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Cytotoxicity Assessment of *Annona muricata* Leave Extracts on (*in vitro*) Cultured Breast Tumour Xenograft Cells, B16F1 Murine Melanoma Cells and Cultured Lymphocytes by MTT Assay

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Abstract:
The ongoing rise in the number of cancer cases increases the concerns regarding the efficacy of the various treatment methods that are currently available. Consequently, patients are looking for alternatives to traditional cancer treatments such as surgery, chemotherapy, and radiotherapy as a replacement. Medicinal plants are universally acknowledged as the cornerstone of preventative medicine and therapeutic practices and (Soursop) *Annona muricata L* is a traditional medicinal plant that has been widely used as an anticancer treatment and requires more thorough study.
This research, inspired by the bioactivity from different studies of the aqueous and alcoholic extract of *A. muricata*, we employed it as a plant-based material for the green synthesis of metal and polymers based nanoparticles to assess their cytotoxic activity on different cultured cells by MTT assay. The synthesized NPs were characterized using double beam UV–visible spectroscopic analysis, and microscopy.
The aqueous extract of *A. Muricata* shows higher level of cytotoxicity with lesser viable cells on each cell culture while ethanolic conjugates have shown moderate cytotoxicity and the extract of 95% methanolic have very poor cytotoxicity effect on breast cell culture as well as lymphocyte culture but have shown moderate effect on B16F1 cells. Therefore, the leaves of the medicinal plant *A. muricata* contained compounds that on extraction exerted an effective activity as an anticancer treatment for cancer. Our data identify graviola extracts and their constituents as promising sources for new chemo preventive and therapeutic agent(s) to be further developed for the control of breast cancer ad melenoma skin cancer. Further in vitro and in vivo studies should be carried out to explore the molecular mechanisms underlying their anticancer activity and more detail toxicity study to ensure they are safe for human consumption.

Keywords: *Annona muricata L.*, Breast xenograft culture, B16F1 cells, melanoma, MTT assay, cell viability.

INTRODUCTION:

According to the Global Cancer Observatory (GLOBOCAN) estimates, there were 19.3 million incident cancer cases worldwide for the year 2020. India ranked third after China and the United States of America. They predicted that cancer cases in India would increase to 2.08 million, accounting for a rise of 57.5 per cent in 2040 from 2020. (Ferlay J *et al*,2020). In India, one in nine people are likely to develop cancer in his/her lifetime. The cancer incidence is continuing to increase in India.

Breast cancer is one of the most common types of cancer, second to lung cancer worldwide. Roughly 2.3 million females were diagnosed with breast cancer around the world in 2020 and with an estimated 685,000 deaths (Arnold M, *et al*, 2022). Breast cancer is a malignancy that originates in breast tissue mainly, ducts and lobules., characterised by uncontrolled cell growth. The most common symptoms include, lumps, changes in breast size or shape, skin alteration, and nipple discharge. Genetic factors are associated with 5–10% of cancers, while other factors also make contributions, such as insufficient diet, certain infections, physical inactivity, obesity, smoking, as well as air and water pollution, which can exert direct or indirect impacts on the activity of key cancer-related genes (Anand, P, *et al*, 2008).

Melanoma skin cancers (MSCs) are the leading cause of skin cancer-related morbidity and mortality. Effective strategies are needed to control MSC occurrence and progression. Non-toxic, plant-derived extracts have been shown to exert multiple anti-cancer effects.

Current treatments modern treatment for cancer includes surgery, chemotherapy, radiation therapy and targeted therapy. However, challenges such as severe side effects, treatment resistance, high costs. Natural plant derivatives contain phytochemicals that can help in treating various diseases. Unlike synthetic compounds, each type of phytochemicals has unique characteristics and complex structures. Over 47% of current anticancer drugs on the market are natural products, their derivatives or natural product synthetic mimics, and more than 25,000 identified phytochemicals have been shown to possess potent anticancer activities (Newman DJ, *et al,* 2016). Graviola (*Annona muricata*), a tropical fruit-bearing plant, has been used in traditional medicine against multiple human diseases including cancer for centuries (Chamcheu JC, *et al*, 2018). The Annonaceae family, to which *A. muricata* belongs, includes Annonaceae Acetogenins (AGEs) that strongly inhibit NADH oxidase, affecting mitochondrial function (Patel M.S, *et al,* 2016). *A. muricata* leaf extract has shown potential in reducing breast cancer cell invasion and promoting cancer cell death, indicating its use in cancer prevention. (Athar M, *et al*,2014).

