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Antioxidant potential of *Annona muricata* (Leaves extract) with metallic conjugates and its cytotoxicity analysis by (*in vitro*) Cytokinesis Block Micronucleus assay

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ABSTRACT: *Annona muricata* is one of the most important traditional medicinal plants which contains numerous chemicals that exhibit various pharmacological properties. Inspired by the bioactivity from different studies of the aqueous and alcoholic extract, we employed it as a plant-based material for the green synthesis of nanoparticles. The NPs was prepared by the addition of isolated *Annona muricata* leaf extract as a reducing agent to metal. The synthesized metal based NPs were characterized using UV–visible spectroscopic analysis and microscopy. The observed colour changes confirmed the successful formation of NPs, Free radicals are inhibited by antioxidant compounds, which can be naturally sourced from soursop (*Annona muricata* L.). Extraction processes affect levels of antioxidant compounds in the extract. Therefore, the present study aimed to evaluate the optimal extraction conditions for soursop leaves using maceration and pressing methods and determine the content of the flavonoid compound rutin in the optimized extract. The analysis was conducted to achieve maximum levels of 2.2-diphenyl 1-picrylhydrazyl (DPPH). This study aimed to evaluate and compare the potential cytotoxic activity of different leaf extracts of *A. muricata* and conjugates using the CBMN assay against the cultured lymphocyte. The cytokinesis-block micronucleus (CBMN) assay is an established method for assessing chromosome damage in human peripheral blood lymphocytes resulting from exposure to genotoxic agents such as ionizing radiation. This study discovered that *A. muricata* crude leaf extracts have the potential to inhibit the cultured lymphocyte cells by inducing cell death. Phytochemicals termed Annonaceae and Acetogenins (AGEs) found in this plant has shown antibacterial, antiviral, and anticancer properties in various *in- vitro* studies done. The objective of this study was to measure cytogenetic DNA damage in-vitro based on MN frequency in peripheral blood these findings contribute to the growing field of eco-friendly nanotechnology and emphasize the significance of plant-mediated approaches in nanomaterial synthesis and biomedical applications.

KEYWORDS: Antioxidant, Metallic NPs, *Annona muricata*, lymphocyte culture, CBMN assay.

INTRODUCTION: Plants are a source of natural ingredients that are widely used as medicines. *Annona muricata Lin*., commonly called soursop, is part of the *Annonaceae* family, which comprises the addition of 130 genera and 2300 species. *A. muricata* L. contains a range of compounds with pharmacological activity. This plant is widely grown in tropical and subtropical areas, such as Southeast Asia, South America, and the rainforests of Africa. The plant produces edible fruit all year around and is widely used as a traditional medicine for skin disease, respiratory disease, fever, bacterial infections, diabetes, hypertension, and cancer (De Souza, E.B.R. *et al.*, 2009). The core active components of *A. muricata* are acetogenin, alkaloids, and flavonoids (Coria-Tellez,A.V. *et al*.,2018). Study of the compounds in *A. muricata* leave extract has seconday metabolites such as flavonoids, terpenoids, saponins, coumarins, lactones, anthraquinones, glycosides, tannins, and phytosterols (Gavamukulya, Y. *et al*., 2014).

Antioxidant: Antioxidants are chemical compounds that can contribute one or more electrons to free Radicals. So, as to neutralize the increase in free radicals, protect cells from the toxic effects Produce and contribute the prevention of diseases (Winarsi H. *et al.*, 2007). The role of antioxidant is very important in neutralizing and destroying free radical which can cause cell damage and also damage inside biomolecules. In small amounts, free radical can be neutralized by the body's enzymatic systems such as the enzyme superoxide, glutathione, catalase, peroxidase, dismutase and glutathione-s-transferase. If amount of free radicals in body is

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excessive, antioxidants from outside the body are needed (exogenic) such as flavonoids, Vitamin A, vitamin C and vitamin E .(Sayuti, K. *et al.*,2015)

Cytotoxicity: Cytotoxicity refers to the capability of a substance or agent to beget damage or death to cells. This can do through colourful mechanisms, including apoptosis (programmed cell death), necrosis, or autophagy (cellular tone- digestion).

Micronucleus Assay: The micronucleus (MN) assay is a extensively used cytogenetic test for detecting chromosomal damage and genotoxicity in cells. Micronuclei are small, membrane- bound structures that form in the cytoplasm of cells when chromosomal fractions or whole chromosomes are not duly incorporated into the son capitals during cell division. The presence of micronuclei in cells is a dependable index of chromosomal damage, which can be caused by exposure to genotoxic agents, similar as chemicals, radiation, or viruses. The most popular interpretation of MN is the cytokinesis- block micronucleus assay (CBMN) (Fenech, M, et al., 1985 & 2000).

Nanoparticles: Nanotechnology evolved as the achievement of wisdom in the 21st century. The conflation, operation, and operation of those accoutrements with a size lower than 100 nm fall under the interdisciplinary marquee of this field. Nanoparticles have significant operations in different sectors similar as the terrain, husbandry, food, biotechnology, biomedical, drugs, etc. like; for treatment of waste water (Zahra Z. *et al*., 2020).

