand oxidative stress (Gothai *et al*., 2017).

The S. *nigrum* L. fruit methanolic extract had a concentration-dependent ability to scavenge hydrogen peroxide, because of phenolics, which transfer electrons to H_2O_2, neutralising it to H_2O (Veerapagu *et al*., 2018).

Annona reticulate Linn. and *Borassus flabellifer* Linn. extracts and fractions scavenged H_2O_2, which could be due to the existence of phenolics and tannins (Tolsarwad *et al*., 2020). Because of the bioactive chemicals that are accountable for their different pharmacological effects, medicinal herbs remain one of the most potent sources of human health. The ethnomedicinal plant *Annona muricata* has acetogenins as one of its main phytoconstituents. (Ogbu *et al*., 2020).

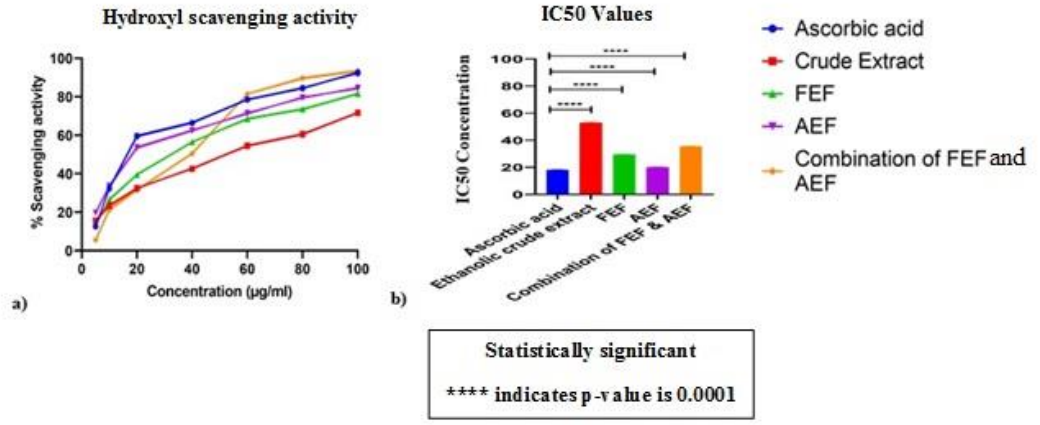
The presence of polyphenolics, flavonoid compounds, and acetogenins in the leaves of *Annona muricata* extract and fractions may be accountable for free radical scavenging activity. The results of the present study are in agreement with the cited literatures, indicating that the phenolic substances acted as H_2O_2 scavenger by transferring electrons to H_2O_2 and causing neutralisation to water. This scavenging action of these phytoconstituents might be responsible for the antioxidant potential of *Annona muricata* there by renders in apposition against cell damage caused by oxidant induced stress.

4.3.1.3. Hydroxyl radical scavenging assay

The hydroxyl radical with the shortest half-life is susceptible of extracting hydrogen anions from cellular membrane and initiating the chain reactions of oxidative degradation of lipids (Nalini *et al*., 2018).

The Hydroxyl radical scavenging results revealed that ethanolic crude extract and all the fractions were able to scavenge hydroxyl radicals (Figure 40a). Among the groups tested combination of FEF and AEF group exhibited greater scavenging activity, and the IC50 values were found to be 18.20µg/ml for Ascorbic acid, 52.87µg/ml for crude extract, 29.46µg/ml for FEF, 20.26µg/ml for AEF and 35.52µg/ml for combination of FEF and AEF (Figure 40b) respectively.

Figure 40: Hydroxyl radical scavenging activity of *Annona muricata* Ethanolic Crude Extract and fractions



a) % Scavenging activity of ethanolic extract and fractions against hydroxyl radical b) IC50 concentration of ethanolic extract and fractions of *Annona muricata*

Agu *et al*. (2017) reported that because of overall flavonoid and alkaloid concentrations, the *Annona muricata* fractions were able to detoxify hydroxyl radicals.

Extracts of *Annona squamosa* seeds are found to possess scavenging effect on hydroxyl radical in a concentration dependent manner. H_2O_2 is not a powerful oxidant, but it can be hazardous to cells when it produces hydroxyl radicals. As a result, H_2O_2 removal is essential. DNA, lipids, and proteins can be oxidised by the highly reactive hydroxyl radicals. The presence of considerable levels of phytochemicals can be associated to the therapeutic potential of plants (Vikas *et al*., 2017).

Various biomolecules, such as proteins, polypeptides, nucleic acids, and lipids, can be affected by hydroxyl radicals. *Manilkara hexandra* (Roxb.) Dubard bark and leaf methanolic extracts, as well as ascorbic acid, were found to successfully scavenge hydroxyl radicals. The leaf methanolic extract scavenged more hydroxyl radicals than the bark extract. Existence of polyphenolic components in extracts of leaf might be accountable for the antioxidant activity when compared to the extracts of bark (Dutta *et al*., 2020).

The *Costus afer* leaf methanol extract possess hydroxyl radical scavenging activity, in living organisms, polyphenolic compounds especially flavonoid groups are thought to have antioxidative properties, serving as scavengers of oxygen species and oxidants (Atere *et al*., 2018).

Annona muricata possess antioxidant properties which might have been due to the rich phytochemicals such as alkaloids, flavonoids, phenols, acetogenins, lipids and tannins (Agu *et al*., 2017).

An aromatic ring containing at least one hydroxyl group is present in natural phenolic and flavonoid compounds. Hydroxyl groups in phenolic compounds are efficient electron donors and can contribute directly to antioxidant activity (Nguyen *et al*., 2020).

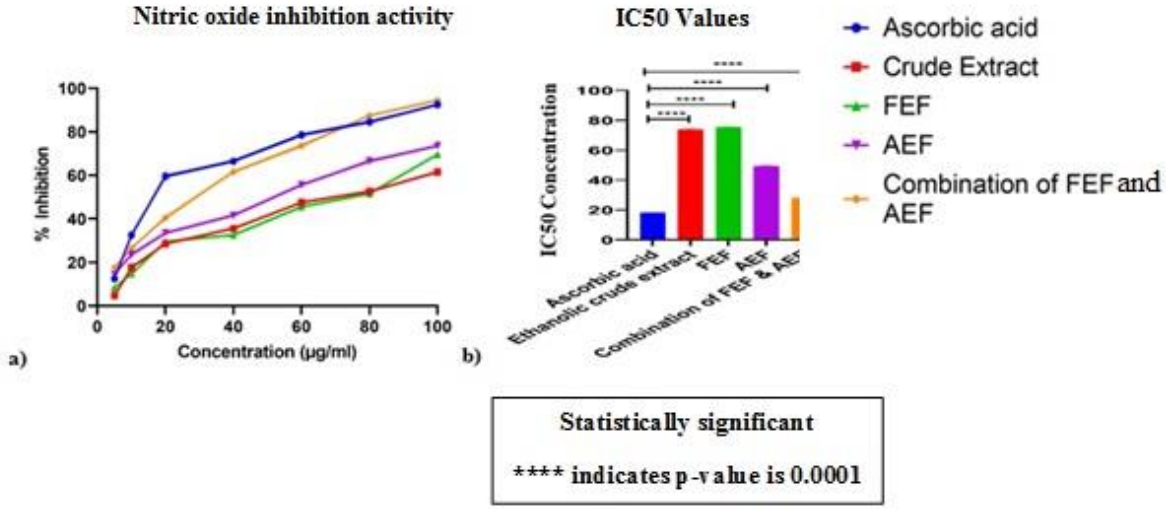
According to the findings, the existence of various secondary metabolites found in the leaf extract and fractions of *Annona muricata*, such as flavonoids and acetogenins, may be responsible for hydroxyl free radical scavenging action that might be attributed by the existence of aromatic ring structures of the phytoconstituents of *Annona muricata*.

4.3.2. Assessment of radical generation inhibition assay

4.3.2.1. Inhibition of nitric oxide generation

The human body produces the NO radical, and excess NO production leads to oxidative damage (Gonzalez *et al*., 2017). The molecule nitric oxide is lipophilic. It interacts with oxygen to create the nitrite ion at physiological pH. Nitric oxide is vital for regulating vasodilation, signal transmission, and the inflammatory response. Nitric oxide scavengers compete with oxygen and inhibit nitric oxide synthesis (Karuna *et al*., 2018).

Figure 41: Inhibition of nitric oxide generation by the *Annona muricata* Ethanolic Crude Extract and fractions



a) % Inhibition activity of ethanolic extract and fractions against NO, b) IC50 concentration of ethanolic extract and fractions of *Annona muricata*

The Nitric oxide inhibition results revealed that ethanolic crude extract and all the fractions were able to inhibit nitric oxide generation (Figure 41a), among the groups tested combination of FEF and AEF group exhibited greater inhibition activity, the half maximal inhibitory concentration were determined to be 18.18µg/ml for Ascorbic acid, 73.81µg/ml for crude extract, 75.40µg/ml for FEF, 49.37µg/ml for AEF and 28µg/ml for combination of FEF and AEF (Figure 41b) respectively.

NO is a free radical produced by mammalian cells that regulates a variety of physiological functions. On the other hand, excess NO generation, is linked to a number of diseases. The hydroethanolic leaf extract of A. *muricata* inhibited NO radicals at 71.98%, which was equivalent to the standard ascorbic acid (73.64%) at 250µg/ml and the antioxidant action of the extract might be ascribed by the presence of phytoconstituents (Durairaj, 2018).

Onohuean *et al*. (2021) reported that *Annona muricata* extracts were able to inhibit NO radicals better than ascorbic acid, and the percentage inhibition of extract increased dose-dependently. Phytochemicals and components found in the plant extract may have synergistically assisted to the antioxidant effects.

Ilango *et al*. (2021) reported that methanol, chloroform, and benzene, leaf extract of *Annona muricata* demonstrated NO inhibition activity. The antioxidants in *Annona muricata* leaves extract may limit the production of nitrite radicals by combating with nitrogen and oxygen oxides in the reaction medium. *Asparagus racemosus* Linn. root extract has a higher antioxidant activity through inhibiting nitric oxide (Karuna *et al*., 2017).

The soluble polyphenols in the *Annona muricata* methanol-acetone extract were found to be efficient against the nitric oxide radical. The OH-substituted functional group in the structure could potentially be responsible for the NO scavenging activity of total soluble polyphenols. Antioxidant activity, as previously indicated, may be able to suppress NO radical species such NO_2, N_2O_3, and N_2O_4 during NO oxidation. (Gonzalez *et al*., 2017).

In accordance with the cited literatures, the current study suggests that polyphenols, flavonoids, and acetogenins found in *Annona muricata* could be accountable for inhibiting nitric oxide radical generation proving it preventive or protection ability against oxidant mediated diseases.

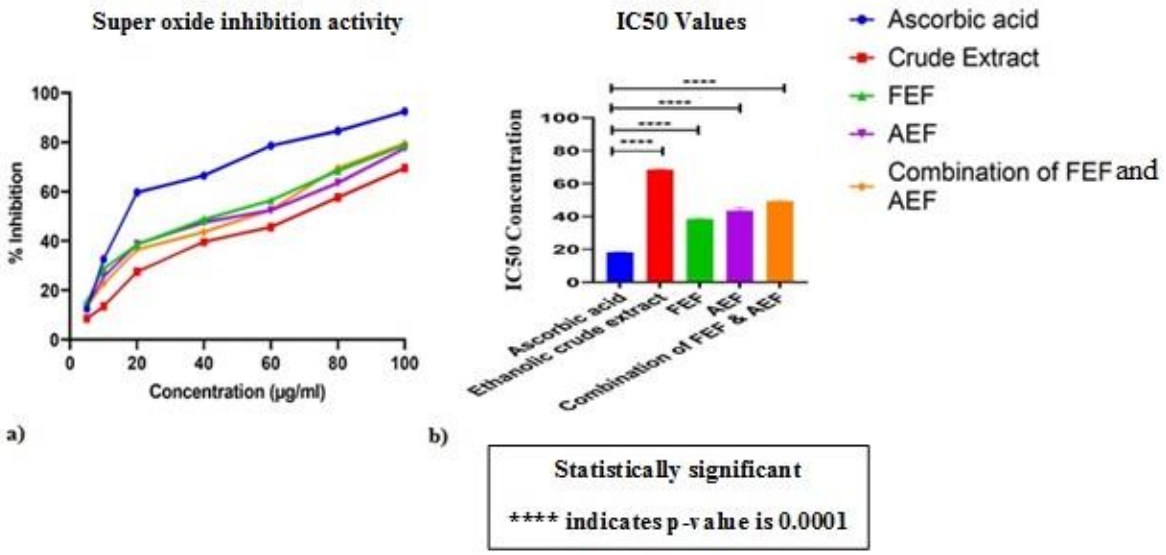
4.3.2.2. Inhibition of super oxide generation

The superoxide anion radical is one of the most strong reactive oxygen species among the free radicals generated. Despite the fact that O_2 is the most common free radical in the biological system, it is relatively unreactive on its own. On the other hand, this system, transforms it into more reactive species, such as H_2O_2 and OH radicals (Bhaskar *et al*., 2021). The combination of AEF and FEF had a higher superoxide inhibition activity than ascorbic

acid, showing that the constituents of the candidate plant are potent superoxide radical inhibitors.

The super oxide inhibition results revealed that ethanolic crude extract and all fractions were able to inhibit nitric oxide production; however, among the groups tested, the combination of AEF and FEF group exhibited greater inhibition activity (Figure 42a), with IC50 values which were found to be 18.17µg/ml for Ascorbic acid, 68.37µg/ml for crude extract, 38.47µg/ml for FEF, 43.60µg/ml for AEF and 49.28µg/ml for combination of FEF and AEF (Figure 42b) respectively.

Figure 42: Inhibition of superoxide generation by the *Annona muricata* Ethanolic Crude Extract and fractions



a) % Inhibition activity of ethanolic extract and fractions against superoxide, b) IC50 concentration of ethanolic extract and fractions of *Annona muricata*

Superoxide radical species generation is important in redox cell signaling and the development of pathophysiological conditions. These species are thought to be relatively unresponsive to the majority of biological substrates. However, as various authors have highlighted, $O_2^{\bullet-}$ is a significant precursor of a range of potent oxidants, making the evaluation of extract inhibitory activity against this species is extremely relevant (Francisco *et al*., 2018).

The inhibitory capacity of *Argyreia pierreana* and *Matelea denticulata* ethanolic extracts, and aqueous extract of *Matelea denticulata* was comparable to that of ascorbic acid in a superoxide free radical inhibition assay, whereas the scavenging activity of aqueous extract of *Argyreia pierreana* was significantly lower than the other extracts and standard. Interestingly, the aqueous extract of *Matelea denticulata* plant leaf exhibited inhibition action against superoxide free radicals that was comparable to ethanol extract and vitamin c, rather than OH and DPPH radicals. The total polyphenolics of ethanol extracts were observed to be greater in plant extracts, suggesting that phenolic and flavonoid content may have a role in therapeutic efficacy. (Gudise *et al*., 2019).

The initial free radical generated by mitochondrial electron transport systems is superoxide anion, which is a reduced form of molecular oxygen and are precursors to activate and generate free radicals and been reported that, it causes the chain reactions of oxidative degradation of lipids on its own. The effect of taxoquinone on superoxide radical generation inhibition was investigated, and the results revealed that taxoquinone inhibited superoxide radicals strongly in a concentration-dependent manner. Some phenolic compounds have been shown to have antioxidant properties that function via inhibiting the generation of superoxide anion radicals (Bajpai *et al*., 2017).

The bioactive compound (Annonacin) showed minimal antioxidant activity when compared to *Annona muricata* leaves and fruits extracts, this may be because of the synergistic action of several phytocompounds in the plant such as terpenes, glycosides, isoquinoline alkaloids, steroids, saponins, tannins, polyphenols and flavonoids

Furthermore, variations in solvent polarity, which selectively extract different hydrophobic or hydrophilic chemical moieties in the sample's phenolic compounds, may account for the variations in antioxidant activity between the extracts (Roduan *et al*., 2019).

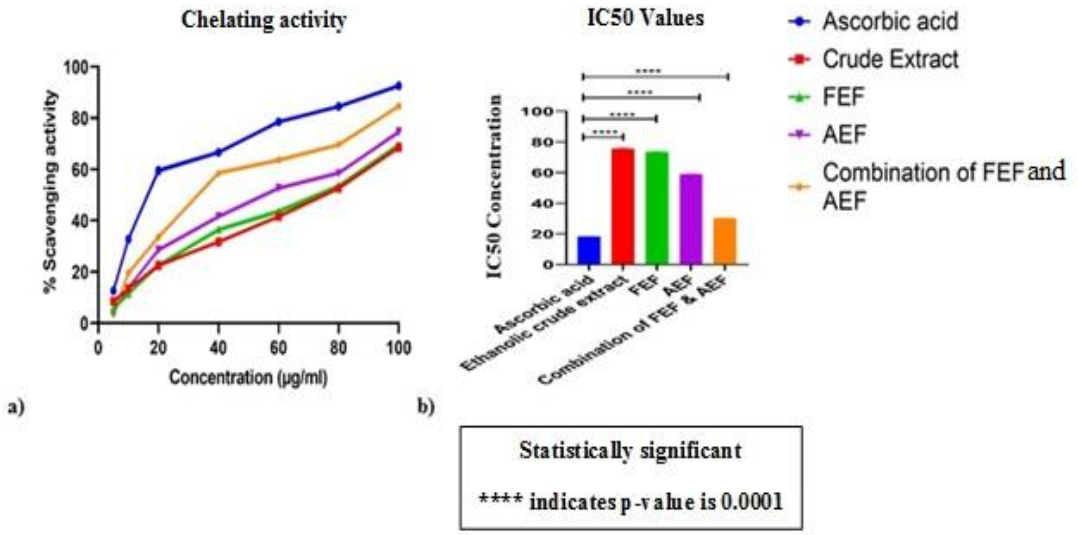
In line with the cited literatures, the current study findings suggest that the several phytochemicals present in *Annona muricata*, especially phenolic compounds and acetogenins, might be responsible for super oxide inhibition, thereby preventing the lipid peroxidation and initiation of free radicals' generation in the mitochondrial electron transport system.

It is clearly evident that the extract with the combination of flavonoids and acetogenins exhibited strong antioxidant potential and scavenging action against the radicals tested in the present study. The reason behind the increased efficacy of the combined fractions might be due to the synergistic action exerted by the team of phytoconstituents present in *Annona muricata* leaves, namely, glycosides, acetogenins, terpenoids, flavonoid and polyphenols. More studies need to be conducted to understand the characteristics of phytoconstituents and their synergistic impact against antioxidant and anticancer activity.

4.3.3. Chelating activity

In cellular lipid peroxidation determination, the fenton reaction is used to reduce the concentration of the catalytic transition metal in metal chelating activity assays. This type of chelating reaction is thought to be important in lowering oxidative stress caused by reactive oxygen species (Abd *et al*., 2020).

Figure 43: Chelating activity of the *Annona muricata* Ethanolic Crude Extract and fractions



a) Chelating activity of ethanolic extract and fractions, b) IC50 of ethanolic extract and fractions of *Annona muricata*

The metal chelating action of the ethanol extract and chromatographically obtained fractions show a significant chelating activity, among the groups tested the combination of FEF and AEF group exhibited greater chelation activity (Figure 43a), and the half maximal

inhibitory concentration were determined to be 18.19µg/ml for Ascorbic acid, 75.84µg/ml for crude extract, 73.48µg/ml for FEF, 59.01µg/ml for AEF and 30.37µg/ml for combination of FEF and AEF (Figure 43b) respectively.

According to Abd *et al*. (2020), *Melaleuca cajuputi* flower extract had substantial chelating activity when compared to *Melaleuca cajuputi* leaf extract, and the chelating activity was concentration-dependent. Agu *et al*. (2020) reported that the iron-chelating abilities of *Annona muricata* leaf and fruit extract possess good chelating action, compared with the standard (EDTA). *Annona muricata* roots, twigs and leaf extract has significant chelating action, according to Nam *et al*. (2017).

Lipid hydroperoxides are decomposed by iron generating reactive free radicals. Chelating drugs inhibit the production of the Fe^{2+}-ferrozine complex, resulting in a reduction of red colour solution. Metal chelating activity was found in A. *nilotica* leaf extracts (Yadav *et al*., 2018).

Annona cherimola Mill peel extract has higher metal chelating activity than pulp extract. The various phenolic hydroxyl group compounds present in the fruit may be responsible for the high antioxidant capacity and free radical scavenging action (Jamkhande *et al*., 2017).

Phenolic chemicals are important bioactive components that can neutralise free radicals, chelate metal catalyst, and prevent the action of oxidising enzymes in biological systems (Gowsalya *et al*., 2021).

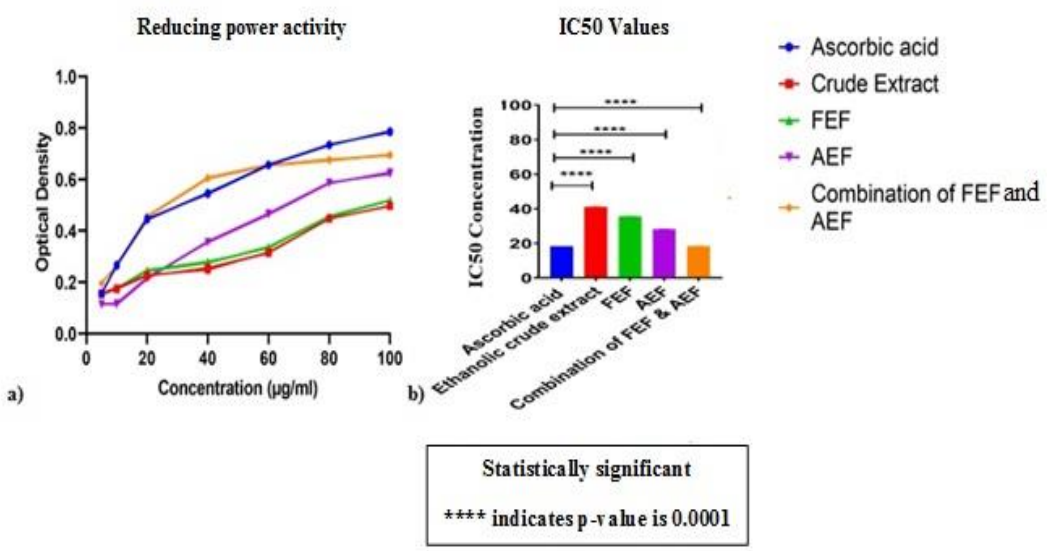
Iron is an important transition element in the body, and too much of it could result in an iron overload. Excess of iron, produce ROS and results in impairment of cellular activities. As a result, chelation of metal ions with chemicals derived from plants can help to reduce the generation of ROS. In line with the aforementioned literature, the current findings suggest that the chelating activity of *Annona muricata* might help in the reduction of ROS formation there by preventing the impairment of cellular functions.

4.3.4. Reducing power assay

Reducing agents are yet another name for antioxidants. Their capacity to give electrons allows them to convert greater valence elements to reduced valence states, as well as eliminate free radicals as well as other oxygen-derived ROS. The reducing power of

antioxidants, which is a key indicator of antioxidant activity, is measured via redox reactions with various metal ions (Shahidi and Zhong, 2015).

Figure 44: Reducing power ability of the *Annona muricata* Ethanolic Crude Extract and fractions



a) Reducing power activity of ethanolic extract and fractions, b) IC50 of ethanolic extract and fractions of *Annona muricata*

The ability of *Annona muricata* ethanolic leaf extract and fractions to convert Fe^{3+} to Fe^{2+} at various concentrations was used to determine their reducing capacity. The results showed that at a concentration of 100µg, all of the treatment groups were able to reduce Fe^{3+} ions (Figure 44a), with IC50 values of 18.21µg/ml for ascorbic acid, 41.12µg/ml for crude extract, 35.52µg/ml for FEF, 28µg/ml for AEF, and 18.39µg/ml for a combination of FEF and AEF (Figure 44b) respectively.

The antioxidant capacity to reduce Fe^{3+} to Fe^{2+} is measured in the reducing power assay. Polysaccharide extracts from *Annona muricata* leaves demonstrated dose-dependent ferric reduction properties (Kim *et al*., 2020).

To evaluate the reductive capability, Fe^{3+} was converted to Fe^{2+} in the presence of ethanolic, methanolic extract of *Cissus quadrangularis* (L.) and the reference compound quercetin and the results revealed that ethanolic extract possess considerable activity (Dhanasekaran, 2020). Polyphenolics, which are electron donors of high quality, have

reducing power, and may give an electron to decrease Fe^{3+} to $Fe^{2+,}$ were shown to have the maximum reducing power in the H. *nepalensis* hexane fraction. (Jafri *et al*., 2017).

The reducing capacity of *Vernonia amygdalina* and *Annona muricata* ethanol leaf extracts converted iron (III) to iron (II), in a dose dependent manner (Usunomena *et al*., 2017).

The reducing power assay is frequently used to assess an antioxidant's ability to donate an electron, which is a key mechanism of phenolic antioxidant action. Presence of antioxidants in the ethanolic leaf extracts of *Vernonia amygdalina* and *Annona muricata*, the ferric cyanide complex (Fe^{3+}) was reduced to the ferrous cyanide form Fe^{2+}. A compound's reducing capacity is usually determined by the presence of reductones (antioxidants), which serve as antioxidants by breaking the free radical chain by donating a hydrogen atom. Reductones are abundant in leaf extracts. As a result, the leaf extracts may function as electron donors, converting free radicals into more stable molecules and thereby stopping radical chain reactions. This action of both plants could suggest their ability to reduce oxidative damage to some of the body's most essential organs (Usunomena *et al*., 2015).