Phytochemicals are often small molecules with many hydrogen bond donors and acceptors, which makes them potential candidates in drug discovery. However, their low water solubility limits their usefulness in the body (Atanasov A.G. *et al,*2021).

The development of strategies to reduce the toxic side effects of chemotherapy drugs is a critical area of research. Nanotechnology, when integrated with biomedicine, holds great promise for transforming drug delivery in modern medicine. Nanomedicine focuses on using nano-carriers—engineered from natural or synthetic polymers—to encapsulate nano-sized drug particles for targeted therapy. These carriers possess essential properties such as targeted delivery, high specificity, improved solubility, controlled drug release, enhanced stability, and better adsorption. Such features enable efficient and precise delivery of therapeutic agents to disease sites, minimizing side effects and enhancing treatment outcomes with maximum efficacy.

Peripheral blood mononuclear cells (PBMCs), including lymphocytes, monocytes, NK cells, and dendritic cells, play key roles in innate and adaptive immunity. It is widely used in disease modelling and immunological researches, PBMCs are isolated via density gradient solvent. Breast tumour xenograft

(PDX) models are patient-derived and mimic the original tumour's gene and protein expression. Maintained in sterile conditions, they are valuable for testing anti-cancer drugs and studying tumor microenvironments. B16F1 murine melanoma cells, which exhibit fibroblast-like morphology and produce melanin, are used to study pigment biology and cancer. In this study, we synthesize metallic and chitosen polymer based nanoparticle explore the cytotoxic capability of a nano-conjugate developed by encapsulating *A. muricata* leaf extract with metallic and chitosan polymer based nanoparticle against B16F1 Murine Melanoma Cell Line, Breast xenograft tumour culture and lymphocytes cell culture.

MATERIAL & METHODS:

Chemicals, reagents and cell lines:

a) **Cell lines:** The B16F1 Murine Melanoma Cell Line had been obtained from the NCCS, Pune. The breast xenograft tumour was obtained from Department of Surgery and the blood sample procured from Department of Pathology, JNCH & RC, Bhopal.

b) **Collection of Plant sample:** Fresh (disease-free) and mature leaves of *A. muricata L*. were collected from the Madan Mohan Herbal Garden of JNCH & RC, Bhopal, M.P.

c) **Samples preparation and extraction**: Aqueous & Alcoholic extraction of the *A. muricata* leaves were prepared. To decolorize a part of crude extract it was filtered by activated charcoal method, where the charcoal absorbs unwanted pigments and we got a clear solution.

d) **Preparation of conjugates:** Silver (Asif M. *et al.* 2022) Copper (Oli H. B. *et al,* 2018), Iron (Alam *et al,*2019), and Chitosan polymer based (Ozdamar B., *et. al*, 2023) nanoparticles were synthesized using *Annona muricata* leaf extracts via modified green biosynthesis methods after reviews taken by above authors**.**

e) **Characterization of NPs:** Prepared nanoparticles were scanned to detect the absorbance peak using an UV/Vis spectrophotometer between the wavelengths of 200 and 900 nm. Further analysis is required on more advance instrumentation like NMR, FTIR and zeta potential is required to characterize the successful synthesis of conjugate

f) **Cell viability assay:** The cytotoxicity test was performed by seeding of the cells in MTT plates with RPMI-1640 media. The cells were treated with the different concentrations of crude extract and different NPs in triplicate set were incubating for 24hrto 48hrs absorbance was determined (547 nm) on a microplate reader.

g) **Statistical Analysis:** The collection of the data from the different biological studies represents the mean ± standard deviation. Two-tailed Student t-test was used to compare differences between two groups. The p-values (≤0.05) was considered statistically significant.

