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SUMMARY AND CONCLUSION

Cancer is a life-threatening and debilitating disease defined by the abnormal multiplication of cells that enter and destroy nearby tissues. Cancer transmits to organs via the bloodstream and lymphatic arteries. The “International Agency for Cancer Research (IARC)” claims that, India's cancer incidence will rise from 1 million to more than 1.7 million in 2035. This means that, over the same time span, the number of people dying from cancer will rise from 680000 to 1-2 million. As a result, cancer continues to be a major medical problem around the world.

Acute lymphoblastic leukaemia (ALL) is a type of white blood cell malignancy that is characterised by uncontrolled development and accumulation of lymphoid progenitor cells. It is most frequent in children, although it can also occur in adults, accounting for 80 percent of all instances of acute lymphoblastic leukemia worldwide. Leukemia (of which >95 percent are acute) is the most prevalent type of children cancer diagnosed worldwide, including in India. Many disorders, including cancer, may benefit from balancing oxidant and antioxidant reactions in the body. Reactive oxygen species are found in high levels in a variety of malignancies and are now being identified as important contributors to ALL leukemogenesis.

Paediatrics with leukemia is experiencing different side-effects which induced by disease and its treatments. Due to the intricacy of the disease, toxicity of chemotherapy, unaffordability of treatment, and significant side effects, cancer treatment and management is currently a challenge. As a result, it's critical to look at complementary and alternative medicine for anticancer drug leads and development. Herbal drugs are considered as being both inexpensive and safe to consume.

One such plant with extensive traditional use is *Annona muricata*. It is a relatively small herbaceous tree located in South America and South East Asia rain forests. The fruits, leaves, stem and roots of *Annona muricata* are known to be rich in “flavonoids, isoquinoline alkaloids and annonaceous acetogenins”. Flavonoids are polyphenolic compounds,

Acetogenins are a class of polyketide natural product, predominantly present in *Annona muricata.* More than 100 *Annonaceous* acetogenins are found to be in the family *Annonaceae* and these are natural antitumor compounds. These acetogenins were revealed to be particularly harmful to a variety of malignant cells while having no effect on healthy cells.

The present study aims to identify the bioactive components present and its synergistic effect of flavonoid and acetogenin enriched fractions of *Annona muricata* leaves and to investigate the antioxidant, anticancer with the focus on the antileukemic activity of the flavonoid and acetogenin enriched fractions of *Annona muricata* leaves.

The present study was classified into four phases. In phase I, an attempt was made to isolate flavonoid and acetogenin enriched fractions from *Annona muricata* leaves and its characterization using various spectral and chromatographic techniques. The second phase was designed to analyse antioxidant activity of flavonoid and acetogenin enriched fractions against a team of radicals. In phase III, antileukemic activity of the flavonoid and acetogenin enriched were performed in Acute Lymphoblastic Leukemia cell line (Molt-3) which was compared with the normal counterpart peripheral blood lymphocytes (PBL). In the final phase, ADME properties and docking studies were performed to find the interactions between the reported flavonoid and acetogenin enriched fraction phytoconstituents against apoptotic and leukemia targets.

The results of UV-Visible absorption spectral analysis showed that distinct peaks at different wavelength of ethanolic crude extract, flavonoid enriched fraction and acetogenin enriched fraction corresponds with the standard Quercetin, Kaempferol and Andrographolide reveals the phytoconstituents present in the fractions and ethanolic crude extract of *Annona muricata* were found to be flavonoids or phenolics and acetogenins in nature.

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.

Thus, it can be inferred that the presence of alkanes, alkenes and aromatic ring in acetogenin enriched fraction confirms the presence of acetogenins. Similarly, the presence of functional groups namely "aromatic, alkane, alkene, alkyne and phenol groups" confirms the presence of flavonoids in flavonoid enriched fractions obtained from the bioassay guided fractionation.

HPTLC analysis performed in the present study clearly evidenced the presence of various phytochemicals including flavonoids and acetogenins in the *Annona muricata* ethanolic crude extract, flavonoid and acetogenin related compounds in the flavonoid and acetogenin enriched fractions of *Annona muricata* leaves. Further, the HPLC analysis confirms the presence of flavonoids and acetogenins in *Annona muricata* leaves, as the results revealed that ethanolic crude extract contain more phytoconstituents, and the bio-assay guided fractionation could help to separate the flavonoid and acetogenin related phytocompounds from crude extract, and the compounds were more similar to the reference standards of flavonoids in FEF and acetogenins in AEF.

The peaks observed in GCMS spectra revealed the presence of "10-Oxodecanoic acid, methyl ester, Pentadecanoic acid, α-Gurjunene, Dodecanoic acid, phytol, pentadecanol, Hexadecatrienoic acid and Oleyl alcohol" in ethanolic crude extract. "Isoferulic acid, Pentanedioic acid, Myricetin, Apigenin-6-C-glucoside, Luteolin 3′7-di-O-glucoside and Glycitein" in flavonoid enriched fraction, and "α-Muurolene, Cis-solamin, Muricatacin and Germacrene B" in acetogenin enriched fractions. However, further studies need to be carried out to elucidate the structure and nature of their bioactive compounds.