According to the reducing power assay, secondary metabolites present in the plant extract act as primary and secondary antioxidants by donating an electron and reducing the oxidised intermediates of the lipid peroxidation process (Rajakumar *et al*., 2018). The reducing power of the *Annona muricata* extract and fractions were found to increase as the concentration of the plant extract and fractions increased, indicating that phytochemicals in the *Annona muricata* act as both electron contributor and has the ability to react with free radicals and terminates sequential reactions., thereby causing a reduction in the oxidative stress mediated cellular damage. Thus, it can be inferred that, the phytoconstituents present in the extracts of *Annona muricata* play major role in the scavenging of oxidants and hence the phytoconstituents of this plant would be a suitable lead molecule for the discovery of drugs with good efficacy against ROS mediated diseases like cancer.

Phase III

4.4. Synergistic effect and assessment of anticancer activity of Ethanolic crude extract, Flavonoid (FEF), Acetogenin (AEF) enriched fraction and combination of FEF and AEF of *Annona muricata* leaves against MOLT -3 and PBL cells

Cancer is one of the world's major causes of death, and the development of new anticancer treatments is essential due to its prevalence. Due to strong population growth and life expectancy, cancer cases are continuously growing, with a projected 75 percent increase by 2030. As a result, it is critical to develop novel cancer treatment procedures in order to reduce patient suffering and expenses associated with today's costly treatments. There are several drugs available to treat various types of cancer, but none of them are perfectly effective or safe. Chemotherapy has a number of drawbacks, including toxicity to the body's healthy cells, which can result in significant side effects such as fatigue, hair loss, anaemia, easy bruising and bleeding. Natural products and their derivatives have shown significant potential for the development of chemotherapeutics, with a wide structural diversity and favourable pharmacological and molecular features (Majolo *et al*., 2019).

Acute lymphoblastic leukaemia (ALL) is a type of cancer that develops in the bone marrow from half-grown cells. Leukemic cells spread quickly through the bloodstream and throughout the body. However, it mostly affects the blood and bone marrow. Various genetic approaches have been employed to increase the malignancy of transformed cells and their offspring in order to clone leukemic cells. Leukemia is the term for an increase of hematogenic cells. In acute leukaemia, the bone marrow contains more than 20% blasts. However, if it is not detected and treated in a timely manner, it can progress quickly and take one's life in a matter of months (Rehman *et al*., 2018).

One of the most common characteristics of cancer is cell death evasion. Apoptosis has the physiological purpose of maintaining a balance between cell death and proliferation. Apoptosis is a multistage process with two distinct pathways, that initiate a series of events that result in chromatin fragmentation and nuclear membrane fragmentation. When this physiological process is disrupted, various pathological alterations occur, including the development of cancer. As a result, researchers are looking for a technique to activate this mechanism in cancer cells, which could have a positive impact on cancer formation and

progression. Plant extracts and natural compounds originating from plants are such novel anticancer treatment prospects (Rajabi *et al*., 2021).

Over the last two decades, patients diagnosed with cancer have been increasingly turning to natural products as supplement or as conventional traditional treatment. Cancer patients are rapidly turning to *Annona muricata* (Chan *et al*., 2020). The active phytocompound present in *Annona muricata* leaves is acetogenin, which kills cancer cells by inhibiting NADH synthesis without damaging healthy cells (Ravi *et al*., 2018). Acetogenins are anticancer compounds that kill tumour cells through a variety of methods. They have the ability to modify chemotherapeutic medicines and are potent inducers of apoptosis. Their pharmacological versatility is evident in their capacity to block cells during the cell cycle, promote apoptosis through protein inhibition, and even induce autophagy (Herrera *et al*., 2019). Thus in the present study, cytotoxic activity, apoptotic inducing property, antiproliferative activity and ROS levels were evaluated using the *Annona muricata* leaf ethanolic extract and it fractions obtained by bio-assay guided fractionation procedure.

4.3.1. Cytotoxic activity

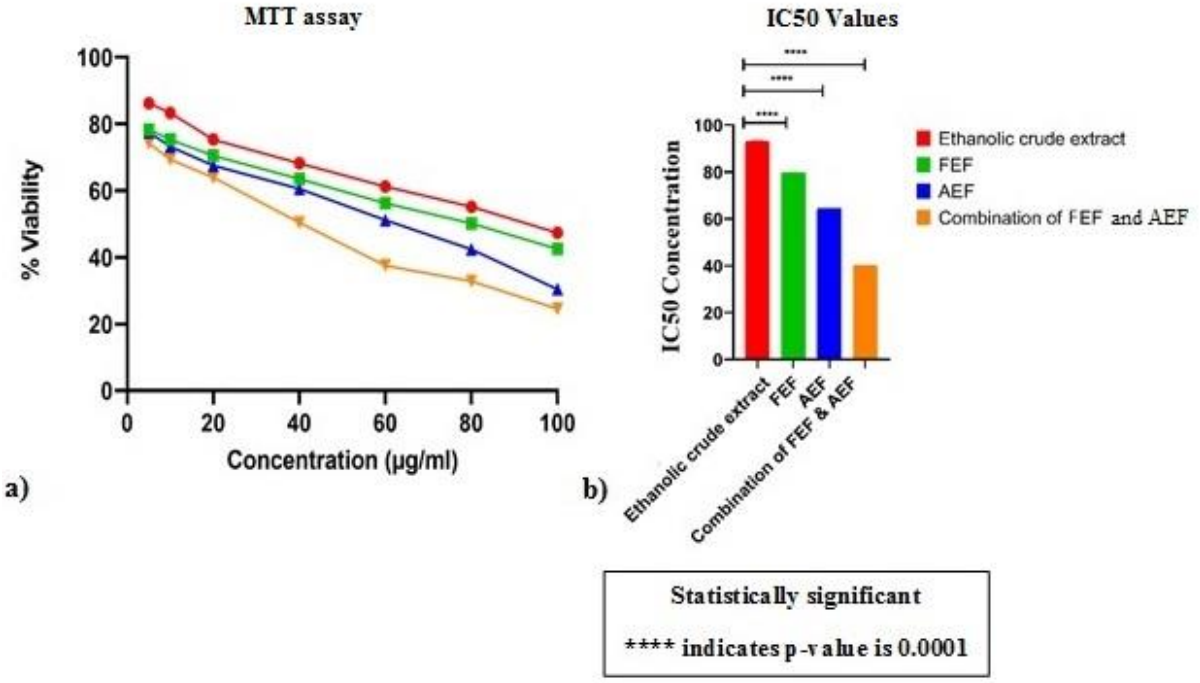
4.3.1.1. Cytotoxic effect of *Annona muricata* Ethanolic Crude Extract and fractions by MTT assay in Molt-3 cells and PBL

The colorimetric MTT assay is based on the conversion of yellow colour soluble MTT tetrazolium salt to a blue colour insoluble MTT formazan product by mitochondrial uptake and succinate dehydrogenase. It determines the functional state of mitochondria, indicating cell viability. MTT is broken by the mitochondrial enzyme dehydrogenase in living cells, releasing the purple product formazan. The amount of formazan generated is inversely proportional to the degree of cytotoxicity and proportionate to the number of live cells (Vajrabhaya and Korsuwannawong, 2018).

To determine the cytotoxic effect of the ethanolic leaf extract of *Annona muricata* and its chromatographically fractions; Flavonoid Enriched Fraction (FEF), Acetogenin enriched fraction (AEF) and Combination of Flavonoid and Acetogenin enriched fraction (FEF and AEF), MTT assay was performed in Molt-3 cell line. Various concentrations of *Annona muricata* ethanolic leaf extract and obtained fractions ranging from 5, 10, 20, 40, 60, 80, and 100g/ml were used in the study. The control cells viability was set to 100%, and the values for the other groups were calculated accordingly.

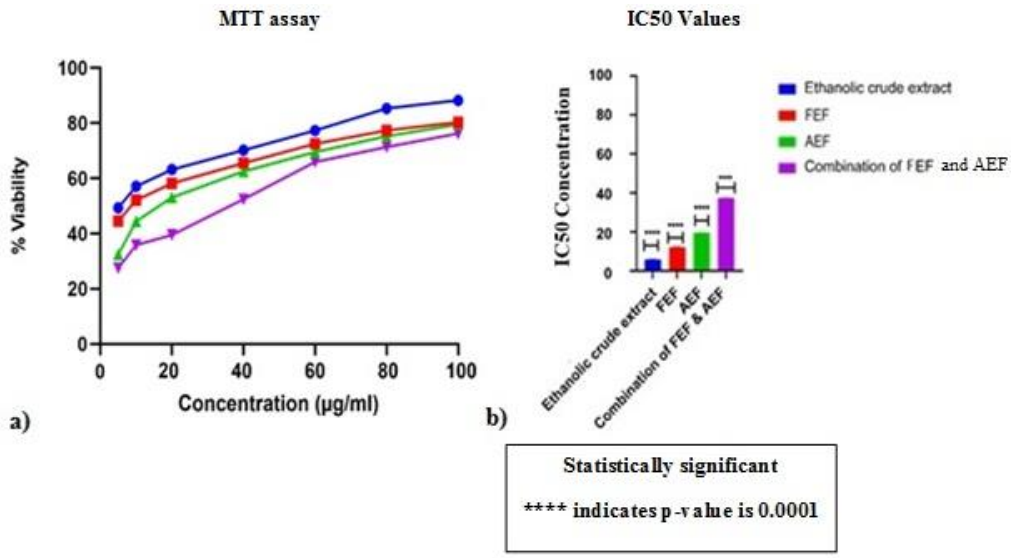
The results reveal that the *Annona muricata* ethanolic leaf extract and its fractions were capable to persuade cell death even at 5µg concentration. When the Molt-3 cell, line was exposed to increasing concentrations of *Annona muricata* ethanolic leaf extract and its fractions for 24 hours, cell mortality was found to be higher than in the control group. Among the treatment groups tested, the combination of FEF and AEF rendered good cytotoxicity, followed by AEF, FEF and crude extract at a concentration of 500µg towards the Molt-3 leukemic cell lines and the IC50 values were found to be 93.28µg/ml for ethanolic crude extract, 79.67µg/ml for FEF, 64.37µg/ml for AEF and 40.21µg/ml for the combination of FEF and AEF respectively (Figure 45).

Figure 45: Cytotoxic effect of *Annona muricata* Ethanolic Crude Extract and fractions by MTT assay in Molt-3 cells



a) Cytotoxic effect of ethanolic extract and fractions in Molt-3 cells b) IC50 concentration of ethanolic extract and fractions of *Annona muricata*

Figure 46: Cytotoxic effect of *Annona muricata* Ethanolic Crude Extract and fractions by MTT assay in PBL



a) Cytotoxic effect of ethanolic extract and fractions in PBL b) IC50 concentration of ethanolic extract and fractions of *Annona muricata*

Since the crude ethanol extract and fraction treated groups could induce cell death in leukemic cells, it became imperative to assess the ability of the extracts and fractions in normal cells (PBL), and hence the cytotoxic effect of the crude extract and fractions were tested against the normal peripheral blood lymphocytes by MTT assay.

The results revealed that when increasing the concentration of the fractions, the cell death was found to be decreased and the IC50 values were found to be 5.28µg/ml for ethanolic crude extract, 10.21µg/ml for FEF, 18.92µg/ml for AEF and 37.67µg/ml for combination of FEF and AEF respectively (Figure 46), which confirms the non-cytotoxic nature of treatment groups in the normal counterpart –PBL and targeting cancerous cells alone.

Roduan *et al*. (2019) reported, dose dependent cell death by the *Annona muricata* leaf ethanolic extract (AMLE), hexane fraction (AMLH), dichloromethane fraction (AMLD), methanolic fraction (AMLM), leaf aqueous extract (AMLA), fruit water extract (AMFA) and annonacin treatment in Raji cell lines. Cytotoxic activity of leaf and fruit *Annona muricata* extracts might be due to the presence of annonacin (acetogenin). As a result, it may have synergistic action with other acetogenins.

Methanolic leaf extract of *Annona muricata* shows the growth inhibitory effect on the proliferation of MCF-7 cells and did not affect the proliferation of non-tumorigenic MCF-10A cells, when compared to tumorigenic cells (Naik *et al*., 2020).

A study by Salsabila *et al*. (2021), showed that leaf extract of *Annona muricata* (AME) possessed a cytotoxic effect on 4T1 cells in a dose-dependent manner, the combination of AME and doxorubicin caused a strong cytotoxic effect, when compared to single treatment with doxorubicin. These effects might be due to the presence of phytocompounds such as acetogenin, annomuricin, annonacin, and flavonoids present in the AME.

Kim *et al*. (2018) studied the cytotoxic activity in MDA-MB-231 cells and the results showed dose dependent activity, the active ingredients in the *Annona muricata* played a major role in cytotoxic effect

Leaf extract of *Simarouba glauca* effectively inhibited the growth of K-562, MOLT-3 and KG-1 in a dose-dependent manner. The inhibitory activities of *Simarouba glauca* leaves extract against MOLT-3 is highest as compared to other two cell lines (K-562 and KG-1). Phytochemicals present in leaves might have played a major role in anticancer activity (Prajapati *et al*., 2018).

Essential oil of C. *odorata* leaves, revealed the anticancer action on the MOLT-3 cell line in a concentration dependent pattern. At lower levels, cancer cell decomposition is minimal, while almost all cancer cells break down at 100µg/ml. The essential oil of C. *odorata* leaves has significant anticancer activity due to the complex mixture of various terpenes present in the essential oil that could act as a potent anticancer agent (Vimaladevi *et al*., 2021).

The extracts and fractions of A. *muricata* leaves were cytotoxic to breast cancer cells (MCF7) but had no effect on non-cancerous CV1 cell growth, demonstrating that they are neither toxic nor suppressive to non-cancerous cell growth. As a result, the chemicals in leaves of A. *muricata* have a specific inhibitory effect on malignant cells. This might be due to the existence of polyphenols, saponins, tannins, phenols, and flavonoids in this fraction, especially acetogenin and flavonoids, which are also prominent cytotoxic chemicals. The inclusion of flavonoids in *Annona muricata*, as well as acetogenin as an active ingredient, gives additional benefits for greatest therapeutic effects (Hadisaputri *et al*., 2021).

Since acetogenins are abundant in numerous plant portions, the A. *muricata* extracts cytotoxic activity could be linked to their presence. These acetogenins are polyketide pathway products of long chain fatty acids that have been proven to be selectively toxic to malignant cells, including multi-drug resistant malignant cell lines. The ATP requirement of cancer cells is greater than that of normal cells, these compounds work by blocking the mitochondrial complex I of the electron transport chain, which is involved in oxidative phosphorylation and ATP synthesis. The absence of an electrochemical gradient causes ATP deficit because no hydrogen ions are forced into the inner mitochondrial space as a result of the inhibition of mitochondrial complex I (Naik and Sellappan, 2021).

The cytotoxicity of the "Squamocin and Bullatacin (Acetogenin) " against three tumor cell lines (A549, HeLa, and HepG2) were evaluated using MTT assay. In comparison to Squamocin and Bullatacin, the majority of these compounds have anticancer efficacy against all three tumour cell lines. Acetogenins are effective inhibitors of mitochondrial complex I in the electron transport system, as well as the NADH oxidases found in cancer cell plasma membranes (Shi *et al*., 2020).

The results of current finding suggest that the presence of flavonoids, acetogenins and other secondary metabolites in the *Annona muricata* ethanolic leaf extract might be accountable for the cytotoxic effect against leukemic cell line (Molt-3) and non-cytotoxic effect against normal counterpart peripheral blood lymphocytes (PBL). The above findings strongly hold up that *Annona muricata* leaf fractions (AEF and FEF) targeting cancerous cells alone (Molt-3), without harming the normal healthy cells (PBL), might have attributed by the presence of more acetogenins and flavonoids which could be responsible either for the inhibition of NADH oxidase or ATP starvation in mitochondria, thereby inhibiting the proliferation rate of leukemic cells compared to PBL as they require more ATP for their survival compared to normal cells.

4.3.1.2. Cytotoxic effect of *Annona muricata* Ethanolic Crude Extract and fractions as determined by SRB assay in Molt-3 cells and PBL

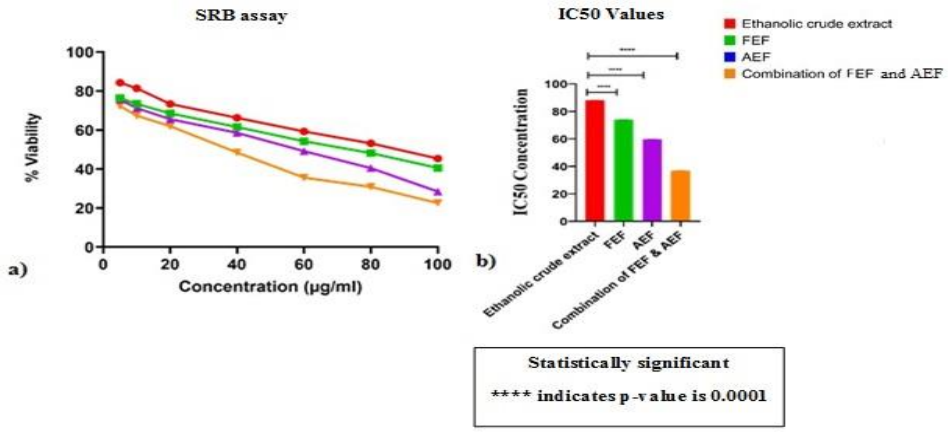
To assess the efficacy of chemical libraries, anticancer drug research programmes use a vast variety of *in vitro* assays. In order to choose potent lead candidates for subsequent (pre)clinical investigations, the accuracy and reliability of these *in vitro* assays are essential.

The sulforhodamine B (SRB) assay has been a common choice among regularly used cell viability assays due to its simplicity, precision, dependability, and reproducibility. The SRB dye interacts with the basic amino acids in proteins, and the number of viable cells is determined by the amount of protein in the cells (Shakil *et al*., 2022).

In SRB assay, the results demonstrated that all the treatment groups exhibited significant cytotoxicity against Molt-3 leukemic cell line. Different concentrations of *Annona muricata* leaf ethanolic extract and fractions ranging from 5, 10, 20, 40, 60, 80, and 100µg/ml were used to treat the cells for a day, cell death was observed in a dose dependent manner. It is evident from the graph (Figure 34) that the combination of FEF and AEF rendered good cytotoxic activity followed by AEF, FEF and ethanolic crude extract.

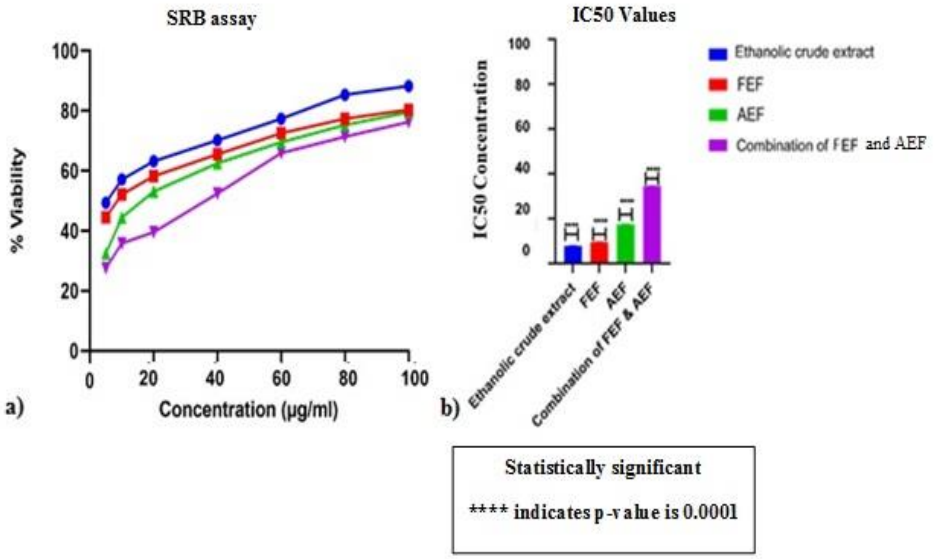
The PBL were treated with varying concentration (5-100µg/ml) of the *Annona muricata* leaf ethanolic extract and fractions for a day, and the results revealed that the cell death was found to be decreased, when increasing the concentration of the extract and fractions (Figure 47), and the IC50 values were found to be 4µg/ml for ethanolic crude extract, 15µg/ml for FEF, 19.8µg/ml for AEF and 39µg/ml for combination of FEF and AEF respectively (Figure 48), which again confirms the non-cytotoxic nature of treatment groups in the normal counterpart –PBL and targeting cancerous cells alone.

Figure 47: Cytotoxic effect of *Annona muricata* Ethanolic Crude Extract and fractions as determined by SRB assay in Molt-3 cells



a) Cytotoxic effect of ethanolic extract and fractions in Molt-3 cells b) IC50 concentration of ethanolic extract and fractions of *Annona muricata*

Figure 48: Cytotoxic effect of *Annona muricata* Ethanolic Crude Extract and fractions as determined by SRB assay in PBL



a) Cytotoxic effect of ethanolic extract and fractions in PBL b) IC50 concentration of ethanolic extract and fractions of *Annona muricata*

SRB assay revealed that the cytotoxic activity was found to be higher in breast cancer cells treated with *K. Africana* methanol extract, when compared to 50 percent methanol in dichloromethane. The inclusion of flavonoids, triterpenes, and phenolic components in the methanol and dichloromethane extracts is thought to be responsible for their anti-cancer properties (Mukavi *et al*., 2020).

In a dose-dependent way, the C. *majus* L. extract reduced the growth of both HeLa and CaSki human cervical cancer cells. The phytochemical component, particularly alkaloids and flavonoids, may have a role in the anti-cancer activity as a cytotoxic-inducing agent (Nile *et al*., 2021).

In colorectal cancer cell lines T84, HCT-15, SW480, and HT-29, ethanolic extracts of M. *oleifera*, T. *tuberosum*, and A. *cherimola* caused cell death. The cytotoxic activity of A. *cherimola* ethanolic extract against T84 cells was found to be higher, followed by *M. oleifera* extract against SW480 and HCT-15 cells, and *T. tuberosum* extract against T84, HCT15, and SW480 cell lines (Fuel *et al*., 2021).

Cytotoxic activity of the S. *fruticosa* hexane extract was tested against the cancer cell lines HCT-116, HepG2 and MCF-7 by SRB assay and the results revealed that the activity was found to be significant in HCT-116 followed by HepG2 and MCF-7 cell lines (Saleh *et al*., 2020).

Using the SRB assay, the cytotoxic impact of the *Senna alata* leaves ethanolic crude extract, fractioned aqueous, butanol, chloroform, dichloromethane and hexane extracts on human breast cancer cells line and non-cancer cell line was determined. Hexane fraction possess good cytotoxic activity against cancer cells, followed by dichloromethane, butanol, ethanol, chloroform, and aqueous extracts. On normal human mammary epithelial cells, the extracts had no cytotoxic impact (Chahardehi *et al*., 2021).

Jurkat and K562 leukemic cell lines were treated with leaves of *Z. rhetsa* in increasing doses of the extract for 24 hours, and the percentage viability of cells was determined by SRB assay. The results of cytotoxicity assay revealed that the viability of Jurkat cells was found to be dose-dependent in a methanol extract of leaves. Similar to that exhibited by the standard doxorubicin. In K562 cell lines, treatment with methanol extract of leaves did not exhibit significant dose-dependent decrease in viability of cell lines. As per the data, it can be concluded that K562 cells are resistant to the treatment with the methanol extract of the leaves of *Z. rhetsa* (Mallya and Bhitre. 2021).

The cytotoxic activity of extracts and fractions from A. *bracteosum* on the proliferation of NCI-H460 and HepG2 cancer cell lines was investigated using the SRB test. The chloroform fraction and ethanol extract had a significant cytotoxic effect on the cells at increasing dosage concentrations (Nguyen *et al*., 2020).

The cytotoxicity of *Mikania cordata* (MC), *Mikania micrantha* (MM), and *Mikania scandens* (MS) extracts against A549, SK-MEL-2, and B16-F1 tested, revealed that M. *micrantha* was shown to have the most potent cytotoxic impact, followed by MC and MM (Khatun *et al*., 2021).

The cytotoxicity of *C. pachystachya* leaves was observed against cell lines- HT-29, OVCAR-3, NCI-H460, MCF-7, U251 and NHI-3T3, the results revealed that the most promising activities were detected against HT-29, OVCAR-3, and NCI-H460 cell lines. The

actions of medicinal plants are generally related to the synergistic effect among several compounds present in the extracts (Pereira *et al*., 2020).

The results of cytotoxic action ethanolic crude extract of *Annona muricata* and fractions as determined by SRB assay in Molt-3 cells and PBL revealed that all the treatment groups were successful in inducing cell death in Molt-3 cell line whereas thay are non toxic to normal peripheral blood lymphocytes with the high performance observed in the combination of FEF and AEF, and this might be due to the synergistic interaction of flavanoids, acetogenins ad other secondary metabolites contained in the *Annona muricata* ethanolic leaf extract and chromatographically separated fractions.

The results of cytotoxic assays revealed that the fractions obtained from *Annona muricata* could exert more cytotoxicity in leukemic cells compared to the crude ethanolic extract, while non-toxic to the normal PBL indicating a possible synergistic action by the acetogenins and flavonoids forwards targeting the cancerous cells.

4.3.2. Synergistic effect of combination of Flavonoid Enriched Fraction (FEF) and Acetogenin Enriched Fraction (AEF)

Combenefit, a tool that evaluates "synergy/antagonism from dose-response data using three classical models, namely the Loewe, the Bliss, and the Highest Single Agent (HSA)", were used to quantify and visualise the level of synergy between two drugs.