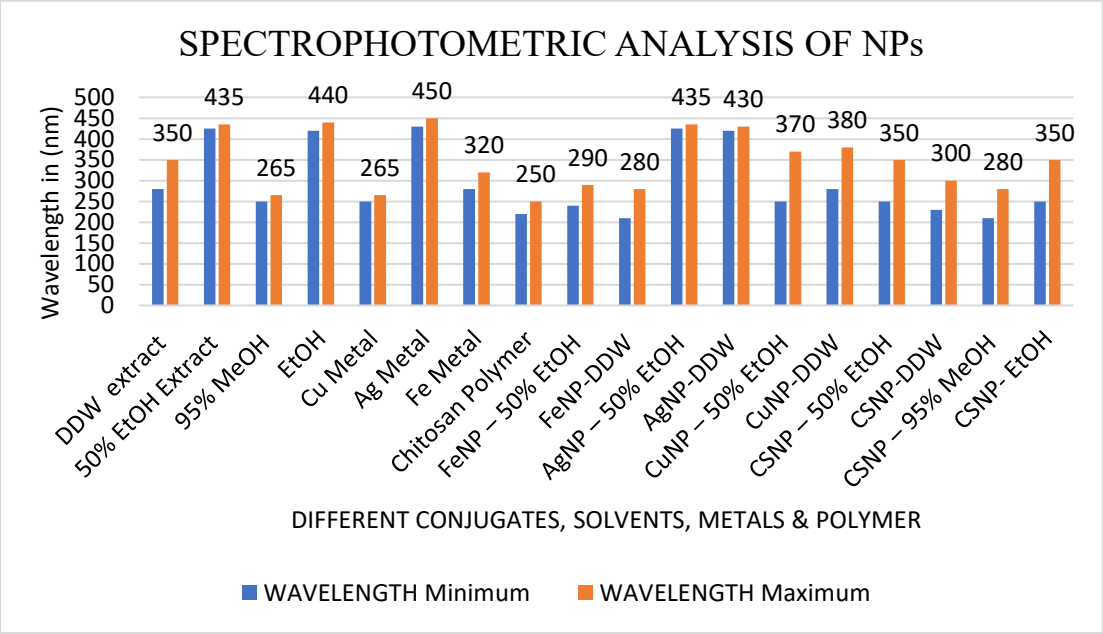
OBSERVATIONS:

(a) Photo aided synthesis of Annona muricata NPs: The synthesis of metal nanoparticles using *Annona muricata* leaf extract was confirmed by characteristic colour changes. CUNPs turned from light brown to greenish dark brown (Rajendran, A.,*et al*, 2018), FENPs from orangish brown to dark brown(Jose, Sr*, et al,* 2020), AGNPs from colourless to dark brown(Ahmed S., *et al,* 2016), and CS from dark greenish to greenish brown, indicating successful nanoparticle formation.

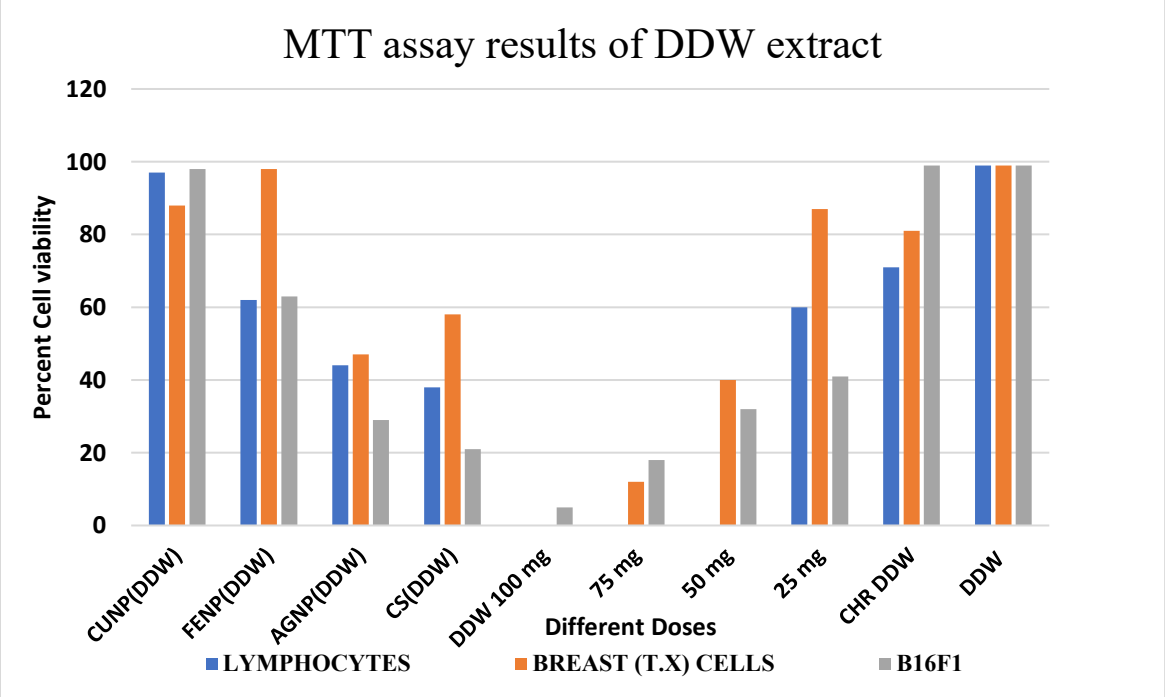
(b) Morphological and Structural Characterization of NPs: Normal photographs were taken by Motic-BA 210 image analyser but TEM microscopy & Zetasizer was proposed.