In phase II, DPPH radical scavenging activity of the *Annona muricata* leaf extract and fractions could be attributed to the existence 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 and thus the results are on par with Ascorbic acid.

ABTS radical scavenging assay revealed that the flavonoids and acetogenins found in *Annona muricata* may be responsible for scavenging action. The combined fractions could cause the potential scavenging of ABTS radical indicating its antioxidant capacity.

Hydrogen peroxide radical scavenging activity reveals that phenolic substances present in the enriched fractions acted as H_2O_2 scavenger by transferring electrons to H_2O_2 and causing neutralisation to water, and the scavenging action could be due to the antioxidant potential of *Annona muricata* there by renders protection against oxidative stress induced damage to cells.

In hydroxyl scavenging activity, the existence of various secondary metabolites in the *Annona muricata* leaf extract and fractions, such as flavonoids and acetogenins, may be responsible for hydroxyl free radical scavenging activity, which might be attributed by the presence of aromatic ring structures of the phytoconstituents of *Annona muricata*.

Nitric oxide inhibition assay suggests that polyphenols, flavonoids, and acetogenins found in ethanolic crude extract, flavonoid and acetogenin enriched fractions of *Annona muricata* might be responsible for the inhibitory action against the generation of NO radicals.

Super oxide inhibition assay revealed 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.

The chelating activity of *Annona muricata* ethanolic crude extract and fractions might help in the reduction of ROS formation there by preventing the impairment of cellular functions. 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 donors and can react with free radicals and terminate chain reactions, thereby causing a reduction in the oxidative stress mediated cellular damage.

In phase III, the cytotoxic MTT and SRB assay revealed that *Annona muricata* leaf fractions targeting cancerous cells alone, without harming the normal healthy cells, might have attributed by the presence of 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.

Synergistic effect of flavonoid and acetogenin enriched fraction indicates strong synergism between the flavonoid and acetogenin enriched fraction against the growth of Acute lymphoblastic leukemia cell line (Molt-3), based on the results and interpretations obtained from Loewe model, Highest Single Agent model and Bliss model.

Measurement of apoptosis by flow cytometry, reveals 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.

Mitochondrial membrane potential by JC-1 staining reveals that in Molt-3 cells, there was a loss of mitochondrial membrane potential to a great extent, whereas in the PBL, minimal loss of MMP was observed and indicated that *Annona muricata* extract and fractions were able to trigger apoptosis through intrinsic apoptotic pathway.

Annona muricata leaf extract and fractions are capable of arresting the cells (Molt-3) in the early phase of the cell cycle and the cells are triggered into apoptotic cell death. 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.

ROS can cause cell death by activating many signaling pathways, leading to cell apoptosis, the measurement of ROS revealed that *Annona muricata* crude extract and fractions elevated the ROS levels in Molt-3 cells while 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.

In phase IV, among 6 compounds chosen from 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. In the AEF, among 4 compounds chosen, only one compound- Muricatacin showed the drug-likeness and these ligands can be used for further studies to develop a potential drug. Molecular docking studies 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 signaling pathway by targeting MDM2 (Leukemia target). To sum up, the *Annona muricata* phytoconstituents with known potent antioxidant and anticancer properties, docking studies suggest that the FEF and AEF phytoconstituents show a good inhibition activity against the apoptotic and leukemia target as per the molecular docking interactions.

To sum up, the findings of this research, 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 thereby minimise the side effects caused during chemotherapy. The interaction results of Flavonoid enriched fraction phytoconstituents exhibited good docking interaction thereby indicating that "Isoferulic acid 3-O glucuronide, Pentanedioic acid, Myricetin, Apigenin 6-C-glucoside, Luteolin-3', 7-di-O-glucoside and Glycitein" 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.

Suggestions for future research

The outcome of our current research work has shown the possibilities of tapping the potential of flavonoid and acetogenin enriched fraction of *Annona muricata* leaves for further research. We hereby, recommend the following points that can be taken up for further investigation.

- The anticancer activity of flavonoid and acetogenin enriched fraction of *Annona muricata* leaves can be further tested using other Acute lymphoblastic leukemia cell lines and also in other cancer cell lines.
- The synergy of flavonoid and acetogenin enriched fraction of *Annona muricata* leaves can be further tested in experimental animals.
- Acetogenin fractions targeting cancer cells specifically can be used as a source for liposome preparation and ease the process of drug delivery as it lacks drug likeliness due to violation of Lipinski's rule of five when arrested *in silico*.

- Animal models can be used to conduct *in vivo* research to validate the drug's anti-cancer efficacy.
- Clinical trials can be carried out using human volunteers for their pharmacological validation.