It is critical to define a non-interactive effect before determining whether a combination of chemicals exhibits an interaction effect. Loewe Additivity and Bliss Independence are two fundamental ideas for non-interactivity that have withstood the critics. Loewe Additivity's appeal originates from its sham combination principle, which assumes zero interaction when a compound is coupled with itself. That assumption is not shared by other null reference models. Bliss Independence, assumes (statistical) independence between the combined compounds (Lederer *et al*., 2019). The combination impact should be equivalent to the greatest single agent effect, according to the highest single agent model (Amzallag *et al*., 2019).

The surface responses of FEF and AEF on Molt-3 cells are shown in Figures 49-51. The level of synergism is indicated by the dark blue coloured regions. The interaction between FEF and AEF was very certainly additive/synergistic over the entire dose-response

matrix, as shown by the Loewe synergy and antagonism surface map (Figure 49), with the largest synergistic effect centred at 60µg/ml of FEF and 80 or 100µg/ml of AEF. The plot, on the other hand, presented some antagonistic pairings. In general, there was a high level of agreement between the Loewe additive approach and the other two methods utilised (Highest Single Agent and Bliss) (Figure 50 and 51), indicating that the overall results for each combination were appropriate.

Figure 49: Loewe model - Synergistic effect of FEF and AEF

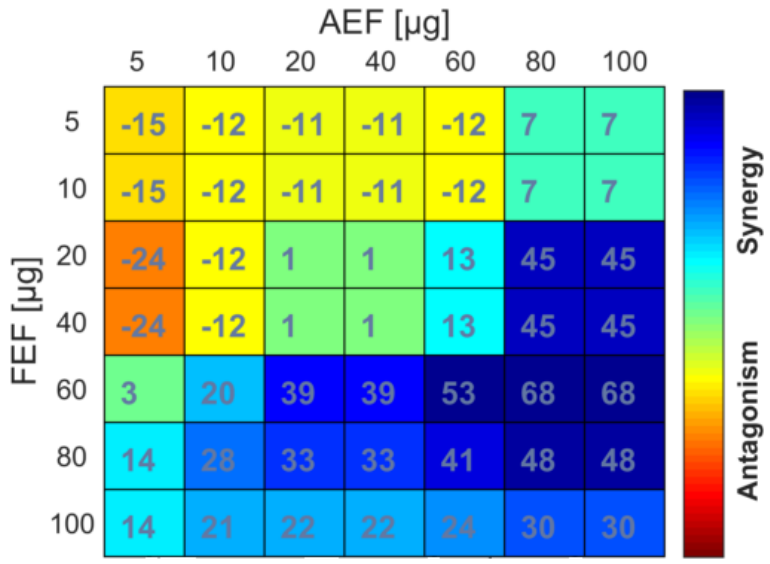


Figure 50: Highest Single Agent model - Synergistic effect of FEF and AEF

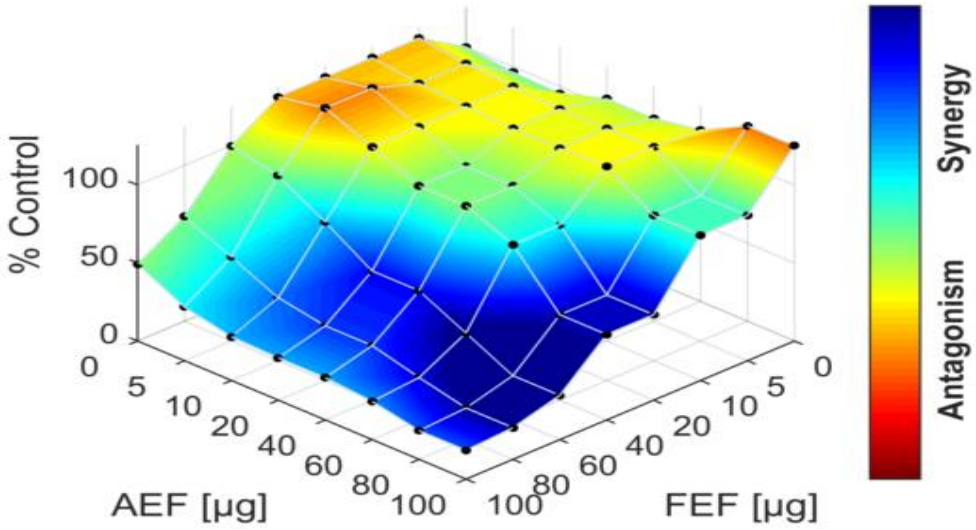
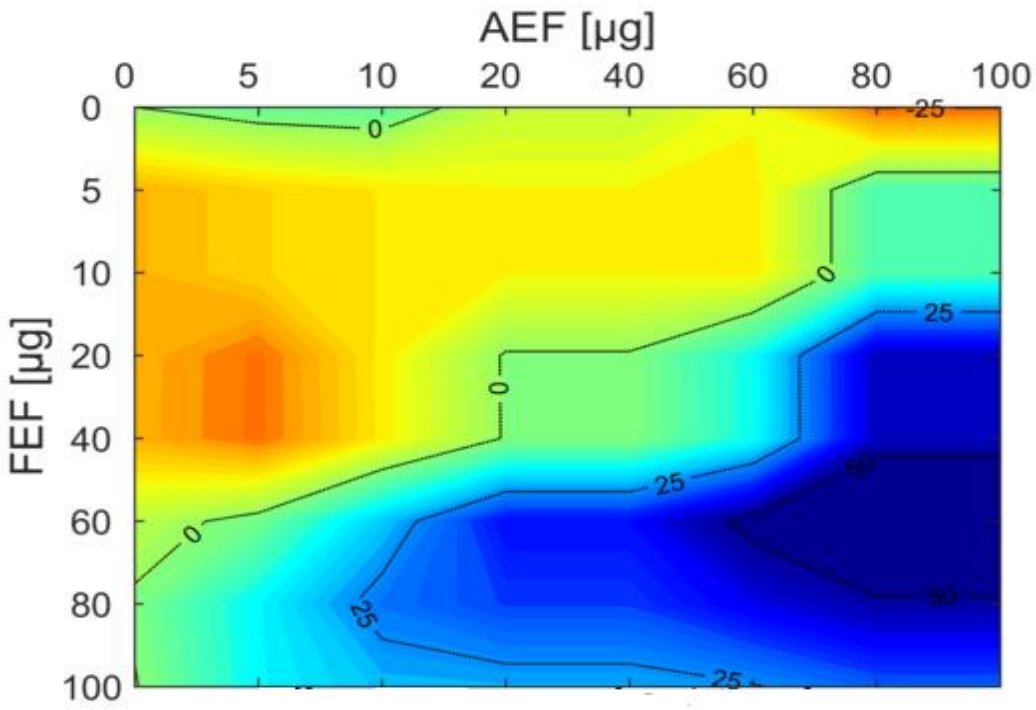


Figure 51: Bliss model - Synergistic effect of FEF and AEF



At inhibition levels of 80%, the low CI values suggest a substantial synergism of anthocyanin and gingerol extracts against the growth of Caco-2 and Hep G2 cells (Abdurrahim *et al*., 2021).

Sigva *et al*. (2019) used "CalcuSyn v 2.1 (BioSoft)" software to calculate the synergetic effect of paclitaxel and sulphoraphane in the PC-3 cell line, and the findings revealed that paclitaxel 3.08µM dose and sulphoraphane 73µM dose had the maximum synergistic effect.

Hii *et al*. (2019) reported that the investigation of synergistic effect between *Clinacanthus nutans* Lindau stem extracts (SN) and gemcitabine, in the pancreatic cancer cells reveals that the combination group produced considerable synergism to suppress the pancreatic cells, according to CI values and combination analyses based on the HSA and Bliss independence models. The β-eudesmol (BE), hinesol (HS), and atractylodin (AT), hinesol (HS) combinations produced a synergistic effect against Cholangiocarcinoma cell line (CL-6) (Martviset *et al*., 2018).

These reports are supportive to our observations, and the results indicates strong synergism between Flavonoid Enriched Fraction (FEF) and Acetogenin Enriched Fraction (AEF) against the growth of Acute lymphoblastic leukemia cell line (Molt-3).

4.3.3. Measurement of apoptosis

4.3.3.1. Detection of apoptotic cell death by flow cytometry

Annexin V and propidium iodide staining were used to determine the mode of cell death in treated cancer cells with *Annona muricata* leaf extract and its fractions.

The mechanism of cell death was evaluated using flow cytometry analysis with Annexin V-FITC/ PI. The results showed that, in the control group, a higher percentage of cells were viable compared to the other treatment groups. Administration of FEF and AEF in combination showed apoptotic cell death and more cells were seen in the early and late apoptosis stage (Figure 52 and 53). The results obtained for ethanolic crude extract, FEF, AEF of *Annona muricata* and combined treatment showed a drastic increase in number of cells undergoing cell death in the early and late apoptotic phase.

Figure 52: Detection of cell death by Annexin V/FITC- PI apoptosis staining by flow cytometry analysis in Molt- 3 cells

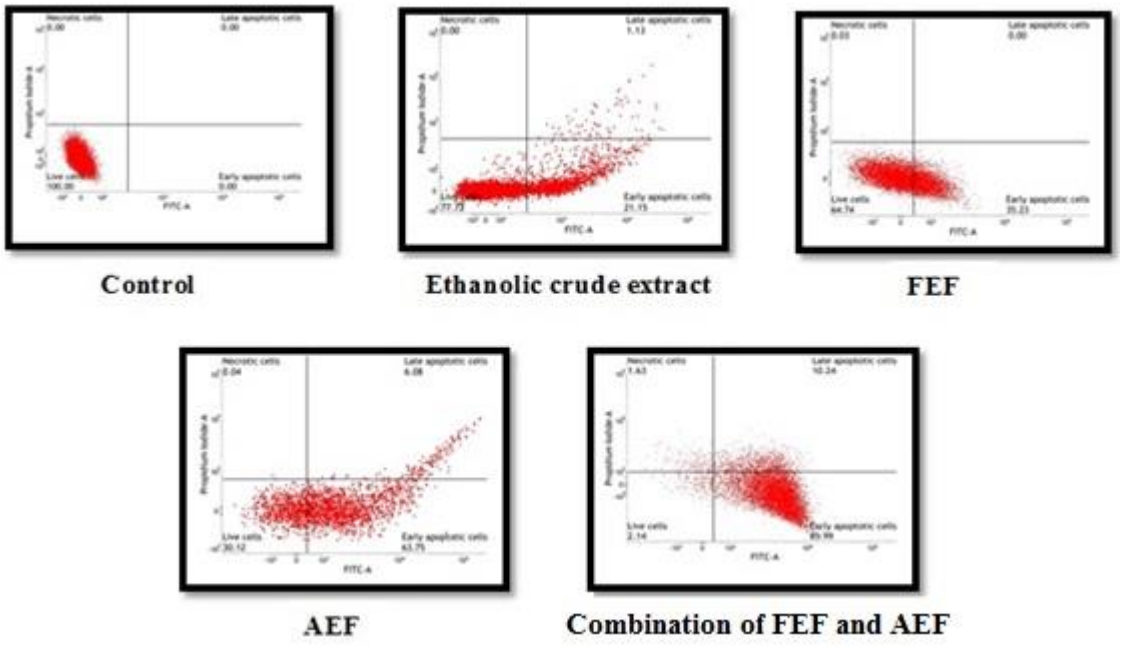
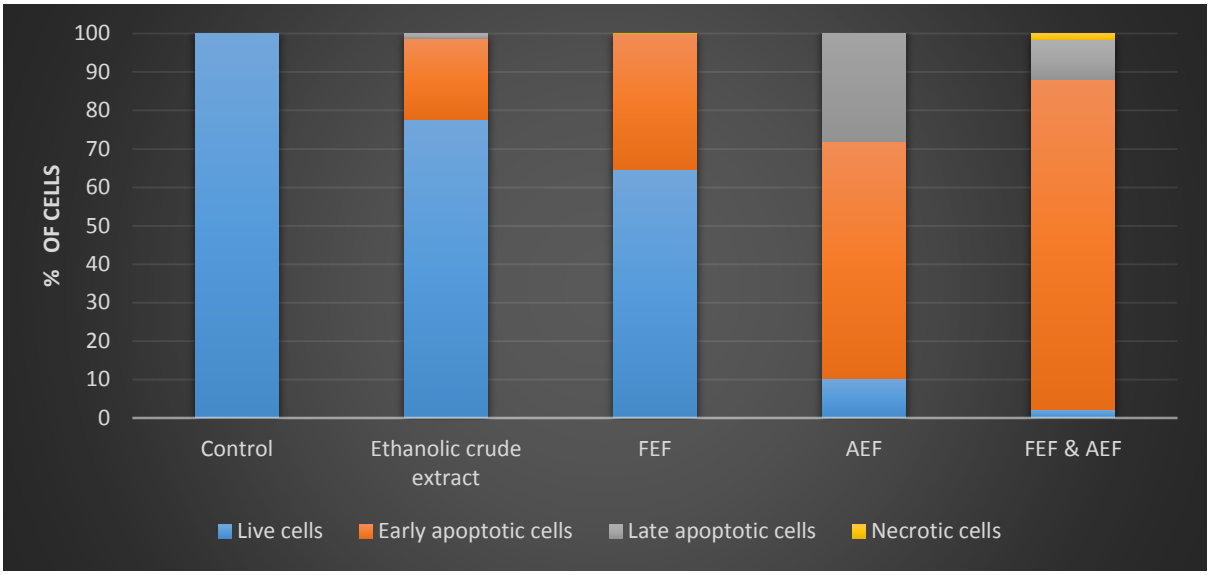


Figure 53: Distribution of Molt-3 cells in apoptosis phase



The results reveal that late apoptotic cells are observed in the group of cells treated with AEF and in the combination of FEF and AEF , indicating that AEF and combined fractions are very effective in their apoptosis inducing ability.

Figure 54: Detection of cell death by Annexin V/FITC- PI apoptosis staining by flow cytometry analysis in PBL

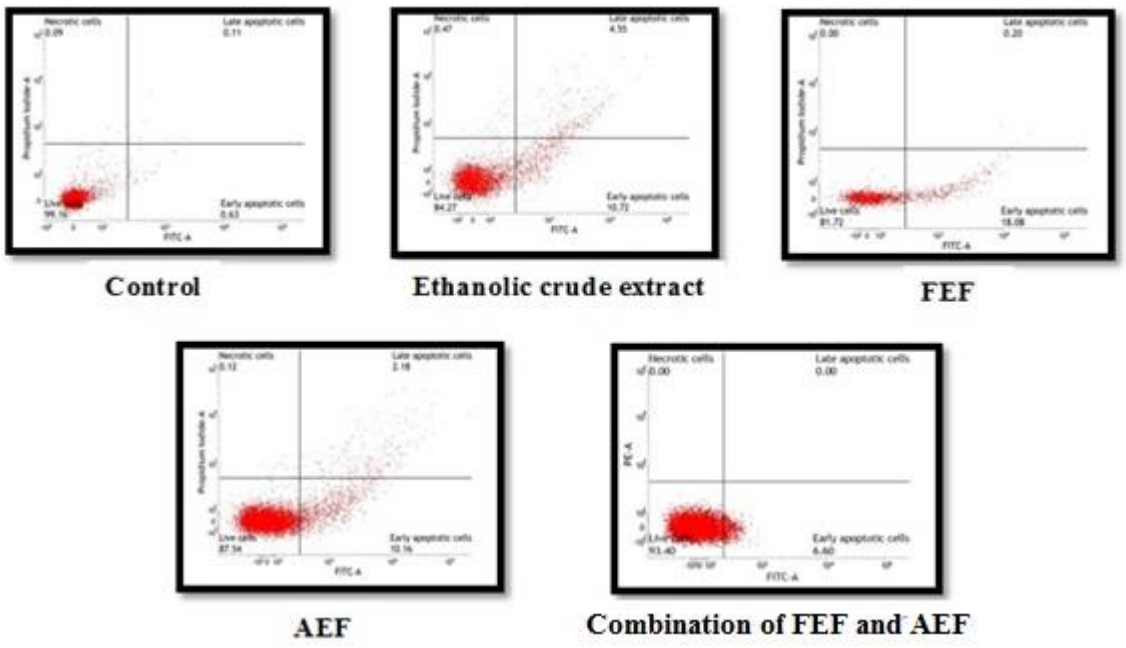
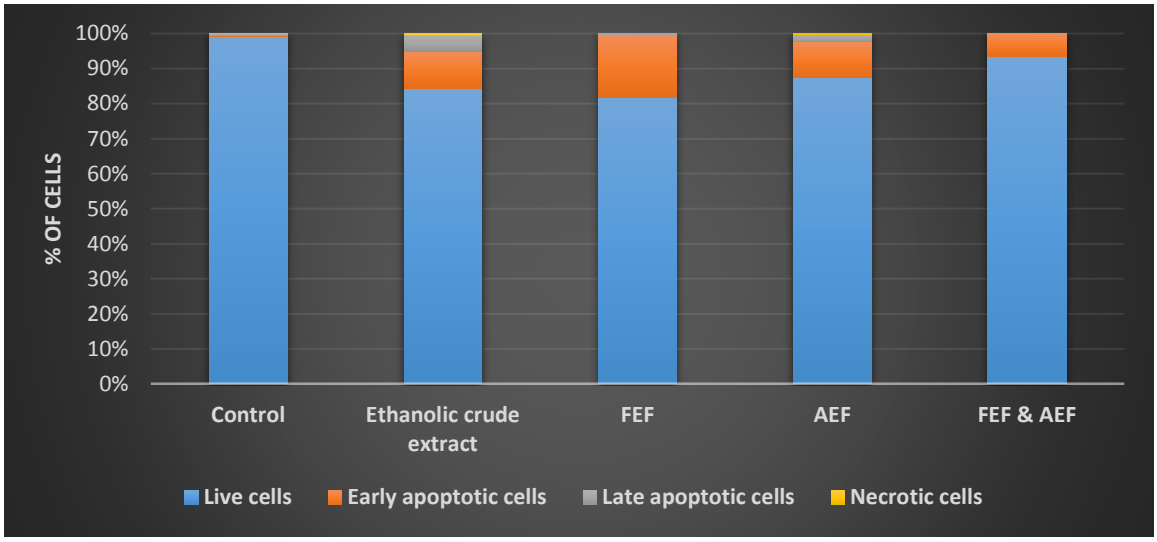


Figure 55: Distribution of PBL in live phase



In the case of PBL treated groups, the number of apoptotic cells were found to be very low, while some of the treated groups showed apoptotic cell death in early and late apoptosis (Figure 54 and 55), with lower proportion of apoptotic cells when compared to treated Molt-3 cell lines, which again confirms the non-toxic nature of the fractions in PBL. In the combined fractions treated group, 93.40% of the cells are in live phase which clearly depicts the protective action in normal cells.

When we compare the results observed in Molt-3 cells, and PBL. It can be inferred that the combined fractions treated group could exert cytotoxic action in leukemic cells and showing non-toxic effect in PBL indicates that the acetogenins could have acted in a synergistic manner with flavonoids. Also, cancer cells are targeted in a specific mechanism thereby inducing apoptosis only in cancer cells and rendering protection to PBL.

Sabapati *et al*. (2019) found that *Annona muricata* fruit extract had more cell death in early apoptotic phase. Acetogenins are particularly powerful against cancer because they interrupt the mitochondrial electron transport system, resulting in death, by depriving cancer cells of their high energy need for adenosine triphosphate (ATP).

To assess the mode of cell death, Ammoury *et al*. (2019), used Annexin V-Propidium iodide counterstaining, and the results showed that "terpene-rich *Annona cherimola* ethanolic leaf extract" triggered early apoptosis in Monomac-1 and KG-1 Acute Myeloid Leukemia cell lines. Soltanian *et al*. (2019) reported that after incubation of MCF-7 cells with the

essential oil of *Semenovia suffruticosa,* majority of cells exhibited cell death at an early apoptotic phase. In line with the previous study, Chothiphirat *et al*. (2019) found that after 48 hours of incubation with the acetone cotyledon extract, majority of HeLa and SiHa cells died in an early apoptotic phase.

B. frutescens methanolic (BME) and hexane extract (BHE) showed early apoptotic cell death in MDA -MB -231 cell population, in comparison with the non-treated cells (Kushwaha *et al*., 2019). According to Samarghandian *et al*. (2019) a quantitative examination of apoptosis using the annexin V/PI assay revealed that after thymoquinone exposure, the ratios of both late and early stage apoptotic cells considerably increased. Apoptosis analysis found that HepG2 cells treated with vernodalin, vernolepin, and vernolide compounds had more Annexin-V positive cell populations than untreated control cells (Thongnest *et al*., 2019). Attri *et al*., (2021) revealed that *Argemone mexicana* chloroform fraction in A431 cell line undergo early and late apoptosis.

The present study revealed that Molt-3 cells on treatment with combination of FEF and AEF *group* induced cell death mediated by apoptosis especially in the early and late apoptotic stage. In the case of PBL treated groups, the number of apoptotic cells were found to be very low, which clearly depicts the protective action in normal PBL cells.

4.3.3.2 Detection of mitochondrial membrane potential (ΔΨm) by – JC-1 staining in Molt- 3 cells and PBL

The loss of mitochondrial membrane potential (MMP) is a distinguishing sign of apoptosis in the early stages of apoptosis and indicative of cellular changes taking place during cell death (Mansour *et al*., 2018).

The untreated group has a greater number of cells existed in the form of J-aggregates. Ethanolic crude extract, FEF and AEF treated groups showed lesser number of cells was observed as monomers. In combination of FEF and AEF treated group, significant numbers of cells were found in the form of monomers when compared to ethanolic crude extract, FEF and AEF treated groups which indicates loss of membrane integrity and mitochondrial membrane potential (Figure 56 and 57).

Figure 56: Detection of mitochondrial membrane potential (ΔΨm) by – JC-1 staining in Molt- 3 cells

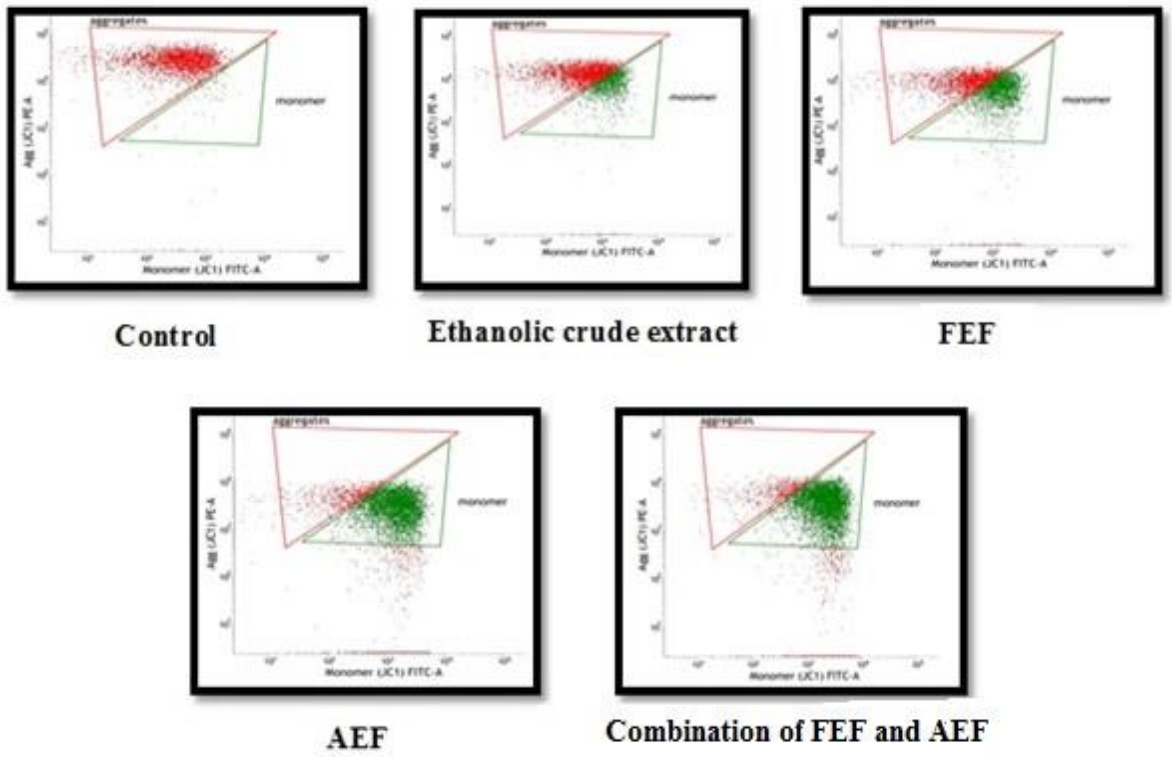


Figure 57: Distribution of monomers in Molt-3 cells

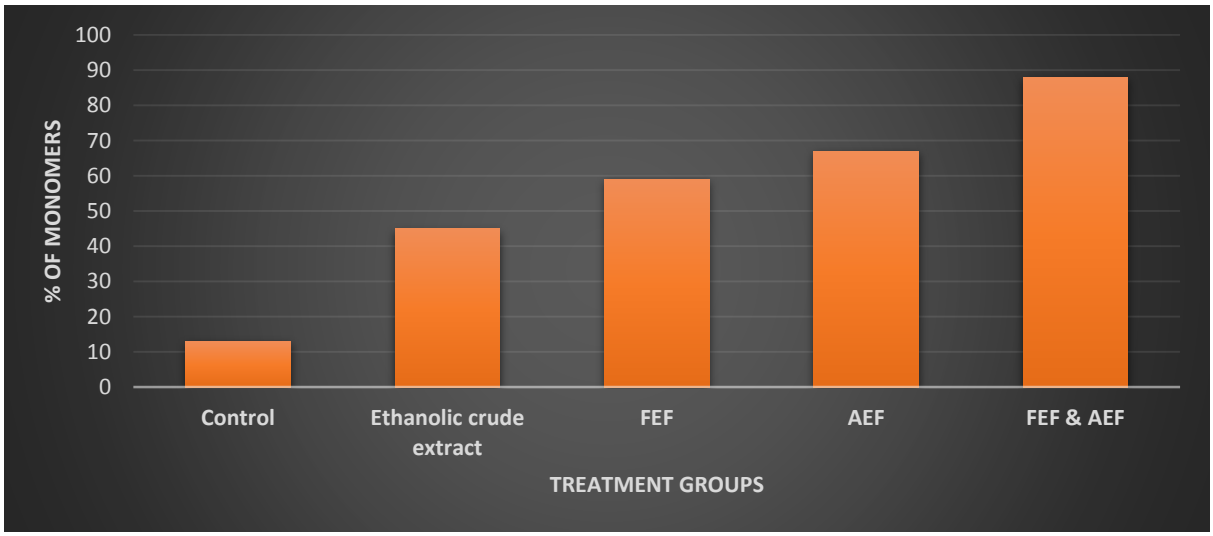


Figure 58: Detection of mitochondrial membrane potential (ΔΨm) by – JC-1 staining in PBL