(c) Characterization of Optical Properties: Wavelength range were scanned by UV-VIS Spectrophotometry. But NMR & FTIR was proposed.

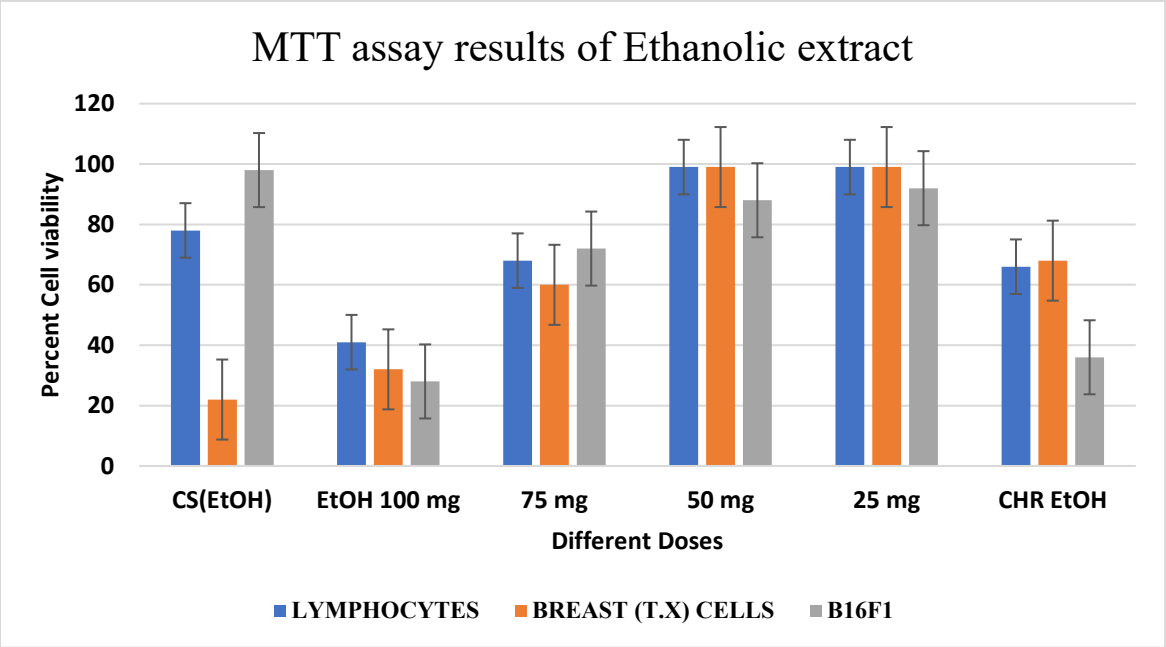
Graph 1: Showing 'Spectrophotometric analysis of prepared NPs absorbance in different wavelength range.



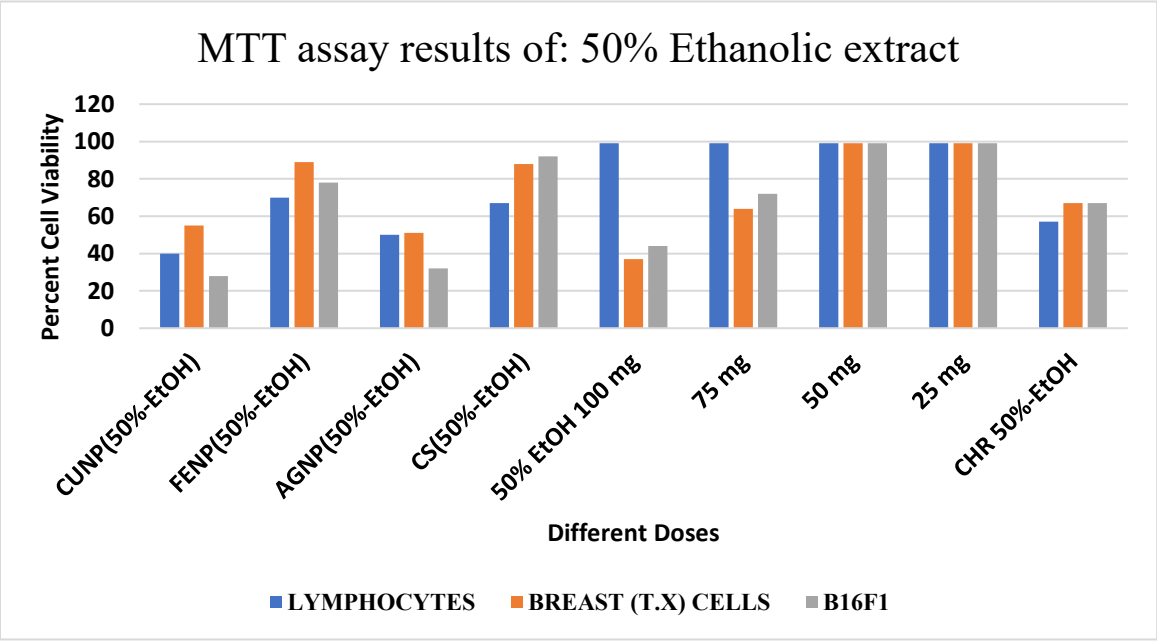
Graph 2: Showing 'Percent Cell viability' after 48 hrs incubation at 37°C (5% CO_2) on different cultured cell, Cytotoxicity effect of *Annona muricata* DDW extract by MTT assay.



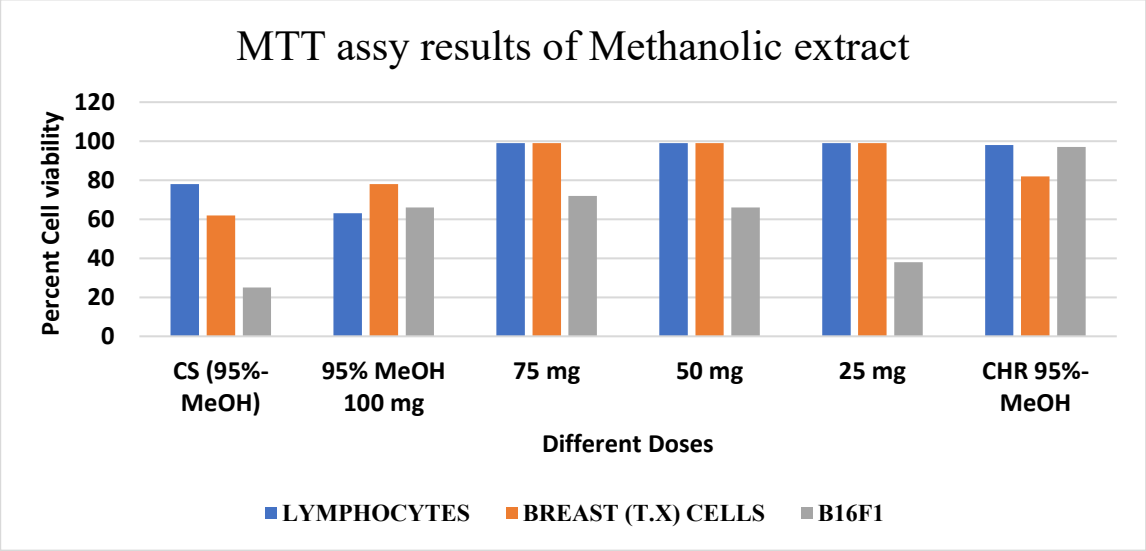
Graph 3: Showing 'Percent Cell viability' after 48 hrs incubation at 37°C (5% CO_2) on different cultured cell, Cytotoxicity effect of *Annona muricata* ethanolic extract by MTT assay.