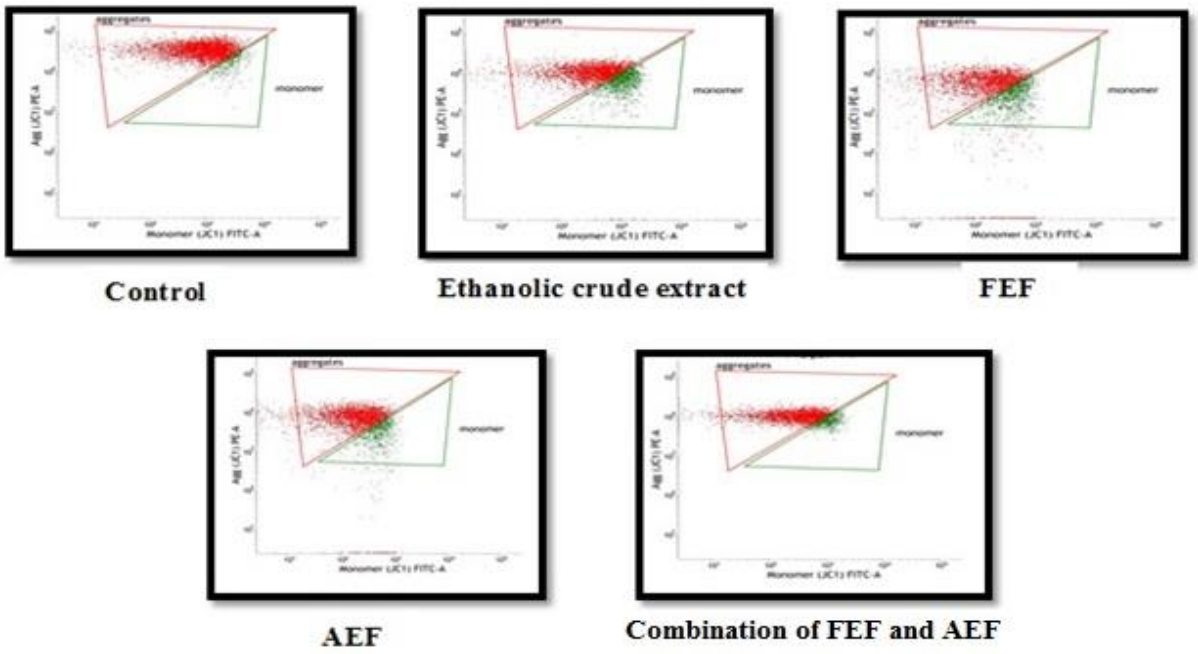
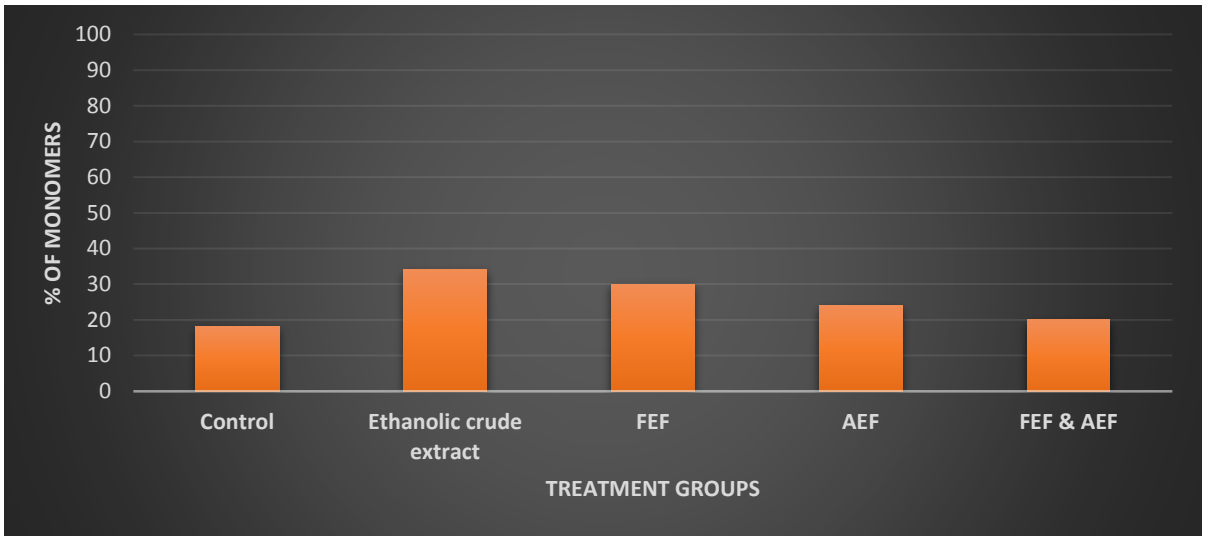


Figure 59: Distribution of monomers in PBL



In Normal PBL, the untreated and treated group has more number of cells existed in the form of J-aggregates which shows the minimal loss of mitochondrial membrane potential (Figure 58 and 59). The results reiterate that *Annona muricata* leaves and the fractions could cause cytotoxicity only in acute lymphoblastic leukemic cells and simultaneously we could observe the peripheral blood lymphocytes are protected by the presence of fractions.

The mitochondrion are one of the most important controllers of cell death and survival mechanisms. The variation in voltage across the inner membrane of mitochondria is known as MMP. A reduction in MMP is a common sign of apoptosis via the intrinsic pathway. The intrinsic signaling system, which involves non-receptor mediated signals, promotes apoptosis by lowering MMP. In comparison to the untreated cells, *Arctium lappa* root ethanolic extract treated cells showed substantial reduction in JC-1 fluorescence ratio (Don and Yap *et al*., 2019).

The ratio of cells treated with acetogenin and ethyl acetate bark extract (EAB) to control healthy cells was comparable, demonstrating that the cytotoxic impact of A. *muricata* bark extract in prostate cells did not entail MMP. Docetaxel, on the other hand, showed a considerably lower ratio of red cells to green cells as compared to untreated cells (Foster *et al*., 2020).

The *Argemone mexicana* chloroform fraction treatment resulted in a dose-dependent increase in membrane potential disruption in A431 cells (Attri *et al*., 2021). The modulation of mitochondrial membrane potential (MMP) by fenugreek seed fraction in SKOV-3 cells confirms the significance of mitochondria in the death process of injured cells (Hajduk *et al*., 2021).

The accumulation of JC-1 staining in mitochondria was membrane potential-dependent, as evidenced by a shift in fluorescence emission from red to green. Prakash *et al*. (2019) reported that control HepG2 cells are red with an intact mitochondrial membrane, whereas HepG2 cells treated with S. *cumini* extract (SC) show a shift in fluorescence emission from red to green, indicating MMP loss.

Mansingh *et al*. (2018) found that gingerol induced a significant decrease of MMP in a human gastric cancer cell line when compared to control. *Vitis vinifera* seed and peel extracts emitted green fluorescence, indicating apoptotic cells with low MMP, but untreated control cells emitted bright red fluorescence, indicating healthy cells with high MMP (Nirmala *et al*., 2018). When compared to untreated MCF-7 cells, seco-chaetomugilin pigment treatment increased depolarization of mitochondrial membrane potential by increasing intensity of green, and decreasing red fluorescence intensity (Wani *et al*., 2021).

The results of JC-1 staining revealed that after treating Molt-3 cells with ethanolic extract and fractions of *Annona muricata*, there was a loss of MMP to a great extent, as more

number of cells existed in the form of monomers when compared to the control group, while in normal peripheral blood lymphocytes, minimal mitochondrial membrane potential was observed, with more number of cells existed in the form of J-aggregates in the control, ethanolic extract and fractions of *Annona muricata* treated groups. This indicated that *Annona muricata* extract and fractions were able to trigger apoptosis through intrinsic apoptotic pathway.

4.3.4. Antiproliferative activity

4.3.4.1. Analysis of cell cycle progression by flow cytometry in Molt-3 cells and PBL

Cell cycle analysis is a useful tool for biologists who want to learn more about the mechanisms of cell division and how they might be used to treat cancer patients. The cell cycle is studied to learn more about the mechanisms that control the time and frequency of DNA duplication and cell division. Flow cytometry is particularly useful for evaluating the efficacy of therapies that prevent cells from progressing through the cell cycle (James *et al*., 2021).

Cancer is a disease caused by a disruption in the cell cycle. Natural products' anti-cancer potential can be enhanced by their capacity to inhibit cancer cells from progressing through the cell cycle (Yajid *et al*., 2018).

The cell cycle is tightly regulated in normal healthy cells, but it is interrupted in cancerous or malignant cells. The quest for cancer treatments focuses mostly on the cancer cells, cell cycle events. One of the most important strategies for controlling cancer cell proliferation is to block the cell cycle stages that control cell growth (Seragy *et al*., 2019).

Cell cycle analysis was performed to find out at which stage of cell cycle, the cells were arrested. The cells in the control were uniformly dispersed throughout the cell cycle, and uninterrupted indication in the normal cell division. Crude extract, FEF and AEF treated cells showed G0-G1 arrest, whereas in the combination of FEF and AEF treated group, more proportion of cells were arrested at G0-G1 phase, attributing that cell were interrupted during the process of cell division (Figure 60 and 61). In case of normal counterpart, peripheral blood lymphocytes, the proportion of cells were evenly distributed, however there is arrest in some of the treated groups with lower proportion of cells when compared to the Molt-3 treated cell lines (Figure 62 and 63). This confirms the non-toxic nature of the fractions of *Annona muricata*.

Figure 60: Cell cycle analysis by flow cytometry in Molt-3 cells

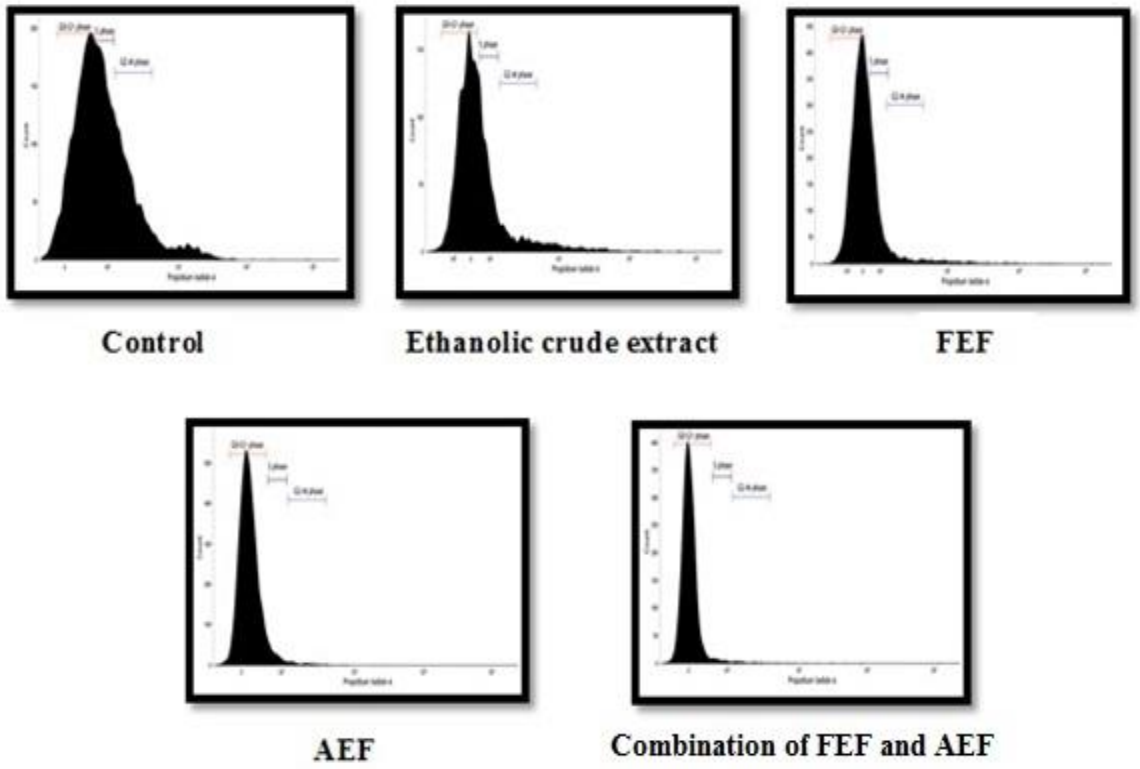


Figure 61: Distribution of Molt-3 cells in various phases of cell cycle

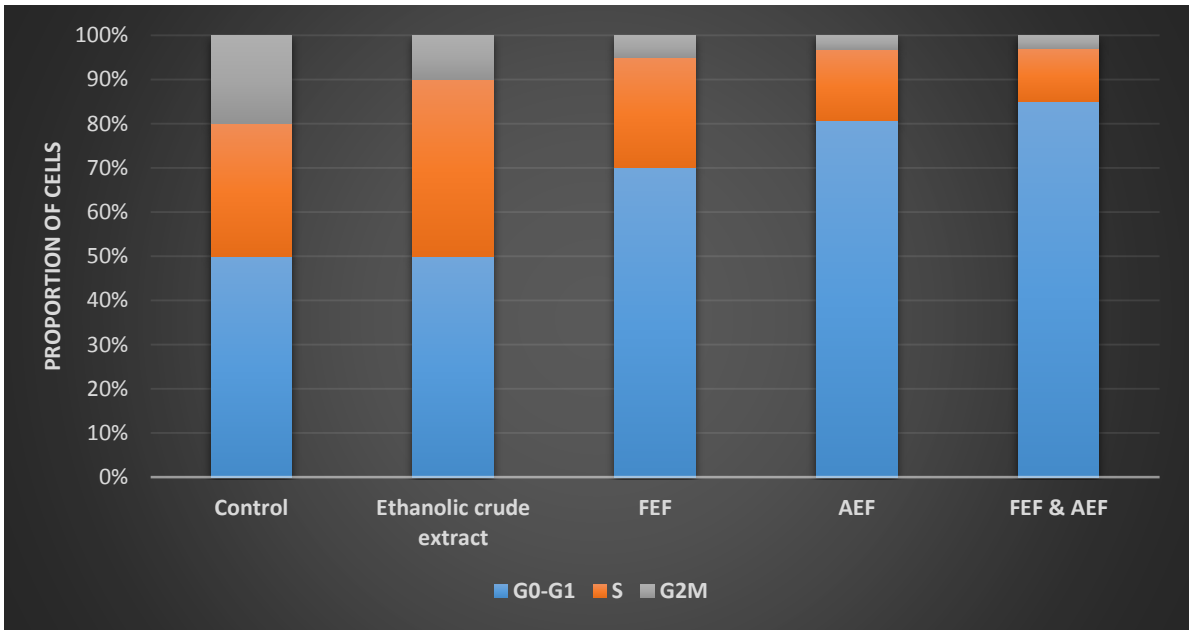


Figure 62: Cell cycle analysis by flow cytometry in PBL

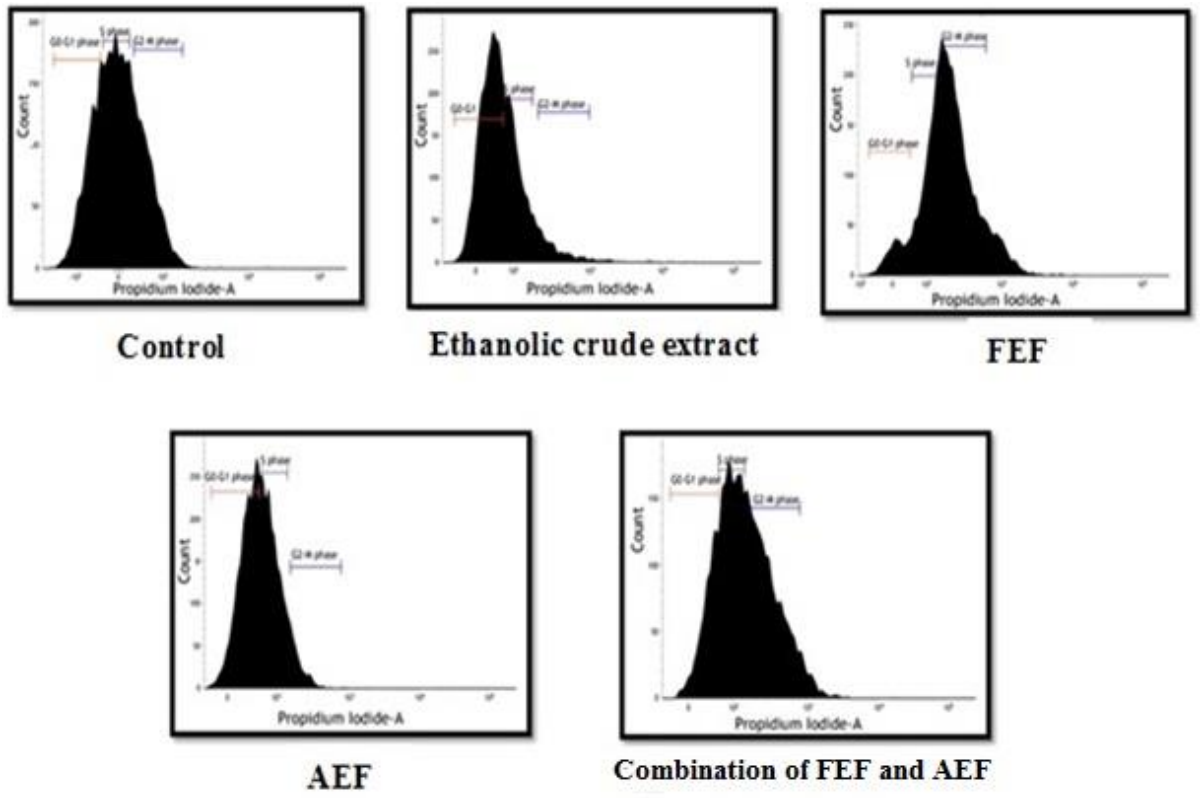
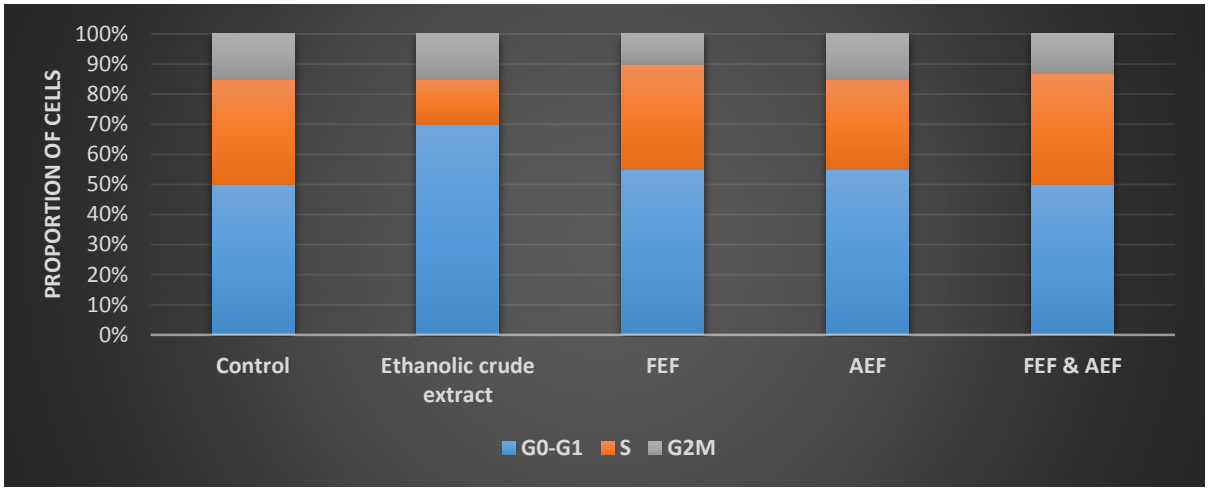


Figure 63: Distribution of PBL in various phases of cell cycle



MCF-7 and MDA-MB-231 cells treated with *Annona muricata* methanolic fruit pulp extract (AMPM) and aqueous fruit pulp extract (AMPW) showed an increase in the G0/G1 phase population. Additionally, both AMPM and AMPW-treated groups showed enhanced S-phase and decreased G2/M phase populations (Prasad *et al*., 2020).

The ethanolic extract of *Annona muricata* caused considerable cell cycle arrest in the G1 phase in 4T1 cells as a single treatment, but its combination with doxorubicin caused accumulation in the G2/M phase (Salsabila *et al*., 2021).

The progression of the cell cycle is closely linked to the cell's proliferation. When compared to the control, the ionic liquid-graviola fruit extract treatment enhanced the number of cells in the G0/G1 phase (Daddiouaissa *et al*., 2019). Zhang *et al*. (2021) found that treatment with *Warburgia ugandensis* extract resulted in G0/G1 arrest, as well as a decline in the S and G2/M phases, when compared to untreated cells. G0/G1 arrest was detected in PC-3 cells after treatment with M. *oleifera* leaves methanolic extract (Khan *et al*., 2020).

Cell cycle analysis was carried out in HepG2 cells to determine the cell cycle arresting potentials of Ethanolic banana leaves extract (EBLE), which resulted in an increase in the cell population at sub G0/G1 apoptotic phase and decrease in the other phases, such as S and G2-M (Singh and Ezhilarasan, 2020). Zhu *et al*. (2020) reported that in a concentration-dependent manner, diosgenin caused G0/G1 cell cycle arrest in HBL-52 cells (optic nerve sheath meningioma cell line).

Our findings are in line with what other researchers had observed. In the G0-G1 phase, all of the treatment groups demonstrated a cell cycle arrest, showing ethanolic *Annona muricata* leaf extract and fractions are capable of arresting in the early phase of the cell cycle and the cells are triggered into apoptosis. This shows that the combination of FEF and AEF fraction can be effectively used to increase the cytotoxicity when exploited for therapy to treat against T- cell acute lymphoblastic leukemia, as they are not targeting the normal peripheral blood lymphocytes.

4.3.5. Assessment of Intracellular ROS levels in Molt-3 cells and PBL

ROS play a role in cell signaling pathways such as proliferation, transformation, metabolism, and death. Low doses of ROS can stimulate cell proliferation and invasion, whereas high doses induce oxidative damage to proteins, triglycerides, RNA, and nucleic acids, leading to cell death. Although a slight increase in ROS has been related to the formation and progression of cancer, large quantities of ROS can trigger cellular damage by activating many signaling pathways, resulting in cell apoptosis. (Zaidieh *et al*., 2019).

The cell permeable dye 2', 7'-dichlorofluorescein diacetate (DCF-DA) can be used to measure oxidative damage in intact cells. When DCF-DA is oxidised, it transforms into the fluorescent chemical 2', 7'-dichlorofluorescein (DCF). The level of ROS in a cell can be determined by measuring this fluorescent molecule (Poongodi *et al*., 2020).

Figure 64 and 65 depicts the ROS levels where, M1 indicates the percentage of cells that are normal, while M2 indicates the percentage of cells that produce increased ROS. DCF fluorescence increased in ethanolic crude extract, FEF, AEF, combination of FEF and AEF in Molt-3 treated cells and shifted the DCF fluorescence histogram from M1(normal cells) towards M2 (% of cells that increase in ROS production). This shift is an indicator of increased ROS production.

In the case of PBL treated cells histogram shift doesn't occur from M1 (normal cells) towards M2 (% of cells that increase in ROS production) and remains in the M1 (normal cells) (Figure 66 and 67), which clearly indicates that ROS has not produced and again proves the non-toxic nature of *Annona muricata* leaf extracts and fractions in normal PBL.