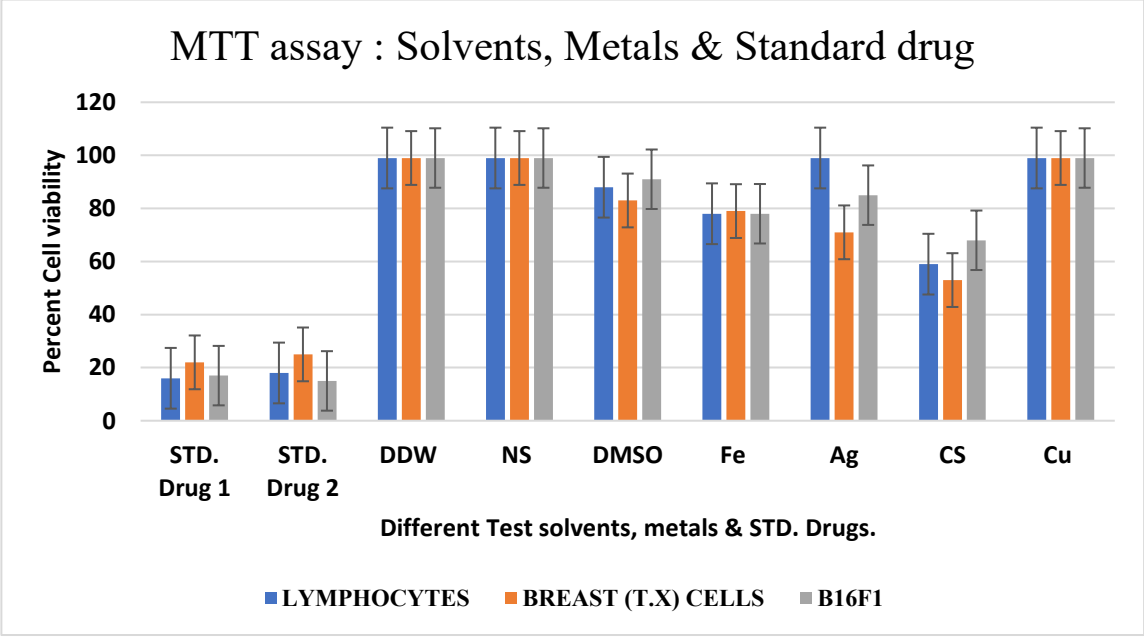
Graph 4: Showing 'Percent Cell viability' after 48 hrs incubation at 37°C (5% CO_2) on different cultured cell, Cytotoxicity effect of *Annona muricata* 50% Ethanolic extract by MTT assay.



Graph 5: Showing 'Percent Cell viability' after 48 hrs incubation at 37°C (5% CO_2) on different cultured cell, Cytotoxicity effect of *Annona muricata* 95% Methanolic extract by MTT assay.



Graph 6: Showing 'Percent Cell viability' after 48 hrs incubation at 37°C (5% CO_2) on different cultured cell, Cytotoxicity effect of Metals, Solvents & Standard drug used in the study by MTT assay.



RESULTS:

The **percentage yields** of *Annona muricata* leaves extracts were higher with 50% EtOH solvent (21.11%) than with DDW (18.77%) and 95% MeOH and EtOH shows 12.66 %, 10.33% yield respectively. Due to presence of Aqua-Alcoholic soluble compounds present in the leaves 50% EtOH shows maximum percent yield with compare to other solvent systems.

The structural properties of the prepared nanoparticles were characterized, by change in colour before and after colour change and its morphology and particle size was calculated by Motic-BA 210 microscope

with image analyse rand its optical properties were determined by using UV-Vis double beam spectrophotometer (SYSTRONICS), diluting of the nanoparticles in deionized H_2O until the equipment cell was filled. It was analysed in a range of 210 to 450 nm, in absorbance mode (Grap:2).

The proliferation of the cells or cell viability was assessed by the 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (**MTT**) dye reduction the cytotoxic potential and the anti-proliferative effect of *Annona muricata* crude extract on PBMCs, Breast Tumour Xenograft cells and B16F1 cells was determined by comparing to the survival of cells in the untreated (negative control) cultures, which was normalised to 100 %. The IC50 results for the anti-proliferative effect of the 39 different test samples on three different cell lines was examined in three repetitions each to get best statically significant data were graphically present in (Graphs). The cells were treated with different dilutions of test samples for 48 hrs. DDW extract of 100mg/ml sample was the most potent among others as it exhibited the maximum cytotoxic activity with zero cell viability for all PBMCs, breast cancer cell lines & very few viabilities of 5% seen in B16F1 cells, as compared to standard drugs. EtOH extract shows second most effective role on cell viability with 41%, 32% and 28% survival rate. On the other hand, other test samples and prepared conjugates have shown moderate cell viability with 30% to 70% cell viability. Most of the conjugates like CS-DDW, CS-95% MeOH and AgNPs shows increase cell toxicity rate as compare to alone. Rest of the test groups were the least potent as both showed weak activity in inhibiting the proliferation of PBMCs and cancer cells as they have higher IC50 compared to the other samples.

CONCLUSION & DISCUSSION:

Natural products have been the target for cancer therapy for many years due to the medicinal values contained in them. In this study, the cytotoxicity effect of the aqueous leaf extract of *Annona muricata* Linn samples were evaluated on three different cell lines; PBMCs, Breast Tumour Xenograft cells and B16F1 cells by MTT assay. Consistent with earlier findings (George VC, et al. 2012) each of the soursop crude extract exhibited the anti-cancer activity as they inhibited the proliferation of the breast cancer cell lines. The cell viability percent values are varied among the samples revealing the influence of the secondary metabolites constituents composed in them.

Based on the results obtained from this study, it is imperative to carefully select the soursop samples from its cultivation area, season as it could determine the potency and anticancer activity of certain soursop sample. Extensive anticancer investigations have been conducted on *A. muricata* due to its reported ethnomedicinal uses against tumors and cancer (Adewole and Ojewole, 2009). Numerous lines of evidence suggest that the leaf extract of *A. muricata* repressed tumor growth in vivo in animal models as well as induced apoptosis of various cancer cells in vitro (Liu *et al.,* 2016).

Cytotoxicity studies on the *A. muricata* leaves have been accomplished on numerous cell lines. The *A. muricata* leaf extracts were investigated for cytotoxicity against different cancer cells and its results manifested a remarkable cytotoxic effect (Oviedo et al., 2009). Our study data shows these effects in similar manner, further study is required in its NPs and other metallic conjugates to prove it as an anti-cancer remedy.

The bioactive compounds contributing to the bioactivities have not been properly identified, qualitatively and quantitatively analysed as chemical markers for standardization and quality control purposes as well as the mechanisms of action have not been well determined. Hence, future research on *A. muricata* should focus on extensive phytochemical investigations in isolating and identifying the active metabolites which contribute to the potent anti-inflammatory and anti-cancer activities. Subsequently, the extract and their

active metabolites should also be subjected to more mechanistic studies, in vivo investigations in various animal models including pharmacokinetic and bioavailability studies.

In addition, more toxicity studies must be conducted before submission to clinical trials to define the safest concentration of the *A. muricata* leaf to subjects. The potential of *A. muricata* leaves to be used as an anti-inflammatory and anticancer agent can clearly be enlightened by understanding its mechanisms of action on the human body system.

FUTURE PROSPECTIVE:

Currently, many people are searching and trying alternate treatment methods for cancer, which is mainly attributed to the limitations of the current treatment methods for combating cancer. Treatments using *A. muricata* might offer an alternative choice besides chemotherapy and radiotherapy, especially for terminally ill patients. The benefits of utilising *A. muricata* extracts, as discussed above, suggest that these plant-based NPs are potential treatment options against cancer. However, it is important to acknowledge that factors such as lifestyle and ignorance towards the symptoms of cancer are still major contributors to the increase in the incidence of cancer. A change to a better and healthier lifestyle and a better understanding and recognition of the signs and symptoms of cancer are needed to increase the chances of fighting cancer successfully.

Further improvements to current treatment methods need to continue to allow the development of more efficient and less expensive treatment procedures against cancer. More stepping stones are require to developing new ways to treat illnesses. In addition, research to integrate plant based NPs, such as *A. muricata*, into mainstream patient management procedures is important to decrease the treatment cost further research should focus on enhancing nanoparticle specificity towards tumor sites by utilizing new linkers and functionalizing agents to optimize their effectiveness as therapeutic agents. Therefore, this plant should be explored for its anticancer properties and its increased efficiency in the form of nanoconjugate.

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