Figure 64: Assessment of Intracellular ROS levels in Molt-3 treated cell lines

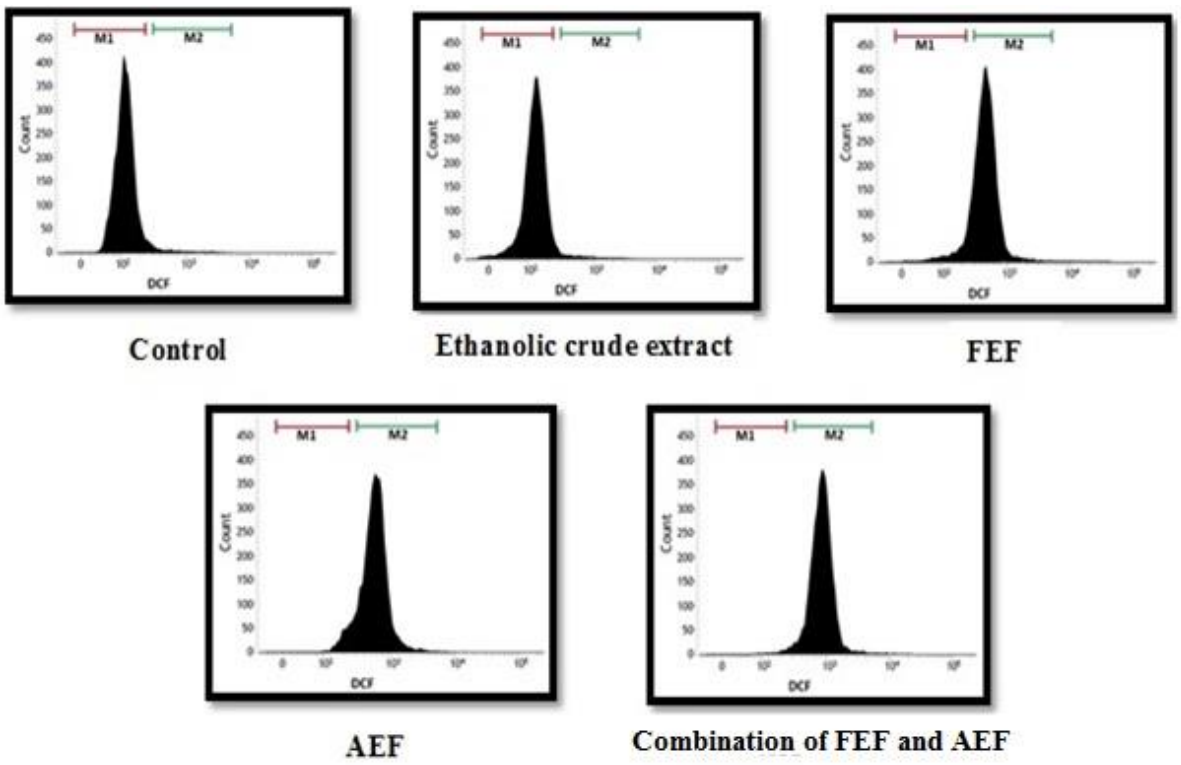


Figure 65: Distribution of normal cells and increased ROS production cells in Molt-3

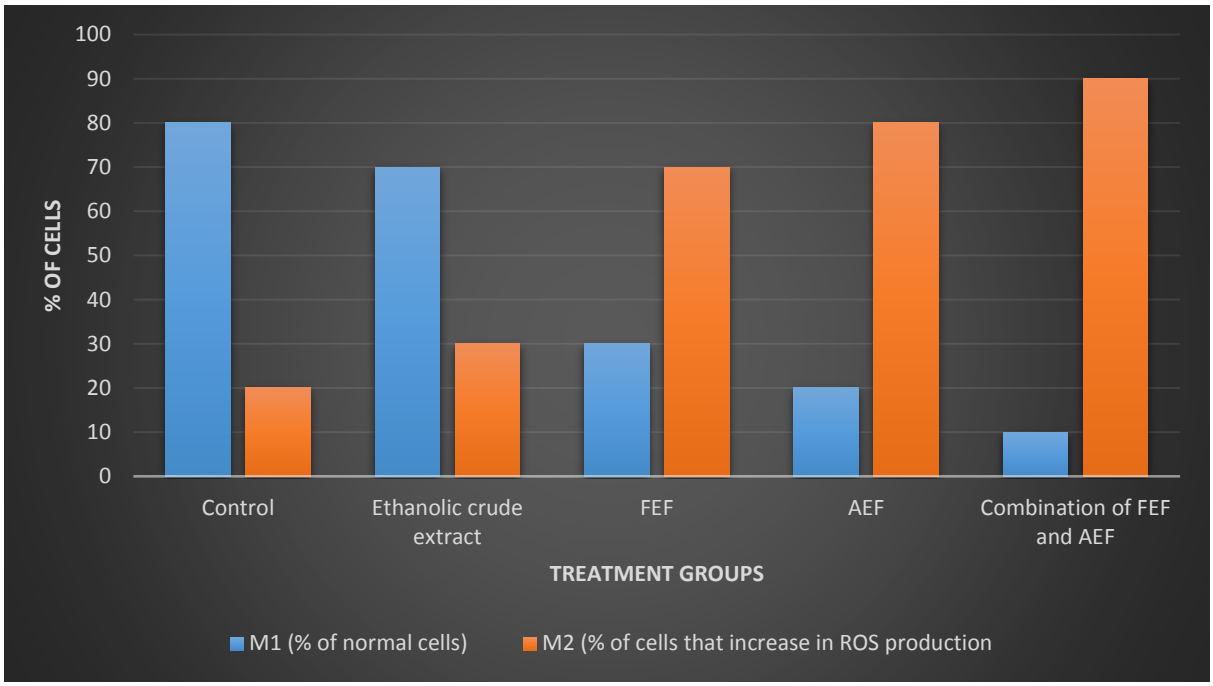


Figure 66: Assessment of Intracellular ROS levels in PBL

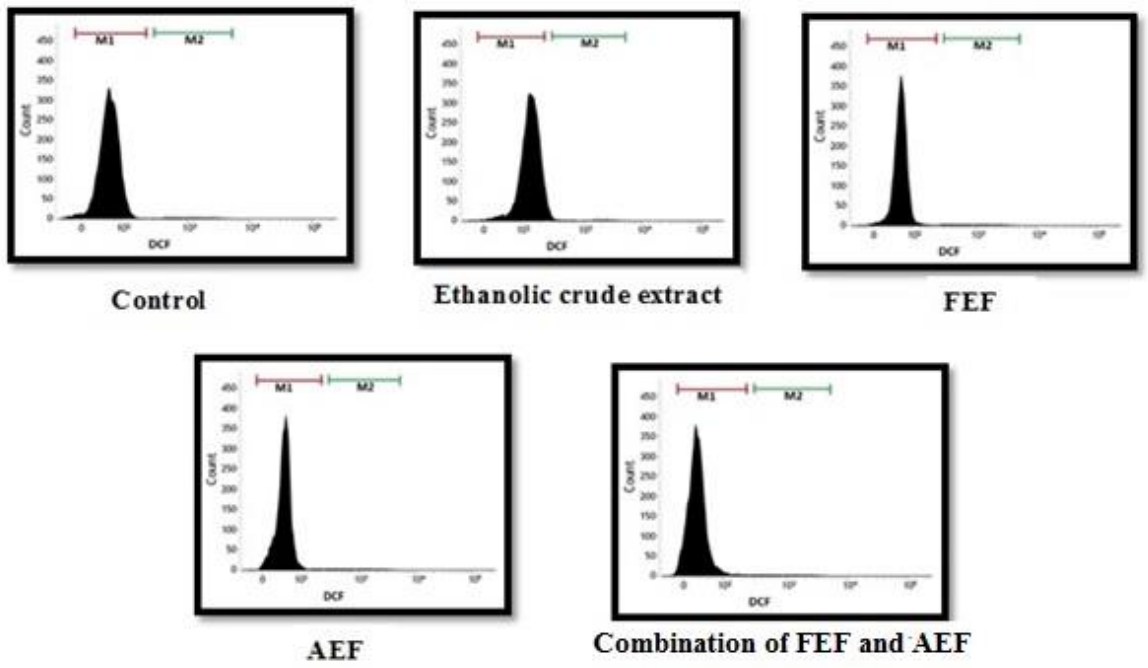
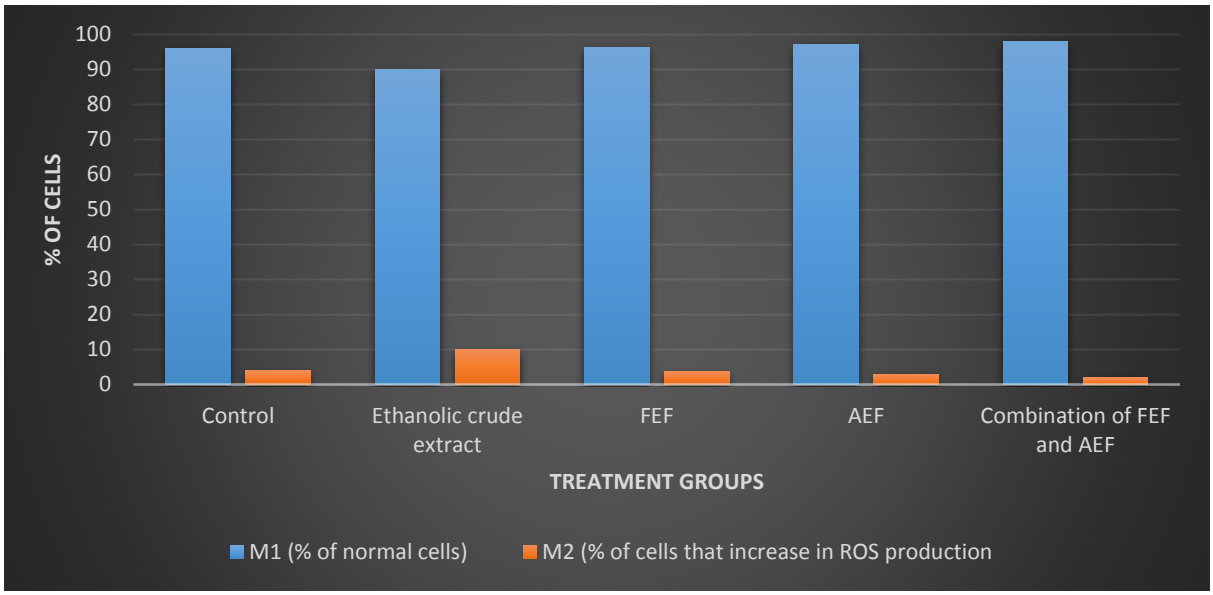


Figure 67: Distribution of normal cells and increased ROS production cells in PBL



Erastin treatment enhanced ROS levels in A549 cells but not in WI-38 cells, showing that NSCLC A549 cells are generally more sensitive than non-tumour WI-38 lung cells (Huang *et al*., 2018). In human colorectal cancer cell line, the DCFH-DA experiment ensures that isolate from ginseng leaves- Rh4 increased cellular ROS levels in a concentration-dependent way. (Wu *et al*., 2018).

Vitamin C treatment enhanced cellular ROS levels in thyroid cancer cells, and this effect was successfully reversed by ROS scavenger N-acetyl cysteine therapy (Su *et al*. 2019). ROS levels were raised after emodin (1, 3, 8-trihydroxy-6-methylanthraquinone) treatment in colon cancer cell lines HCT116 and LOVO cells (Wang *et al*., 2018). Su *et al*. (2019) reported that Zingerone treatment, elevated ROS generation in HCT116 cells.

Ovarian cancer cells were treated with "apigenin, luteolin, and myricetin (Flavonoids)" to determine the intracellular ROS levels, results indicated that changes in ROS production were found to be concentration-dependent (Tavsan and Kayali, 2019). DCFH-DA staining was used to determine the intracellular redox status of gall bladder cancer (GBC) cells after hesperidin treatment. As a result, intracellular ROS levels in treated cells were found to be considerably higher than those in untreated cells (Pandey *et al*., 2018).

According to Foster *et al*. (2020), A. muricata extract nor annonacin caused a substantial rise in intracellular ROS levels in prostate cancer cells. High amounts of ROS can cause cells to lose their mitochondrial membrane potential irreversibly, leading in the release of cytochrome C from the mitochondria and subsequent signalling of executioner caspases, which leads to programmed cell death via the apoptosis pathway.

Intracellular ROS levels are intriguing since they represent a cell's proliferative status. Intracellular ROS works as a second messenger that promotes cell proliferation on a subcellular level. ROS can phosphorylate mitogen-activated protein kinase and extracellular signal-regulated kinase directly. As a result, massive cell division and proliferation occur, resulting in gene alterations and metabolic abnormalities. This results in elevated intracellular ROS levels, which is followed by a rise in antioxidant enzyme expression. Cancer cells, on the other hand, create a lot of ROS and antioxidants and as a result of this state, tend to be more susceptible to changes in ROS levels. An inherent apoptotic pathway can be triggered by a high quantity of ROS (Salsabila *et al*., 2021).

The reduction of MMP triggers the generation of reactive oxygen species. ROS are key intercellular mediators that cause cell damage and death by maintaining regular cellular activities. Many anticancer drugs cause cell damage by releasing a large amount of reactive oxygen species. The levels of ROS in Molt-3 cell lines were measured after staining with 2′, 7′-dichlorofluorescein. The levels of ROS in the group treated with combination of FEF and AEF fractions showed a significant increase when compared to the ethanolic crude extract, FEF and AEF fraction treated groups. In the case of normal PBL, ROS levels were not elevated, which confirms the non-toxic nature of *Annona muricata* leaf extracts and fractions and proves Molt-3 cells are much more sensitive than normal PBL.

The results of various spectrophotometric, antioxidant, cytotoxic and staining assays performed in the current study are in concord with these findings that the *Annona muricata* ethanolic crude extract and fractions showed differential response against Molt-3 leukemic cells and PBL. The anticancer action of the crude ethanol extract and fractions could be due to the presence of phenols, flavonoids and acetogenins present in the *Annona muricata*, and their cytotoxic potential were also recorded in the present study. Cell cycle analysis performed to find out whether the treatment with crude extract and fractions, induced apoptosis by cell cycle arrest in Molt-3 cells and PBL**,** revealed G0-G1 cell cycle arrest, in

case of PBL, the proportion of cells were evenly distributed in all the phases. The induction of apoptotic effect by the crude extract and fractions was further confirmed by Annexin V-FITC/ PI staining, the results revealed that late apoptotic cells are observed in the group of cells treated with AEF and in the combination of FEF and AEF, indicating that AEF and combined fractions are very effective in their apoptosis inducing ability. In case of PBL, the number of apoptotic cells were found to be very low, which again confirms the non-toxic nature of the fractions in PBL. The loss of MMP is putatively the initial event leading to apoptosis, the analysis of MMP by JC-1 staining reveals that there was a great loss of MMP in Molt-3 cells; and in the PBL, minimal loss was observed and confirming that apoptosis is via intrinsic pathway in Molt-3 cells. ROS can cause cell death by activating many signalling pathways, culminating in cell apoptosis, the measurement of ROS revealed that *Annona muricata* crude extract and fractions elevated the ROS levels in Molt-3 cells and in PBL, ROS levels were not elevated, confirming the non-toxic nature. Thus, from the above results it is clearly evident that *Annona muricata* ethanolic crude extract and fractions could very well act as an anticancer agent.

Phase IV

It is evident that, the *Annona muricata* ethanolic extract and fractions exhibit anticancer property towards Molt-3 leukemic cell line which might be attributed by the antioxidant property as well as by the presence of various phytochemicals. In order to validate the results obtained in *in vitro* studies and to ascertain whether the apoptosis inducing ability of the extracts were due to the proteins Bcl2, Mcl1, Bax and MDM2, *in silico* studies were done to confirm the interactions of the FEF and AEF phytoconstituents and the proteins involved in apoptotic and cell signaling pathway related to cancer and leukemia.

4.4. *In silico* docking of apoptotic and leukemia targets with Flavonoid (FEF) and Acetogenin enriched fraction (AEF) phytoconstituents

The most prevalent computational structure-based drug design tool is molecular docking, which was developed to work between a small molecule (ligand) and a macromolecule target (protein). Molecular docking has a wide range of uses in drug discovery, including structure-activity investigations, lead optimization, and virtual screening for new leads (Stanzione *et al*., 2021).

As a result, the current research was carried out in order to predict ADME properties of reported phytoconstituents and to dock phytoconstituents of *Annona muricata* fractions against apoptotic and leukemia targets**.** Around 6 phytoconstituents in the Flavonoid Enriched Fraction (FEF) and 4 phytoconstituents in the Acetogenin Enriched Fraction were identified by the GCMS analysis, which were chosen and prepared for molecular docking studies.

4.4.1. Prediction of ADME properties of the compounds using QikProp module of Schrodinger

Among the following 6 compounds chosen from Flavonoid enriched fraction (FEF), only 3 compounds, namely Isoferulic acid 3-O glucuronide, Pentanedioic acid, Glycitein showed the drug-likeness as they have '0' violations with Lipinski's Rule of five and in the Acetogenin Enriched Fraction (AEF), among 4 compounds chosen, only one compound-Muricatacin showed the drug-likeness (Table 20) and these ligands can be used for further studies to develop a potential drug.

Table 20: ADME properties of the FEF and AEF compounds

Compound	No. of Donor H bond	No. of acceptor H bond	Molecular mass	Log P	Human oral absorption	Blood-Brain Barrier permeant	Lipinski's violations
Flavonoid Enriched Fraction Phytoconstituents							
Isoferulic acid 3-O glucuronide	**5**	**10**	**370.31**	**1.32**	**High**	**No**	**0**
Pentanedioic acid	**2**	**4**	**132.11**	**0.45**	**High**	**No**	**0**
Myricetin	6	8	318.24	1.08	Low	No	1
Apigenin 6-C-glucoside	7	10	432.38	1.94	Low	No	1
Luteolin-3',7-di-O-glucoside	10	16	610.52	2.07	Low	No	3
Glycitein	**2**	**5**	**284.26**	**2.36**	**High**	**No**	**0**
Acetogenin Enriched Fraction Phytoconstituents							
α-Muurolene	0	0	204.35	3.38	Low	No	1
Cis-solamin	2	5	564.88	7.78	Low	No	2
Muricatacin	**3**	**1**	**284.43**	**3.98**	**High**	**No**	**0**
Germacrene B	0	0	204.35	3.27	Low	No	1

4.4.2. Molecular docking of Flavonoid (FEF) and Acetogenin Enriched Fraction (AEF) phytoconstituents with apoptotic and leukemia targets

A notable feature of most cancer cells is that they do not undergo apoptosis, giving them an advantage over normal cells in terms of survival. Targeted cancer therapy tries to interfere with the functioning of proteins that are essential in the progression of cancer. BCL2, an antiapoptotic protein, is one such protein that is increased in many tumours when compared to normal cells, making it an excellent target for cancer therapy (Rosdi *et al*., 2018).

Apoptosis is a strictly controlled cell death process that follows two distinct pathways: extrinsic and intrinsic. B-cell lymphoma-2 (Bcl-2), an antiapoptotic protein, is a key regulator of the intrinsic pathway. Bcl-2, a proto-oncogene, inhibits apoptosis and promotes tumour proliferation, whereas Bax regulates apoptosis and aids tumour regression (Raghav *et al*., 2019).

A group of proteins known as the B cell lymphoma-2 (Bcl-2) family of proteins regulates the mitochondrial intrinsic pathway, which is a critical signalling mechanism in apoptosis. Antiapoptotic, proapoptotic activators, and proapoptotic effectors make up this class of proteins, which interact in some way to function.

Activators like as tBid protein, Bim, and Puma bind to the antiapoptotic and proapoptotic Bcl-2 proteins during apoptosis. The effector proteins Bmf, Bnip3, Hrk, Bik, Noxa and Bad liberate the activators from antiapoptotic proteins (A1, Mcl-1, Bcl-XL, Bcl-w, Bcl-2), causing MMP and the release of biomarkers like cytochrome C and apoptosis inducing factor. The apoptotic machinery is then activated as a result of this process (Radha and Raghavan, 2017). MDM2 (murine double minute 2) is a proto-oncogene that has been found to be elevated in a number of malignancies, including leukaemia (Liu *et al*., 2020).

To ascertain whether the apoptosis inducing ability of the *Annona muricata* fractions were due to the proteins Bcl2, Mcl1, Bax and MDM2, *in silico* studies were performed to confirm the interactions of Flavonoid and Acetogenin enriched fraction phytoconstituents with apoptotic and leukemia targets.

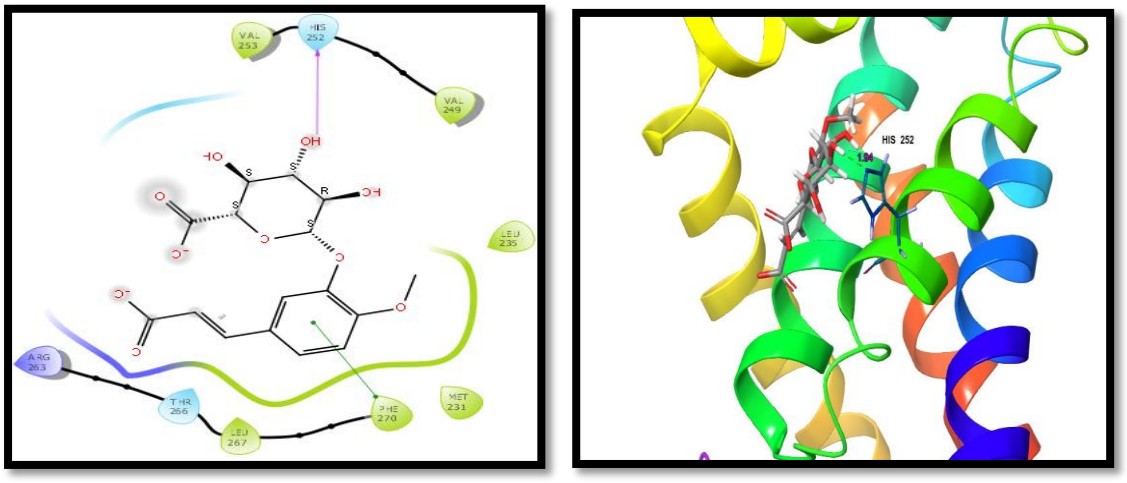
Table 21: Glide SP docking of the Flavonoid Enriched Fraction (FEF) phytoconstituents with Mcl 1

Target and PDB ID	Ligand and PubChem ID	Glide score	Glide energy
Mcl 1 (6VBX)	Isoferulic acid 3-O glucuronide (49844484)	-6.109	-27.309
	Pentanedioic acid (3811953)	-5.938	-30.159
	Myricetin (5281672)	-5.488	-25.644
	Apigenin 6-C-glucoside (131750832)	-7.404	-42.849
	Luteolin-3',7-di-O-glucoside (133611799)	-8.294	-45.417
	Glycitein (5317750)	-4.704	-23.156

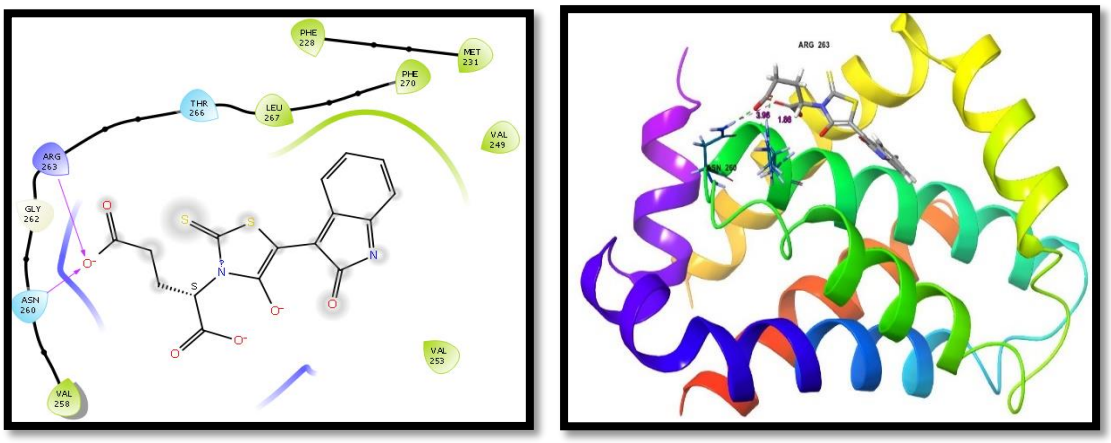
Molecular docking studies of Flavonoid enriched fraction (FEF) phytoconstituents with Mcl 1 target revealed that Luteolin-3',7-di-O-glucoside exhibited best glide score of -8.294 (Table 21 and Plate 4) followed by Apigenin 6-C-glucoside, Isoferulic acid 3-O glucuronide, Pentanedioic acid, Myricetin and Glycitein.

Plate 4: Docking interactions of Flavonoid Enriched Fraction (FEF) phytoconstituents with Mcl 1

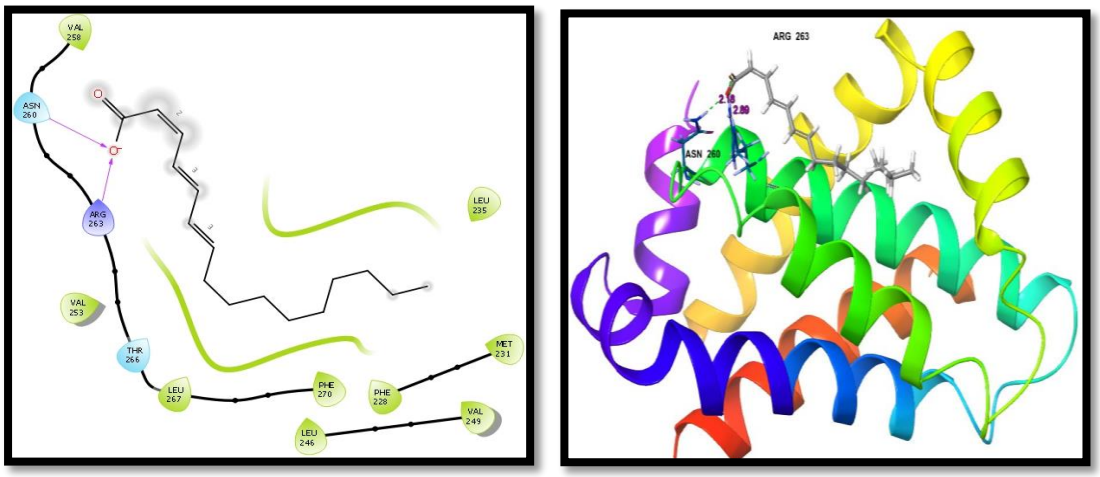
2D and 3D interaction of Isoferulic acid 3-O glucuronide with Mcl 1



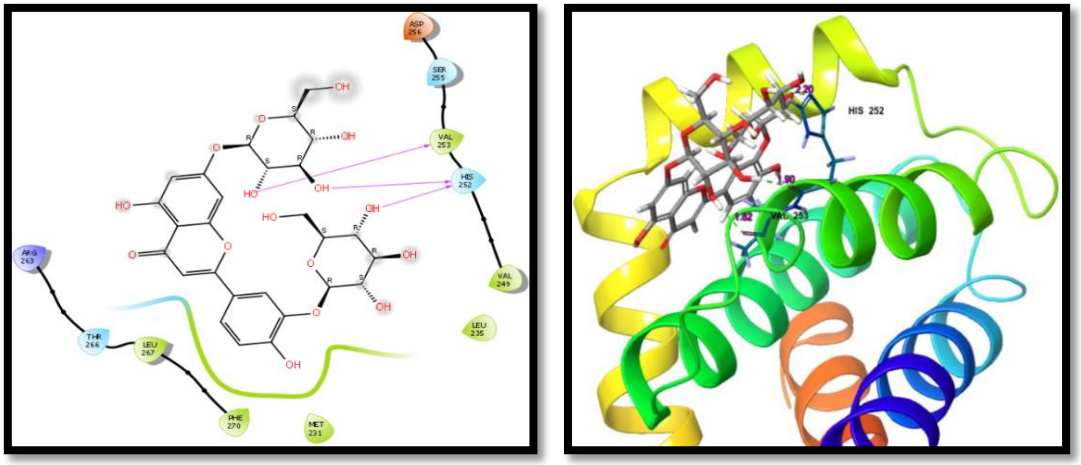
2D and 3D interaction of Pentanedioic acid with Mcl 1



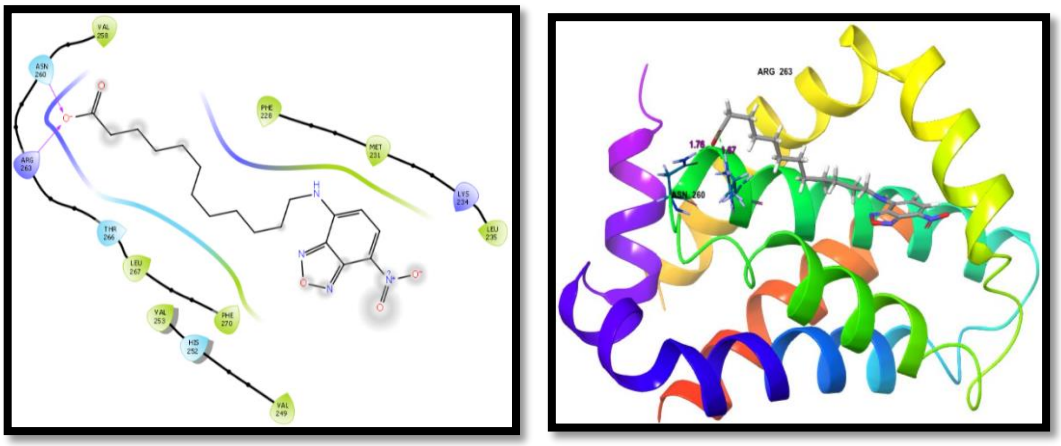
2D and 3D interaction of Myricetin with Mcl 1



2D and 3D interaction of Apigenin 6-C-glucoside with Mcl 1



2D and 3D interaction of Luteolin-3', 7-di-O-glucoside with Mcl 1



2D and 3D interaction of Glycitein with Mcl 1

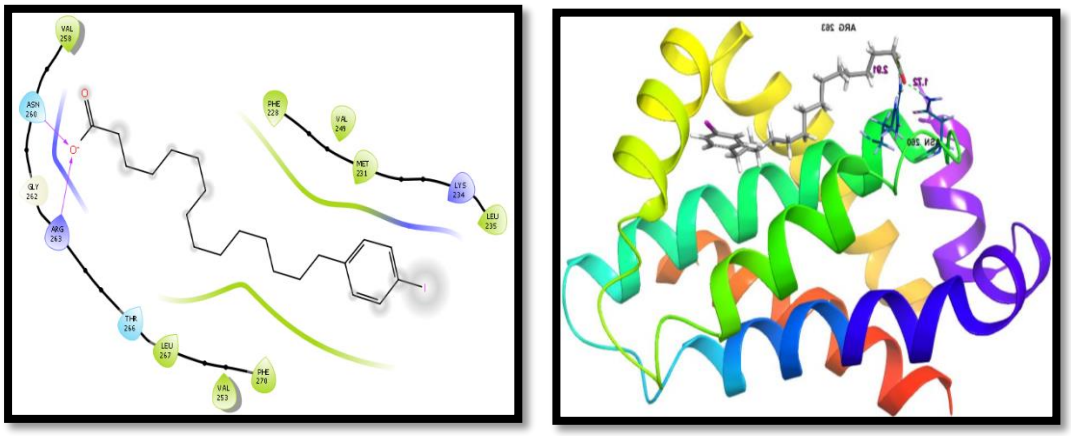


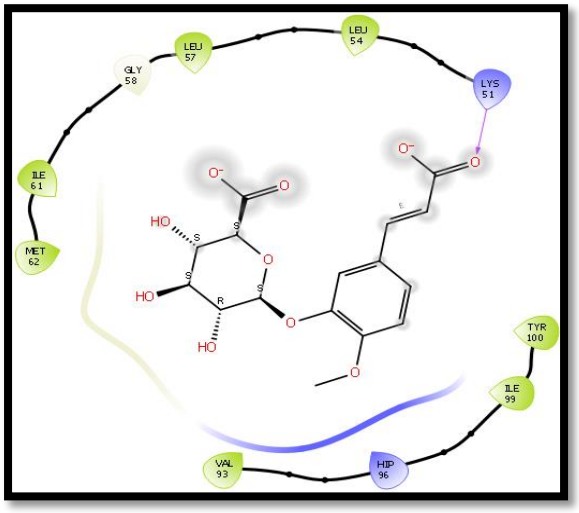
Table 22: Glide SP docking of the Flavonoid Enriched Fraction (FEF) phytoconstituents with MDM2

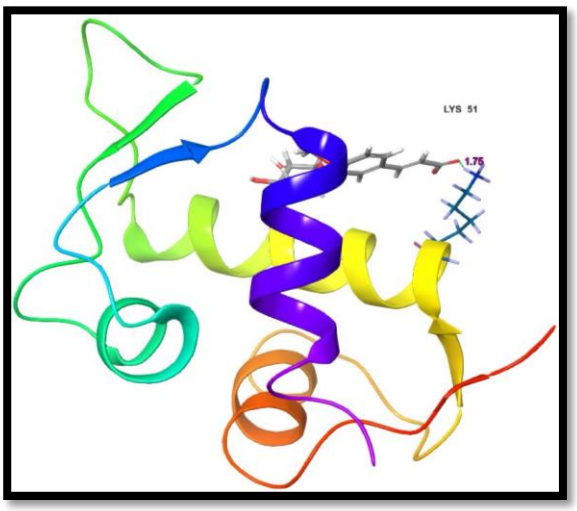
Target and PDB ID	Ligand and PubChem ID	Glide score	Glide energy
MDM2 (5AFG)	Isoferulic acid 3-O glucuronide (49844484)	-4.751	-26.884
	Pentanedioic acid (3811953)	-6.64	-40.212
	Myricetin (5281672)	-6.093	-33.8
	Apigenin 6-C-glucoside (131750832)	-5.82	-42.307
	Luteolin-3',7-di-O-glucoside (133611799)	-7.65	-49.736
	Glycitein (5317750)	-5.687	-31.938

Molecular docking studies of Flavonoid enriched fraction phytoconstituents with MDM2 target revealed that Luteolin-3', 7-di-O-glucoside exhibited best glide score of -7.65 (Table 22 and Plate 5) followed by Pentanedioic acid, Myricetin, Apigenin 6-C-glucoside, Glycitein and Isoferulic acid 3-O glucuronide.

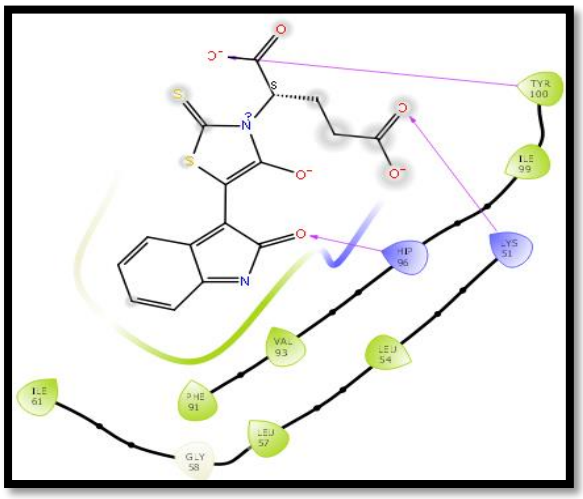
Plate 5: Docking interactions of Flavonoid Enriched Fraction (FEF) phytoconstituents with MDM2

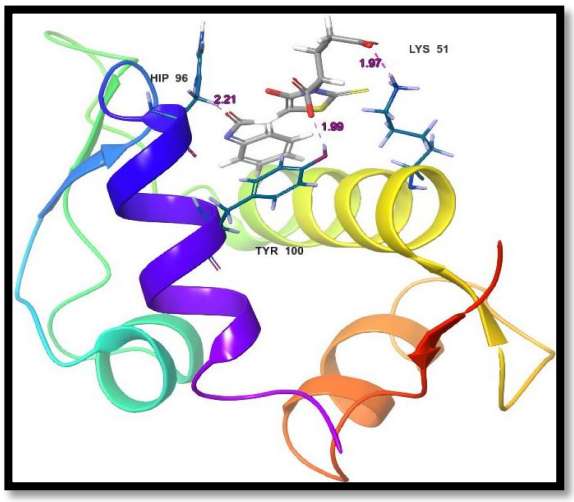
2D and 3D interaction of Isoferulic acid 3-O glucuronide with MDM2



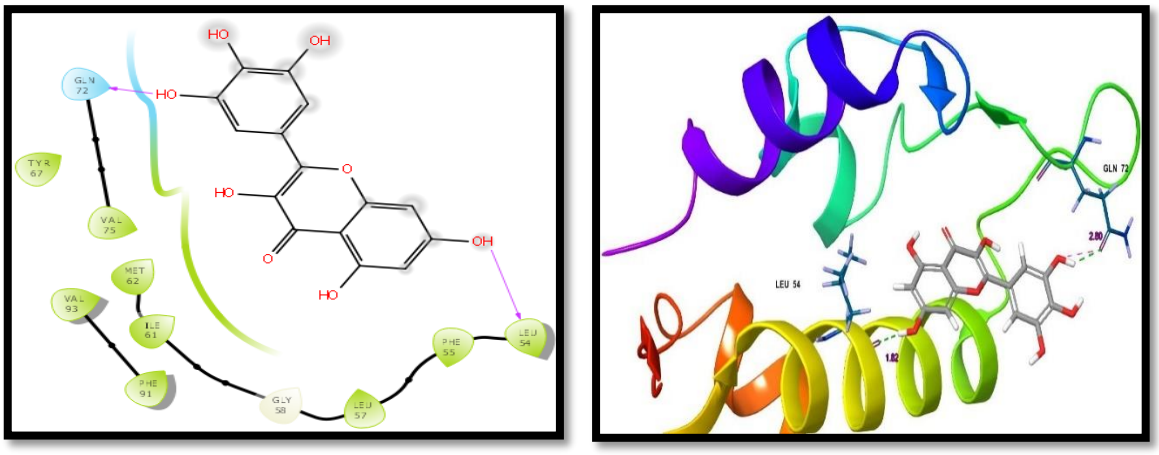


2D and 3D interaction of Pentanedioic acid with MDM2

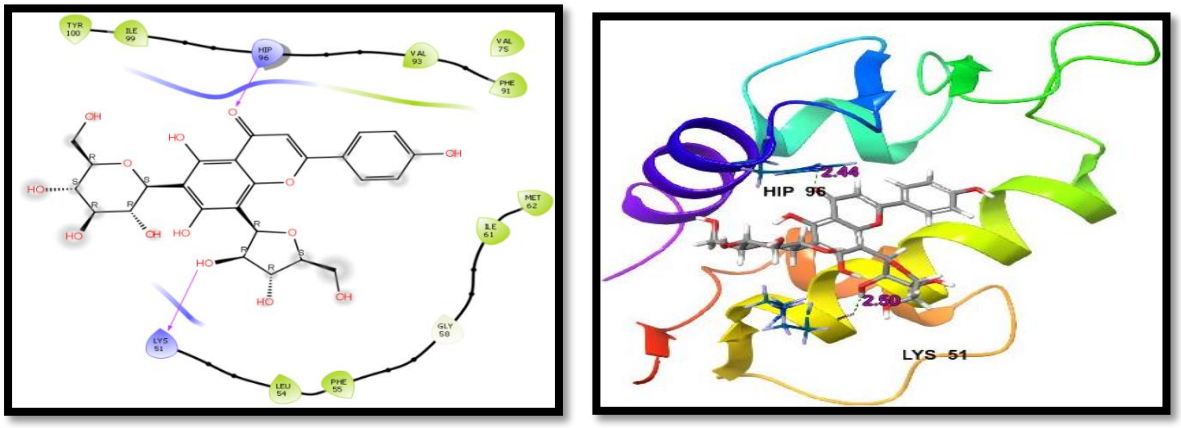




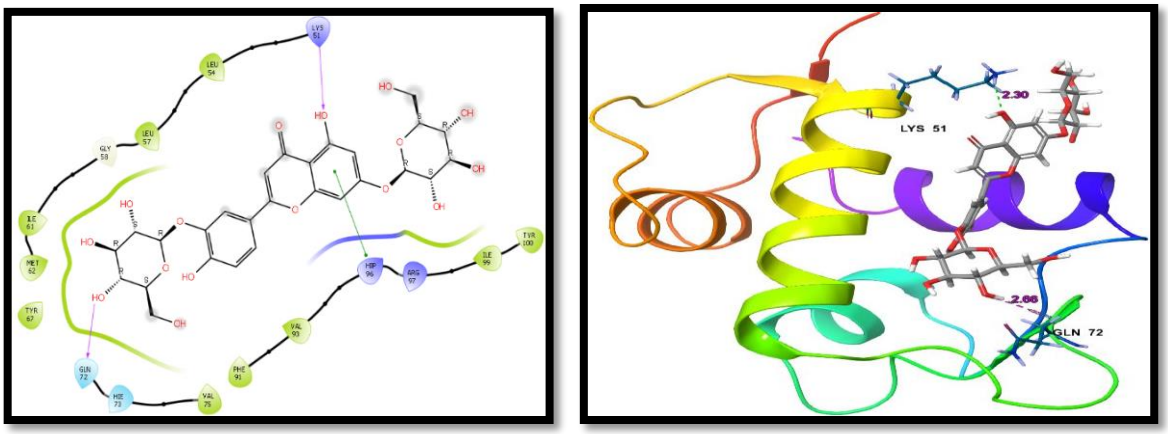
2D and 3D interaction of Myricetin with MDM2



2D and 3D interaction of Apigenin 6-C-glucoside with MDM2



2D and 3D interaction of Luteolin-3', 7-di-O-glucoside with MDM2



2D and 3D interaction of Glycitein with MDM2

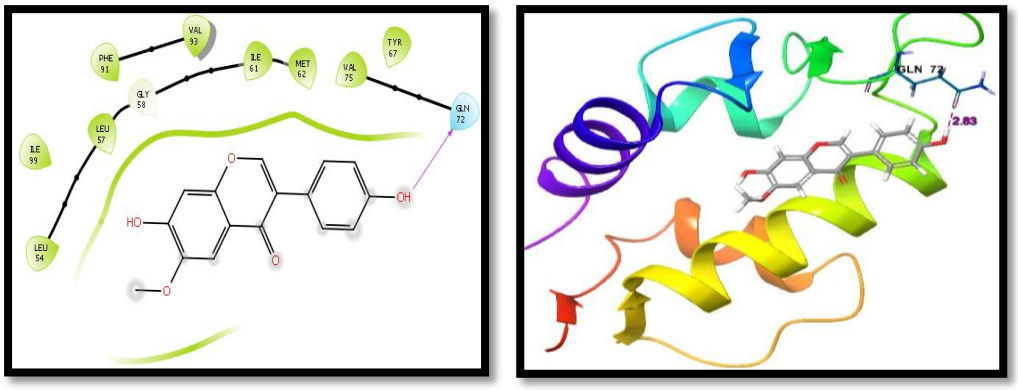


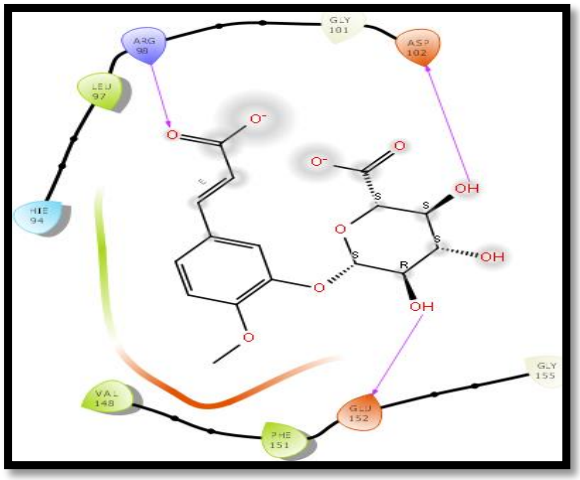
Table 23: Glide SP docking of the Flavonoid Enriched Fraction (FEF) phytoconstituents with Bcl2

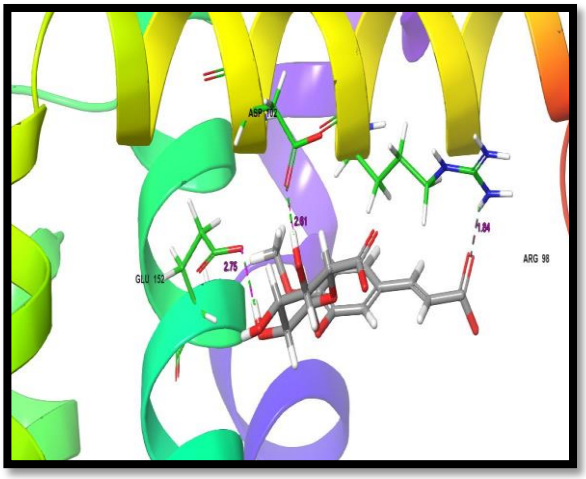
Target and PDB ID	Ligand and PubChem ID	Glide score	Glide energy
Bcl2 (6QGG)	Isoferulic acid 3-O glucuronide (49844484)	-4.811	-33.508
	Pentanedioic acid (3811953)	-4.387	-38.221
	Myricetin (5281672)	-4.95	-29.538
	Apigenin 6-C-glucoside (131750832)	-6.644	-47.102
	Luteolin-3',7-di-O-glucoside (133611799)	-8.249	-59.113
	Glycitein (5317750)	-3.918	-29.33

Molecular docking studies of Flavonoid Enriched Fraction (FEF) phytoconstituents with Bcl2 target revealed that Luteolin-3', 7-di-O-glucoside exhibited best glide score of -8.249 (Table 23 and Plate 6) followed by Apigenin 6-C-glucoside, Myricetin, Isoferulic acid 3-O glucuronide, Pentanedioic acid and Glycitein.

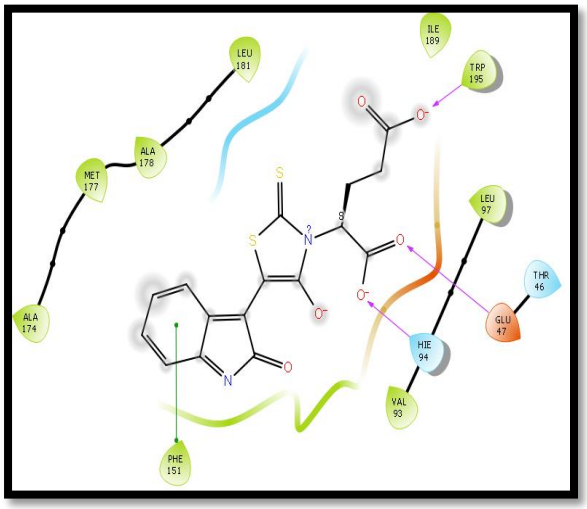
Plate 6: Docking interactions of Flavonoid Enriched Fraction (FEF) phytoconstituents with Bcl2

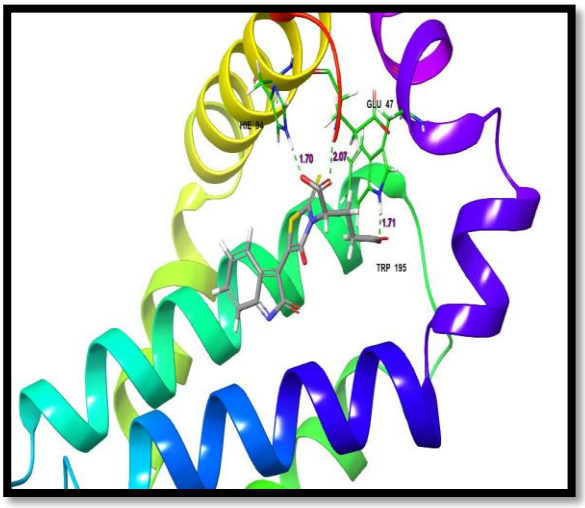
2D and 3D interaction of Isoferulic acid 3-O glucuronide with Bcl2



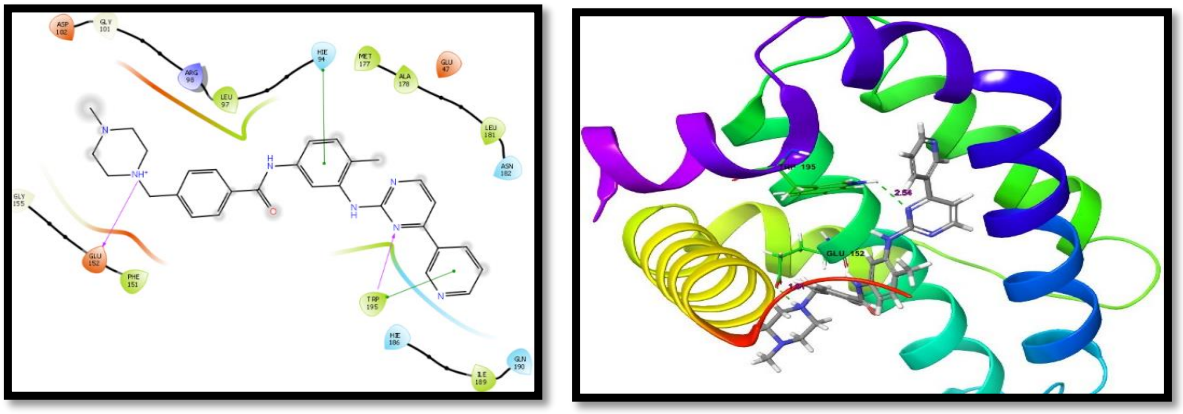


2D and 3D interaction of Pentanedioic acid with Bcl2

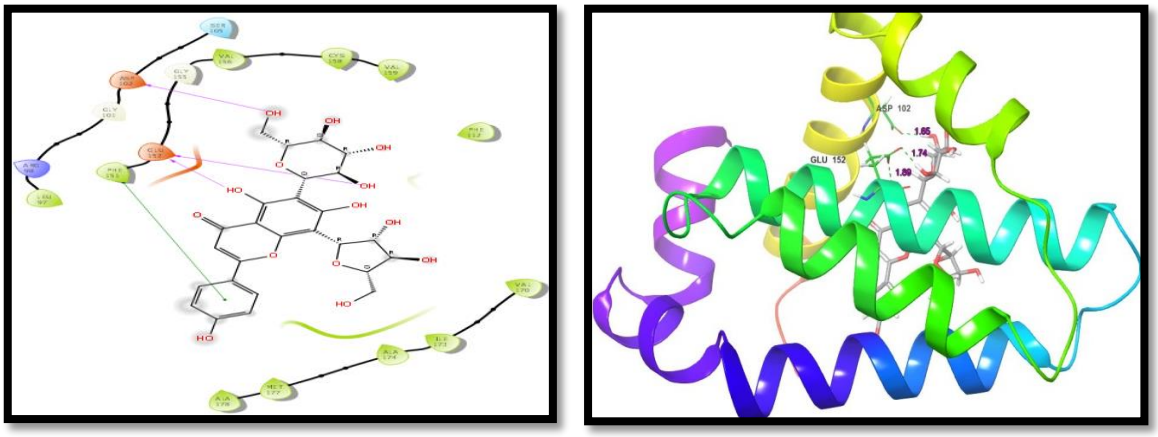




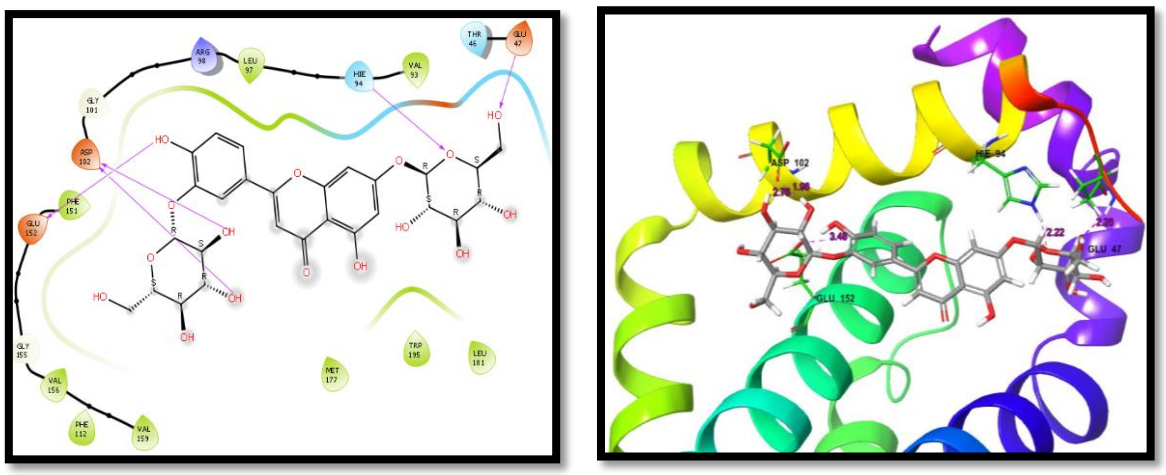
2D and 3D interaction of Myricetin with Bcl2



2D and 3D interaction of Apigenin 6-C-glucoside with Bcl2



2D and 3D interaction of Luteolin-3', 7-di-O-glucoside with Bcl2



2D and 3D interaction of Glycitein with Bcl2

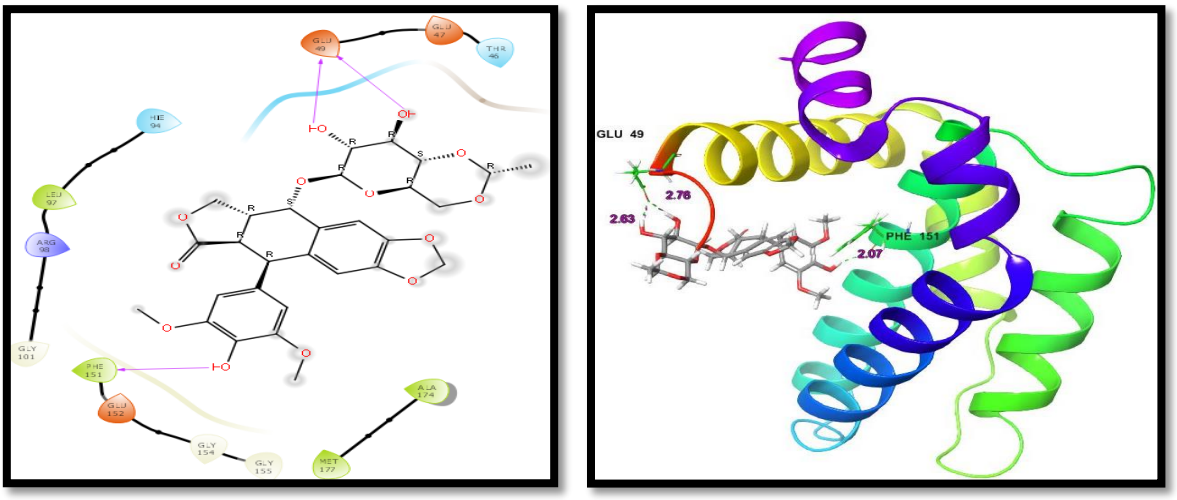


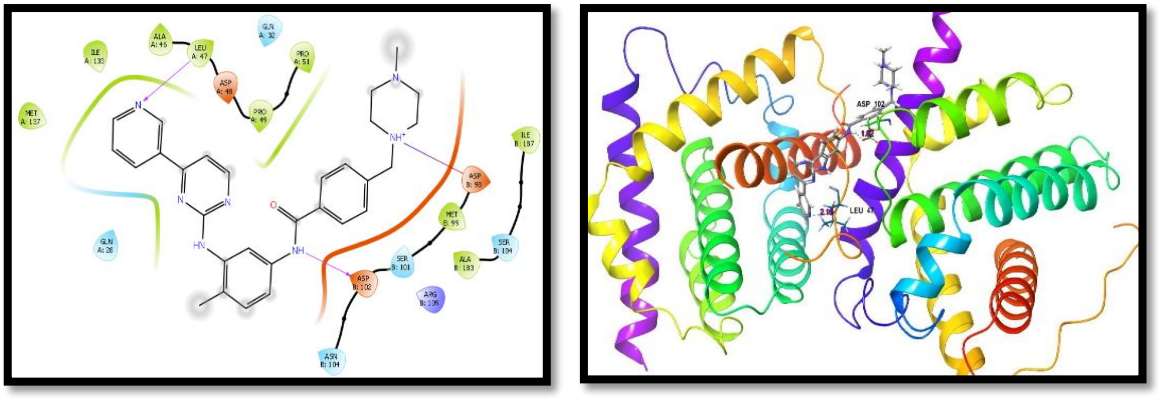
Table 24: Glide SP docking of the Flavonoid Enriched Fraction (FEF) phytoconstituents with Bax

Target and PDB ID	Ligand and PubChem ID	Glide score	Glide energy
Bax (4S0O)	Isoferulic acid 3-O glucuronide (49844484)	-2.845	-27.098
	Pentanedioic acid (3811953)	-3.008	-23.145
	Myricetin (5281672)	-8.927	-29.352
	Apigenin 6-C-glucoside (131750832)	-3.581	-21.741
	Luteolin-3',7-di-O-glucoside (133611799)	-8.354	-53.645
	Glycitein (5317750)	-4.475	-28.963

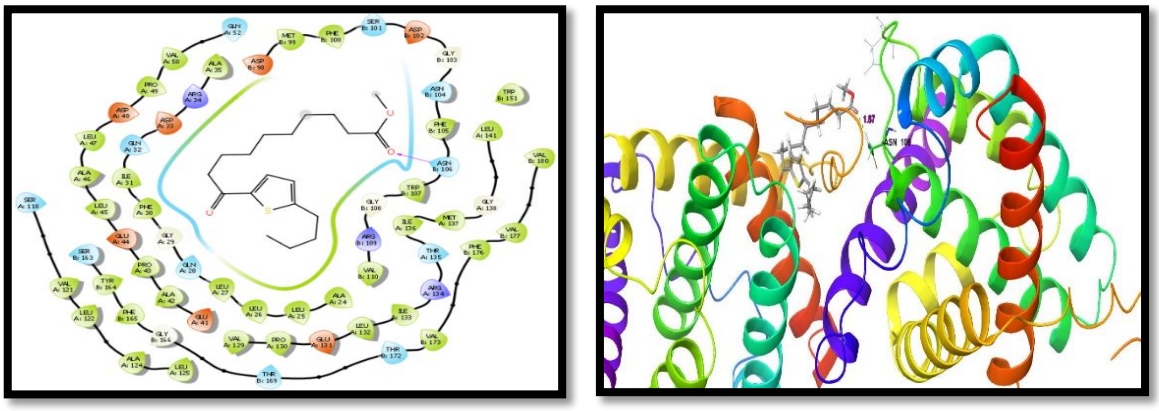
Molecular docking studies of Flavonoid Enriched Fraction (FEF) phytoconstituents with Bax target revealed that Myricetin exhibited best glide score of -8.927 (Table 24 and Plate 7) followed by "Luteolin-3', 7-di-O-glucoside, Glycitein, Apigenin 6-C-glucoside, Pentanedioic acid and Isoferulic acid 3-O glucuronide".

Plate 7: Docking interactions of Flavonoid Enriched Fraction (FEF) phytoconstituents with Bax

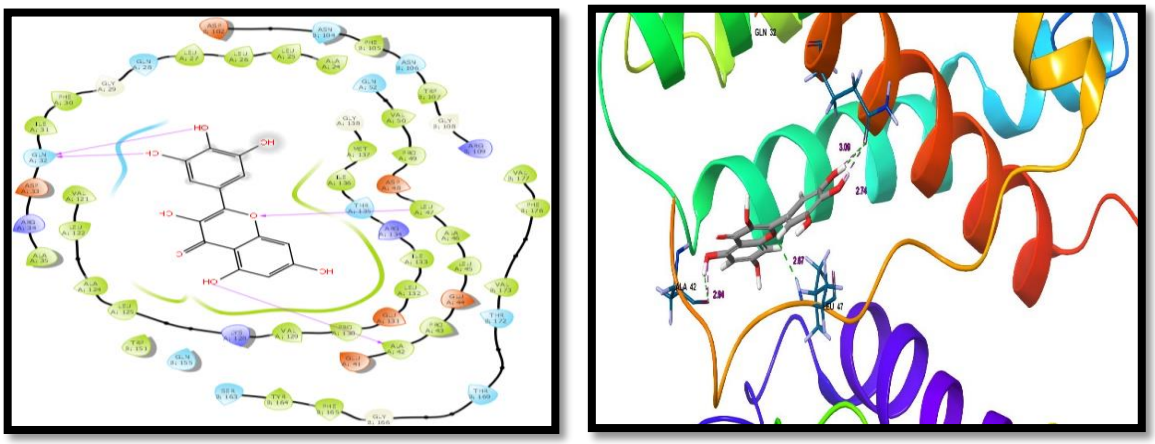
2D and 3D interaction of Isoferulic acid 3-O glucuronide with Bax



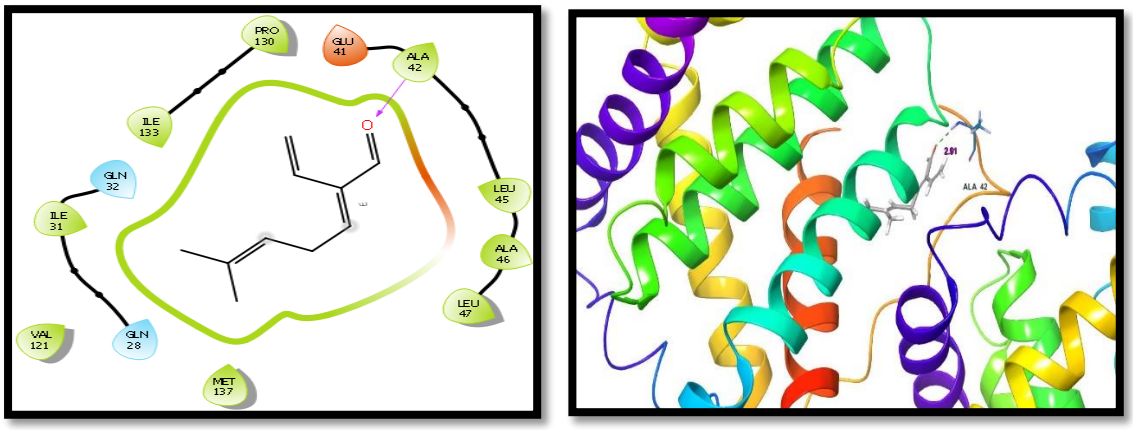
2D and 3D interaction of Pentanedioic acid with Bax



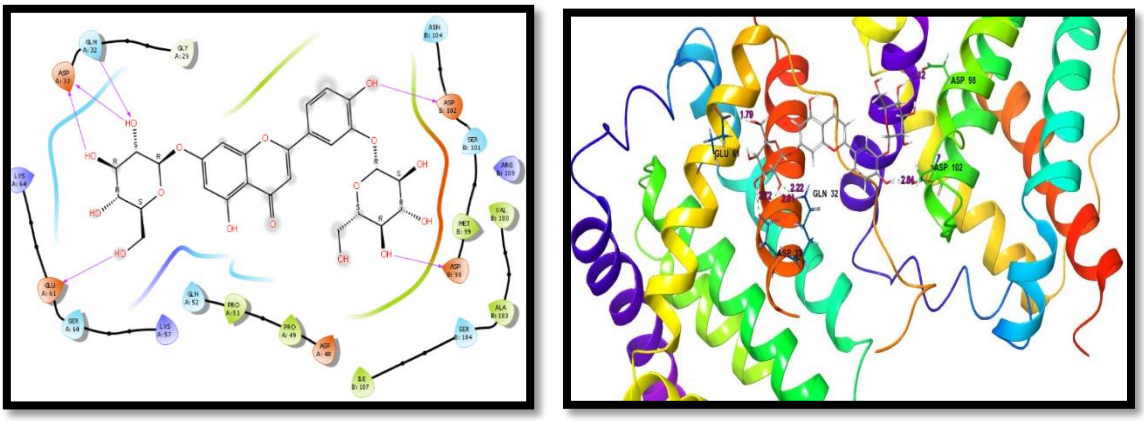
2D and 3D interaction of Myricetin with Bax



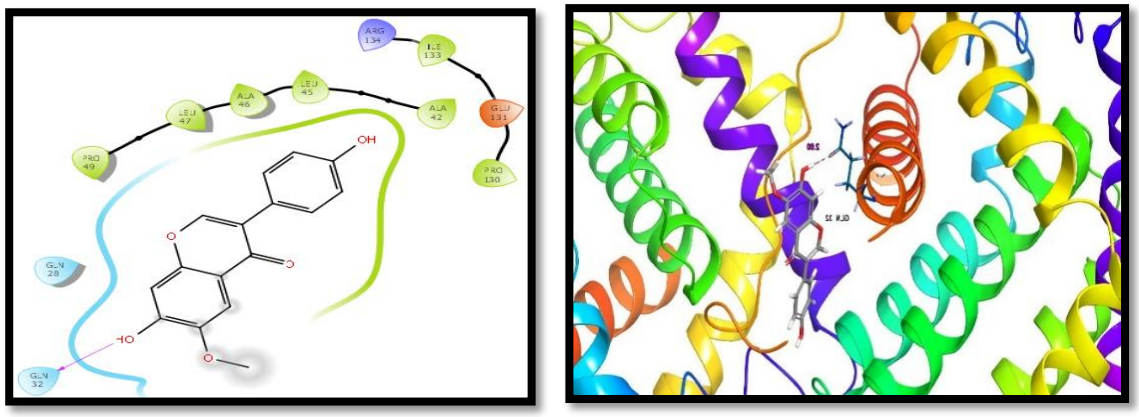
2D and 3D interaction of Apigenin 6-C-glucoside with Bax



2D and 3D interaction of Luteolin-3', 7-di-O-glucoside with Bax



2D and 3D interaction of Glycitein with Bcl2



The results of docking interactions revealed that Isoferulic acid 3-O glucuronide has '0' violations and it has good docking interactions against antiapoptotic proteins namely Bcl2, Mcl1, Bax and the proto-oncogene MDM2, and this can be used for drug designing against cancer and leukemia. Similarly, "Luteolin-3', 7-di-O-glucoside, Apigenin 6-C-

glucoside" exhibit an excellent glide score and glide energy, indicating FEF fractions are potential source of compounds for drug designing as reflected by the docking interaction score against antiapoptotic proteins and proto-oncogenes. The one disadvantage of using these two compounds for drug development is that they violate Lipinski's rule of 5 by 3 and 1 respectively which includes "MW>500, N or O>10, NH or OH>5 and NH or OH>5", this can be overcome by the use of nanocarriers and other formulations for the targeted delivery.

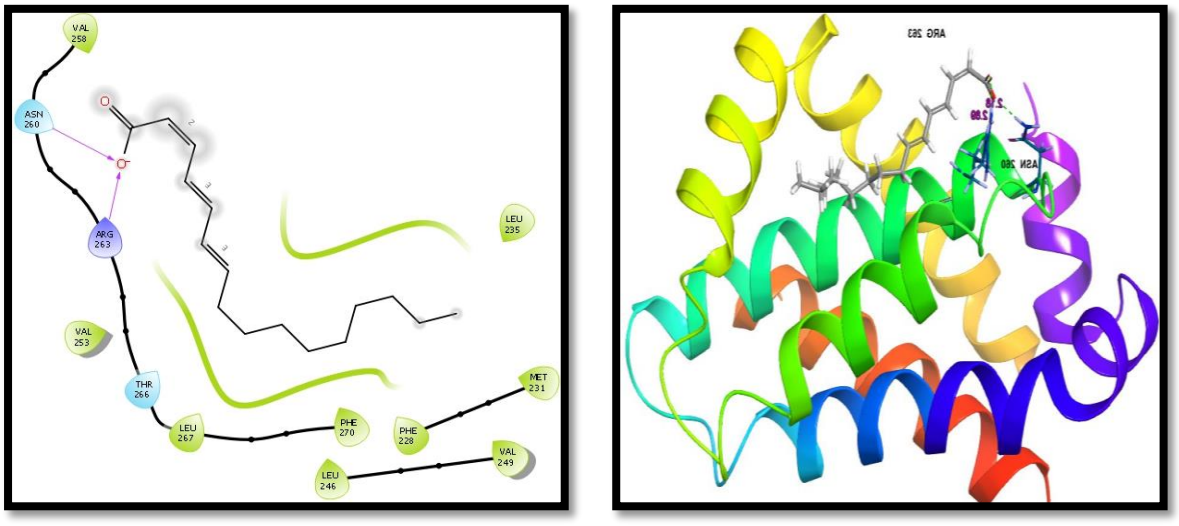
Table 25: Glide SP docking of the Acetogenin Enriched Fraction (AEF) phytoconstituents with Mcl 1

Target and PDB ID	Ligand and PubChem ID	Glide score	Glide energy
Mcl 1 (6VBX)	α-Muurolene (12306047)	-4.787	-15.506
	Cis-solamin (11376469)	-5.475	-32.948
	Muricatacin **(3035657)**	-3.139	-35.908
	Germacrene B (5281519)	-3.866	-15.715

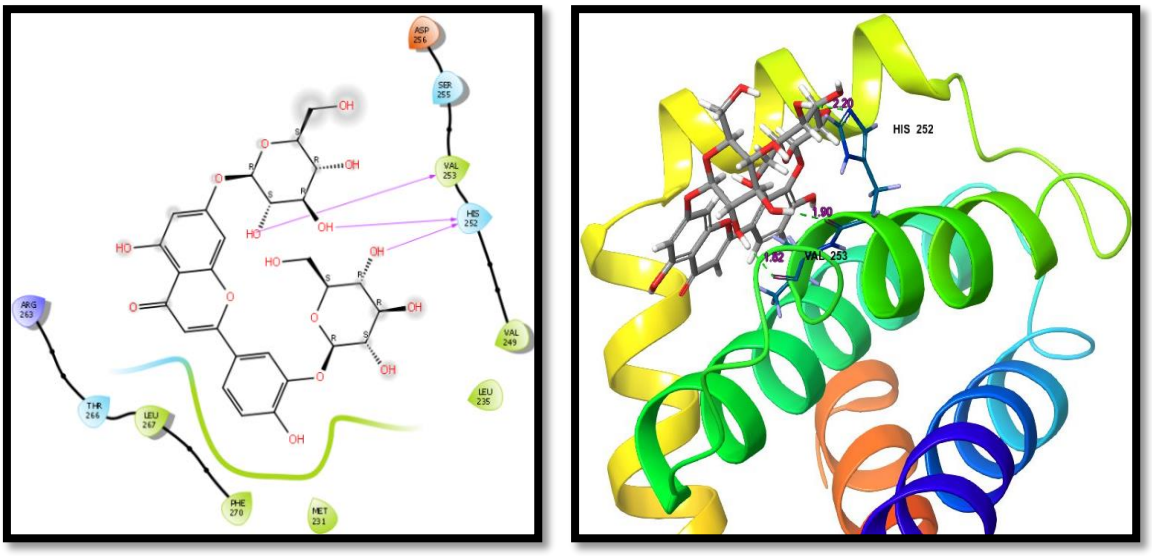
Molecular docking studies of Acetogenin Enriched Fraction (AEF) phytoconstituents with Mcl1 target revealed that Cis-solamin exhibited best glide score of -5.475 (Table 25 and Plate 8) followed by α-Muurolene, Germacrene B and Muricatacin.

Plate 8: Docking interactions of Acetogenin Enriched Fraction (AEF) phytoconstituents with Mcl 1

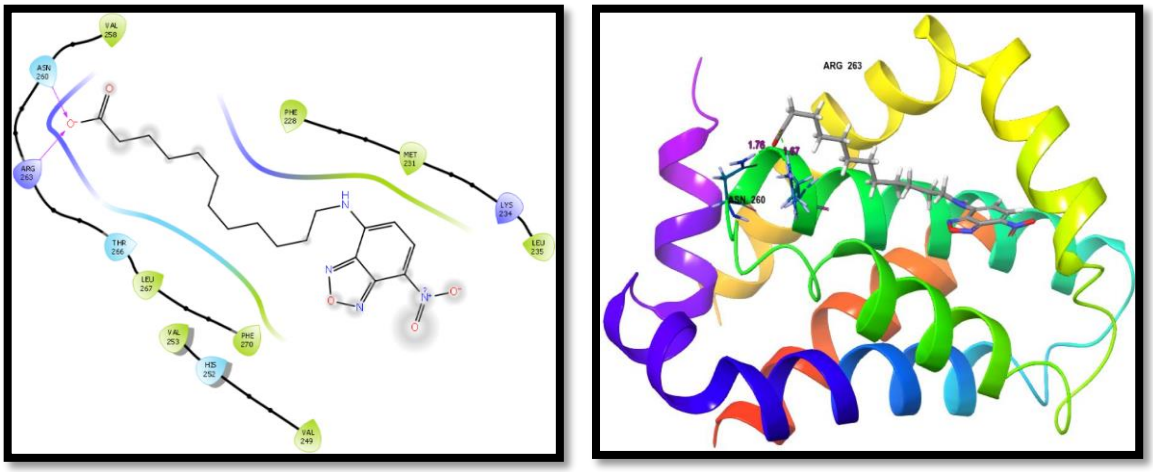
2D and 3D interaction of α-Muurolene with Mcl 1



2D and 3D interaction of Cis-solamin with Mcl 1



2D and 3D interaction of Muricatacin with Mcl 1



2D and 3D interaction of Germacrene B with Mcl 1

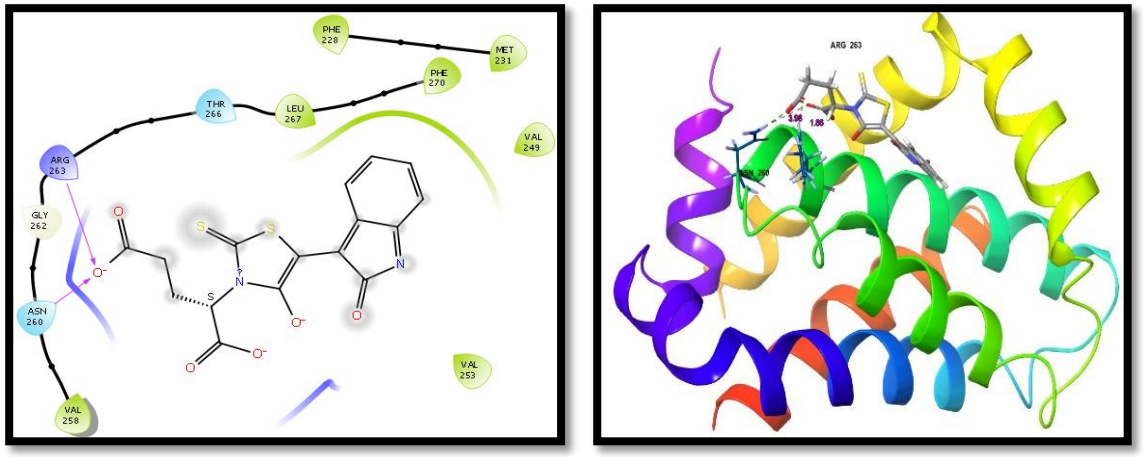


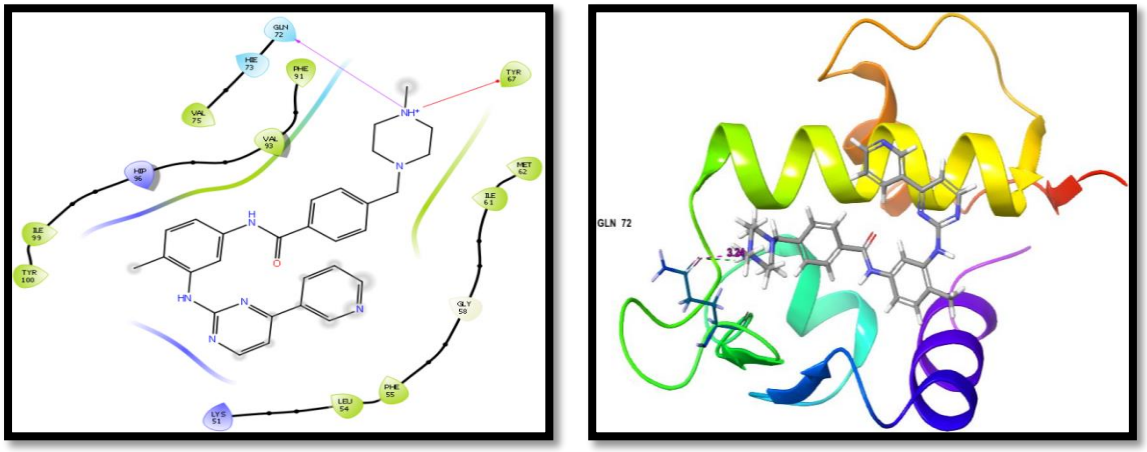
Table 26: Glide SP docking of the Acetogenin Enriched fraction (AEF) phytoconstituents with MDM2

Target and PDB ID	Ligand and PubChem ID	Glide score	Glide energy
MDM2 (5AFG)	α-Muurolene (12306047)	-4.5	-17.071
	Cis-solamin (11376469)	-4.842	-34.064
	Muricatacin **(3035657)**	-5.213	-46.576
	Germacrene B (5281519)	-3.393	-13.882

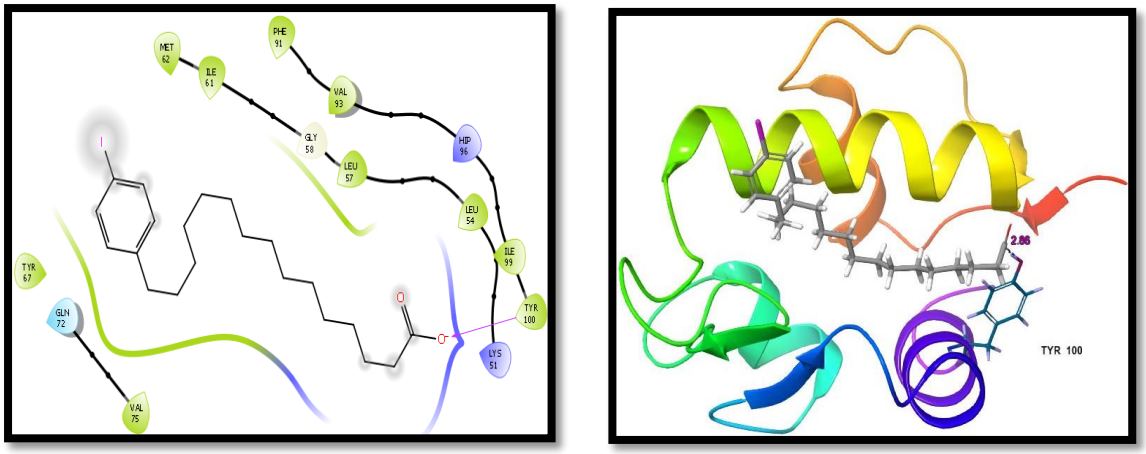
Molecular docking studies of Acetogenin enriched fraction (AEF) phytoconstituents with MDM2 target revealed that Muricatacin exhibited best glide score of -5.213 (Table 26 and Plate 9) followed by Cis-solamin, α-Muurolene and Germacrene B.

Plate 9: Docking interactions of Acetogenin enriched fraction (AEF) phytoconstituents with MDM2

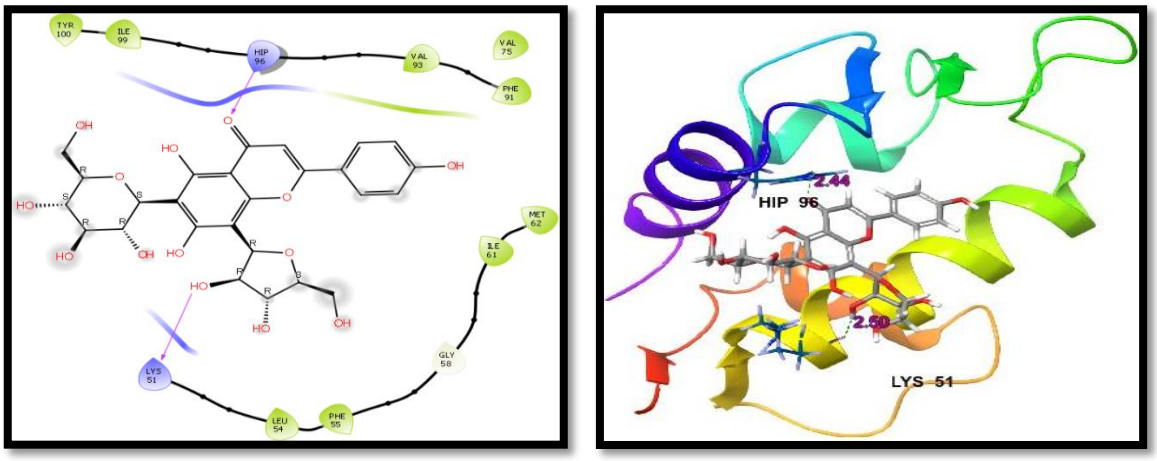
2D and 3D interaction of α-Muurolene with MDM2



2D and 3D interaction of Cis-solamin with MDM2



2D and 3D interaction of Muricatacin with MDM2



2D and 3D interaction of Germacrene B with MDM2

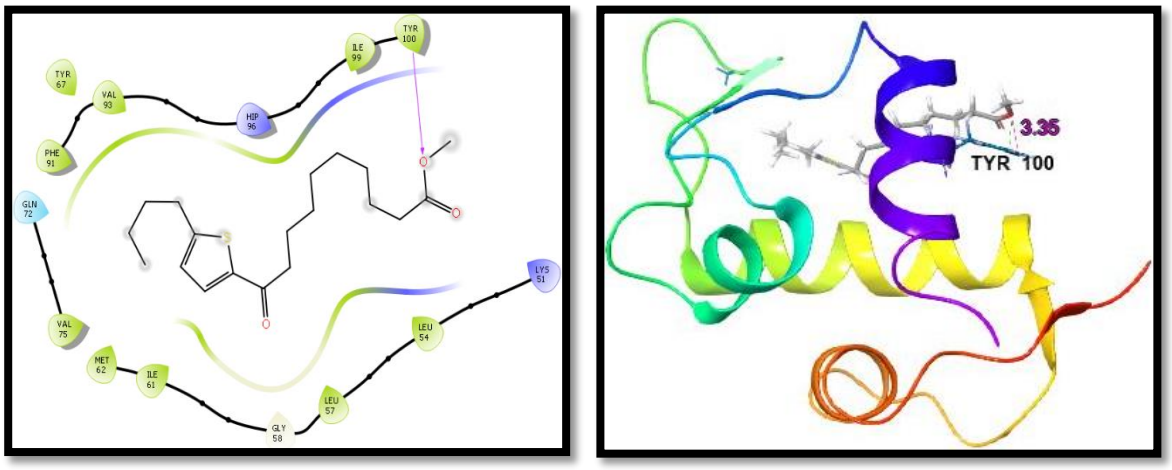


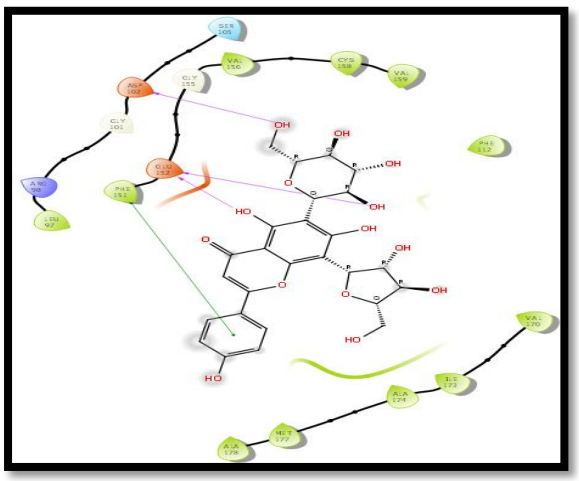
Table 27: Glide SP docking of the Acetogenin Enriched Fraction (AEF) phytoconstituents with Bcl2

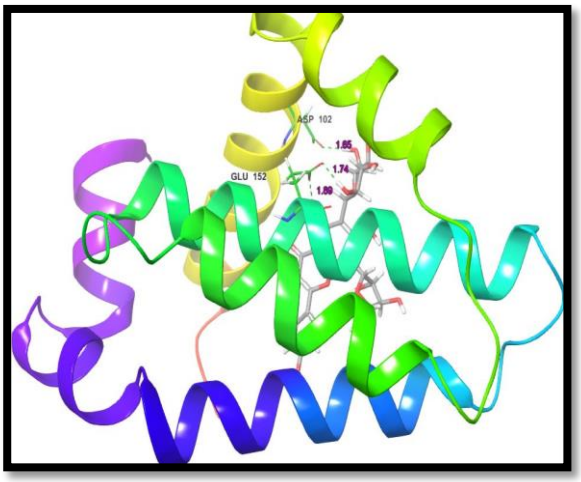
Target and PDB ID	Ligand and PubChem ID	Glide score	Glide energy
Bcl2 (6QGG)	α-Muurolene (12306047)	-2.663	-16.393
	Cis-solamin (11376469)	-3.297	-32.857
	Muricatacin **(3035657)**	-6.879	-54.213
	Germacrene B (5281519)	-2.621	-18.664

Molecular docking studies of Acetogenin enriched fraction (AEF) phytoconstituents with Bcl2 target revealed that Muricatacin exhibited best glide score of -6.879 (Table 27 and Plate 10) followed by Cis-solamin, α-Muurolene and Germacrene B.

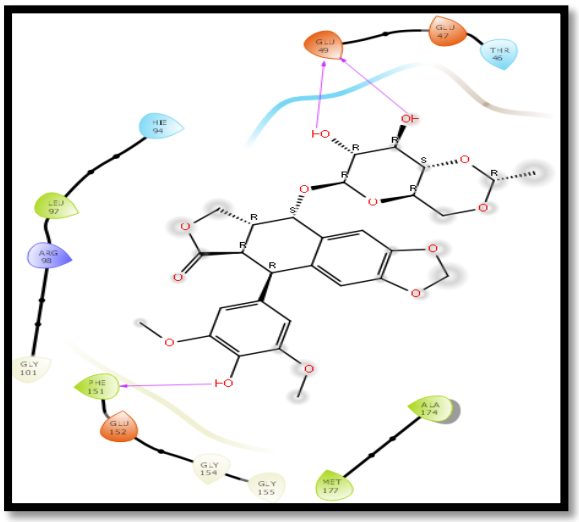
Plate 10: Docking interactions of Acetogenin Enriched Fraction (AEF) phytoconstituents with Bcl2

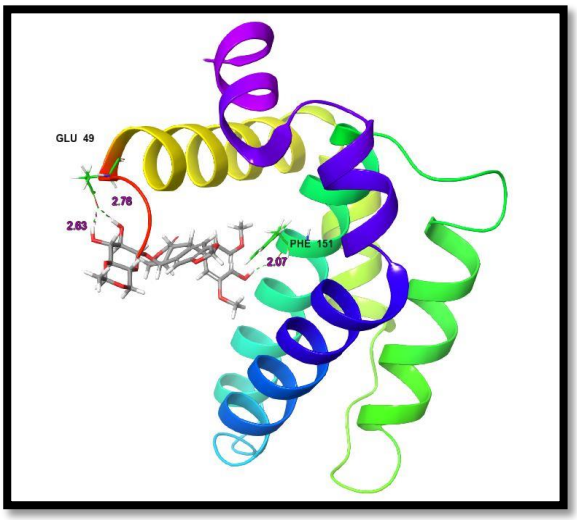
2D and 3D interaction of α-Muurolene with Bcl2



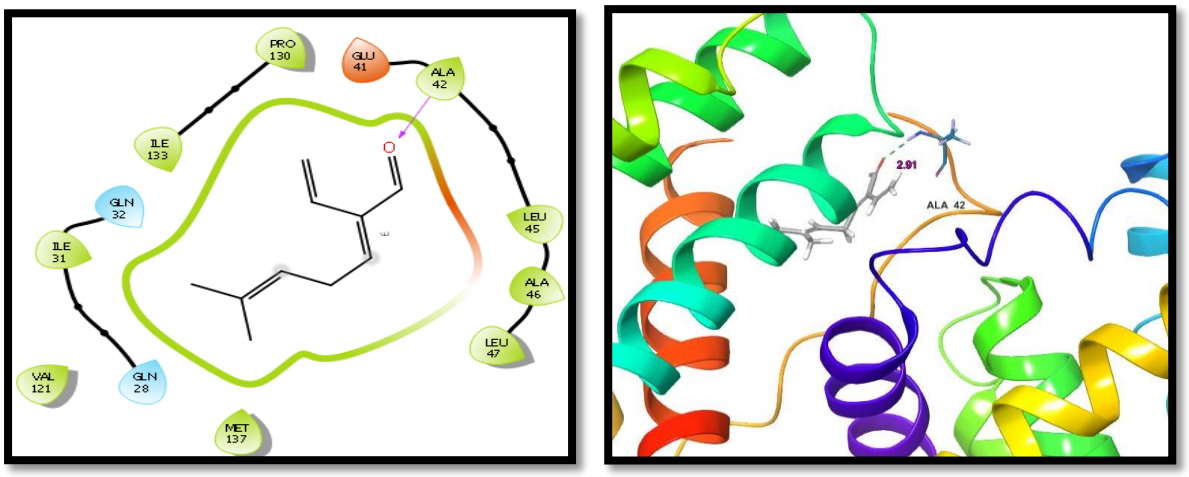


2D and 3D interaction of Cis-solamin with Bcl2





2D and 3D interaction of Muricatacin with Bcl2



2D and 3D interaction of Germacrene B with Bcl2

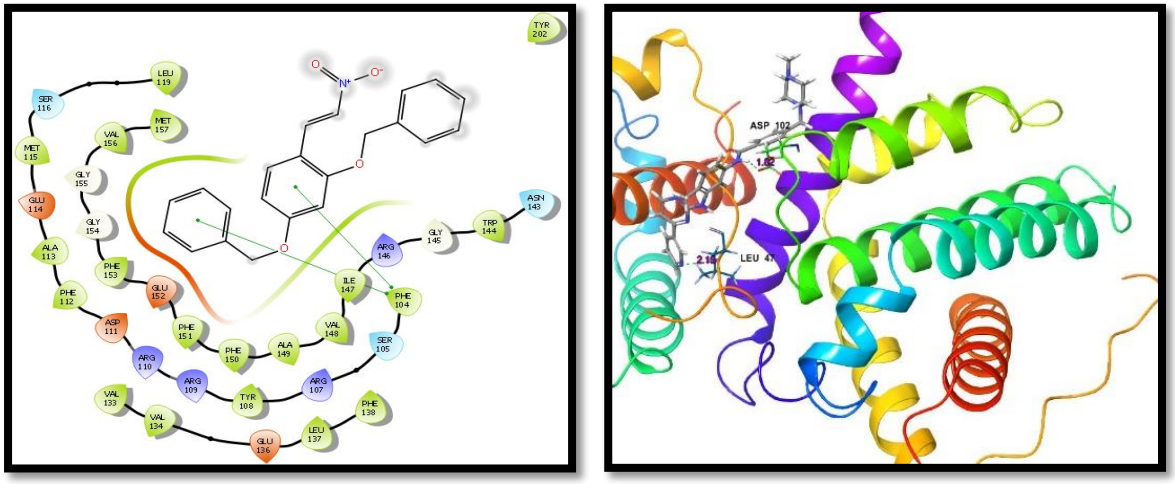


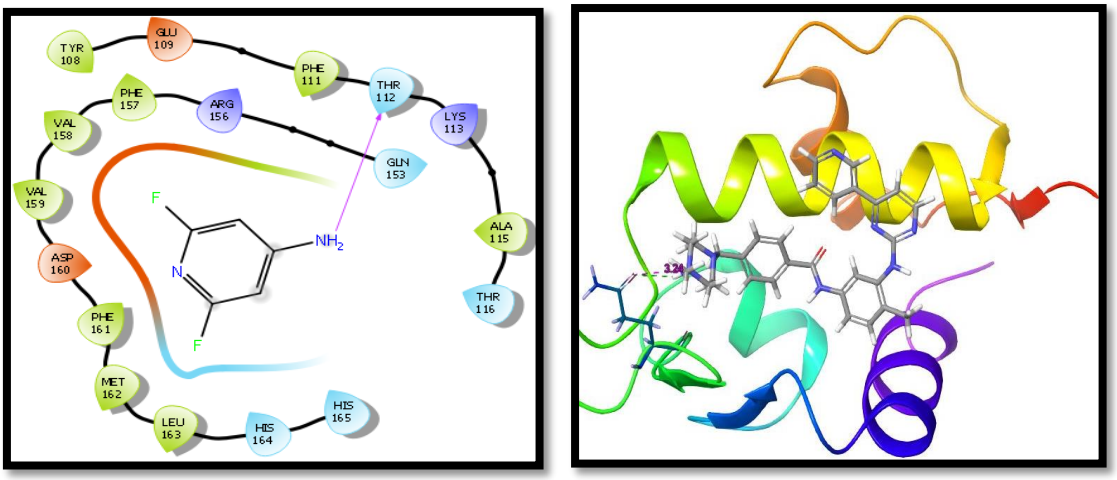
Table 28: Glide SP docking of the Acetogenin Enriched Fraction (AEF) phytoconstituents with Bax

Target and PDB ID	Ligand and PubChem ID	Glide score	Glide energy
Bax (4S0O)	α-Muurolene (12306047)	-3.893	-20.834
	Cis-solamin (11376469)	-4.124	-15.506
	Muricatacin **(3035657)**	-5.594	-47.912
	Germacrene B (5281519)	-4.787	-16.779

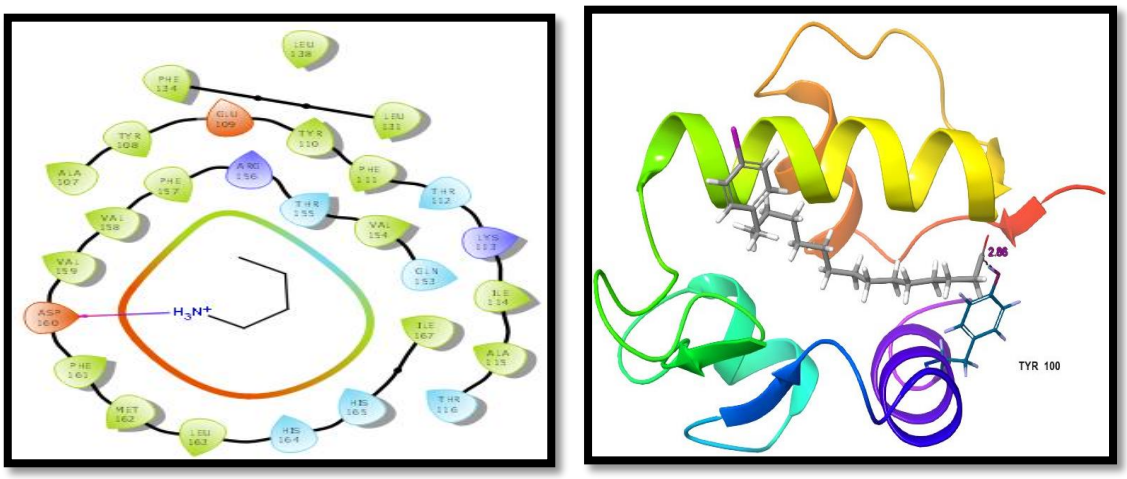
Molecular docking studies of Acetogenin enriched fraction (AEF) phytoconstituents with Bax target revealed that Muricatacin exhibited best glide score of -5.594 (Table 28 and Plate 11) followed by Germacrene B, Cis-solamin and α-Muurolene.

Plate 11: Docking interactions of Acetogenin Enriched Fraction (AEF) phytoconstituents with Bax

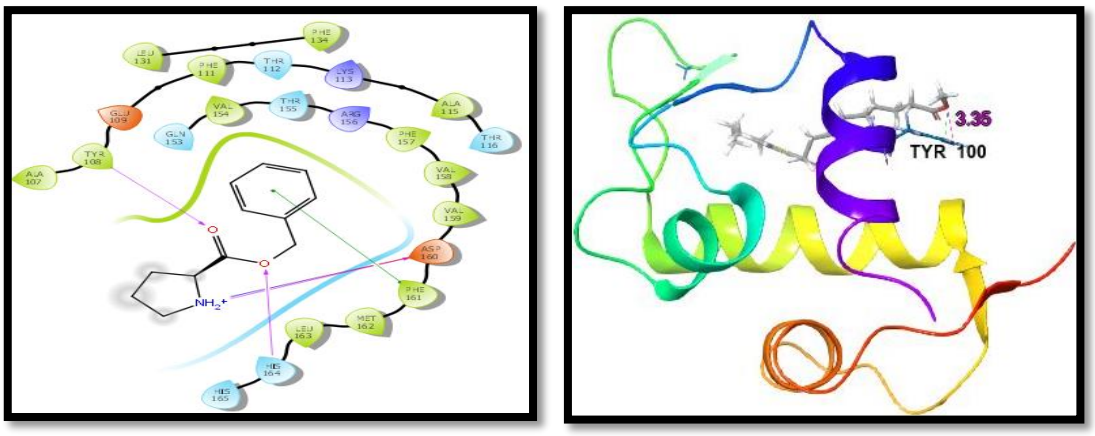
2D and 3D interaction of α-Muurolene with Bax



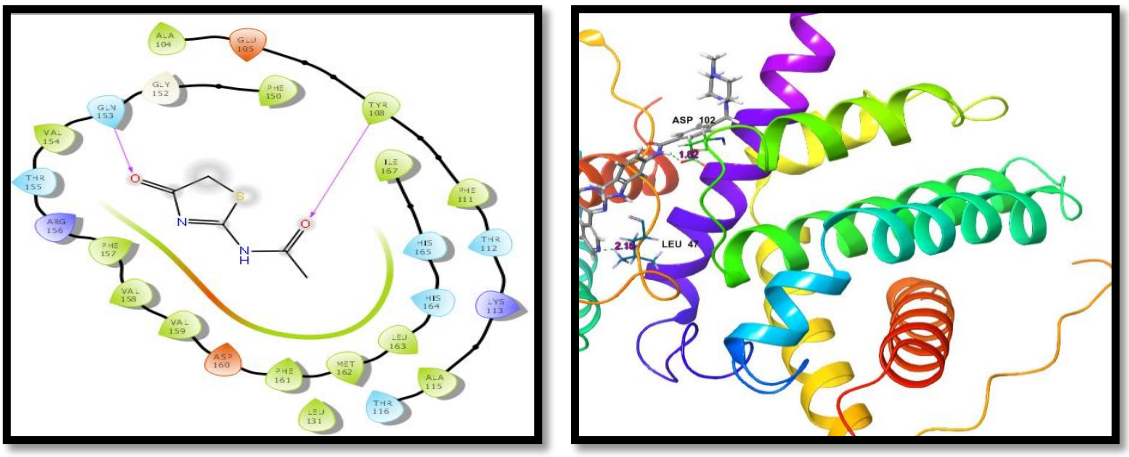
2D and 3D interaction of Cis-solamin with Bax



2D and 3D interaction of Muricatacin with Bax



2D and 3D interaction of Germacrene B with Bax



Ginsenosides isolates were found to be successful in controlling the overexpression of anti-apoptotic proteins such as "BCL-2, BCL-XL, and MCL-1" by Sathishkumar *et al*. (2012), indicating that these drugs could be employed in cancer treatment. Ghani *et al*. (2020) found that "naturally derived flavonoids (biochanin A, myricetin, apigenin, galangin, and fisetin)" could operate as potential inhibitors of the Bcl-2 and Bcl-xl proteins. According to Saffari et al. (2019), thymoquinone forms more persistent connections with BCL-XL than BCL-2 and MCL-1 and promotes apoptotic effects by blocking the anti-apoptotic factor BCL-XL protein, as compared to other factors investigated. Acetogenins - BCL-XL protein complex, notably muricin B, muricin F, muricin H, muricin I, xylomaticin, annomontacin, annonacin, squamocin, squamostatin A, bullatacin, and annoreticulin, displayed substantial binding affinities, according to a molecular docking research by Nordin *et al*. (2021).

Jeevitha *et al*. (2020) intends to screen a powerful multidrug resistance (MDR) inhibitor among acetogenins from the plant *Annona muricata* in an *in-silico* analysis. The compounds were then docked with the ATP binding site and the drug binding site of modelled human P-glycoprotein (P-gp). When compared to conventional medications, Annonacin, Annohexocin, and Annomuricin E docked better with high binding energy.

The above-mentioned reports support our study and the current study reveals that Flavonoid enriched fraction phytoconstituents- "Isoferulic acid 3-O glucuronide, Pentanedioic acid, Myricetin, Apigenin 6-C-glucoside, Luteolin-3', 7-di-O-glucoside and Glycitein" and Acetogenin enriched fraction (AEF) phytoconstituents- "α-Muurolene,

Cis-solamin, Muricatacin and Germacrene B" was able to dock with all the selected apoptotic targets (Mcl 1, Bcl 2, Bax) and leukemia target (MDM2). Among the Flavonoid enriched fraction phytoconstituents docked with apoptotic and leukemia targets "Luteolin-3', 7-di-O-glucoside" showed highest binding efficiency with Mcl 1, Bcl 2 and MDM2. Whereas in the Acetogenin enriched fraction "Muricatacin" showed highest binding efficiency with Bcl 2, Bax and MDM2. The current study shows that Luteolin-3', 7-di-O-glucoside (flavonoid) and Muricatacin (acetogenin) was able to interact with apoptotic targets (Mcl 1, Bcl 2, Bax), demonstrating that these compounds induced apoptosis via intrinsic apoptotic pathway and it also might be able to influence the cell signalling pathway by targeting MDM2 (Leukemia target). These *in silico* results would be the platform for the drugs to be focused in future for exploring therapeutic strategies against Acute lymphoblastic leukemia.

The results of the current study clearly reveal that combination of flavonoid and acetogenin possess increased antioxidant activity than ethanolic crude extract, flavonoid and acetogenin enriched fraction. Similarly in the anticancer activity, the combination group exert considerable effect. Acetogenin enriched fraction induce apoptosis via ATP starvation in cancer cells and acetogenins gain importance as it could specifically target cancer cells without harming healthy cells, thereby minimise the side effects caused during chemotherapy. The interaction results of Flavonoid enriched fraction phytoconstituents exhibited good docking interaction thereby indicating that FEF phytoconstituents could favourably interact with target compounds to induce apoptosis in cancer cells and also downregulate proto-oncogenes like MDM2. Therefore, it might act as a potential source for drug development against leukemia and further studies need to be carried out to validate the results.