MATERIAL & METHODS:

Sample collection and authentication: The present *in-vitro* study was conducted in the Department of Research & Clinical Genetics, Jawaharlal Nehru Cancer Hospital & Research Centre, Idgah Hills, Bhopal, M.P, India, after obtaining the ethical approval from the Institutional Ethics Committee of JNCH & RC, Bhopal, M.P (**Ref. No. 1130 d/JNCH/RES/20/12/24**). Fresh (disease-free) and fully expanded leaves of *A. muricata L*. were collected from the Madan Mohan Herbal Garden of JNCH & RC, Bhopal (23.2730˚N and 77.3806˚E) in the month of February'2025.The plant was identified and authenticated by potent authority. Experimental research on the plant used for the study complies with relevant institutional, national, and international guidelines and legislation.

Preparation of extracts: Ethanolic & Aqueous extracts of the Soursop leaf were prepared in the Pharmacology lab, Department of Research & Clinical Genetics, JNCH & RC, Bhopal, India. Maceration method is used for extraction as it is a convenient, simple in expensive, and favorable technique, especially in the case of small-scale extraction, such as that at laboratory scale.

Preparation of metal- extract conjugates:

- **Copper conjugation synthesis:** Biosynthesis of Copper Nanoparticles with extracts of *A. muricata*, a modified method adopted from (Oli, H. B. *et al*.,2018).
- **Iron conjugation synthesis:** Biosynthesis of Iron Nanoparticles with extracts of *A. muricata*, a modified method adopted from (Alam *et al*.,2019)

***In-vitro* Antioxidant Activity:** Free Radical Scavenging Activity by DPPH Method: (Mensor *et al.*,2001) and (Manigauha *et al*.,2009).

CBMN assay: Micronucleus modified method adapted from (Fenech, M, et al., 1985 & 2000).

Microscopic observation: Motic- BA210 with Image analyser (40X for observation and 100X for Photography).

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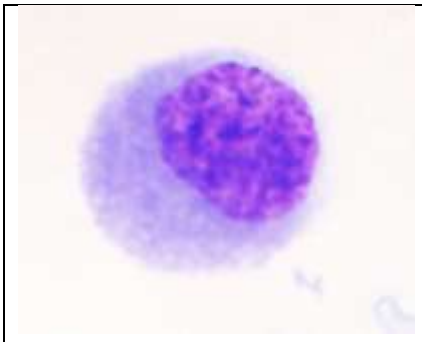
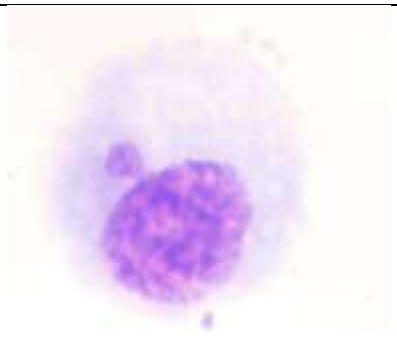
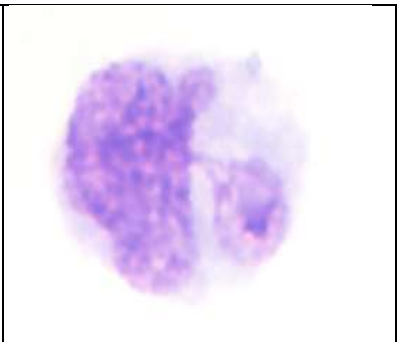
OBSERVATIONS:



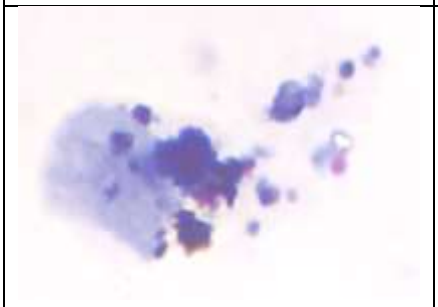
Normal Mononucleated Cell



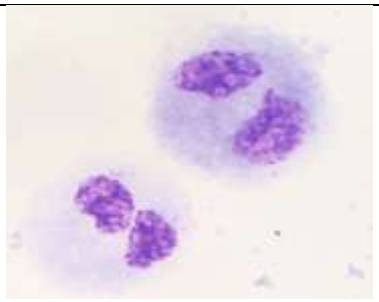
Mononucleated Micronucleus

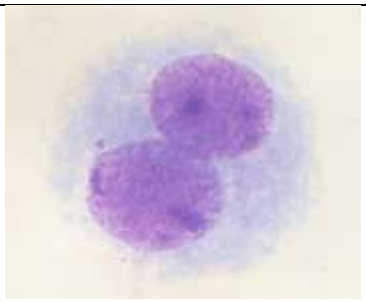


Nucleoplasmic bridge

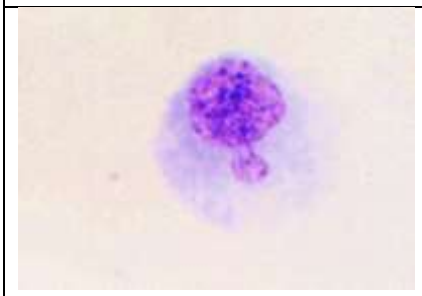


Nacrotic Cell

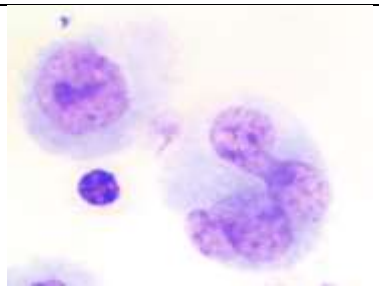




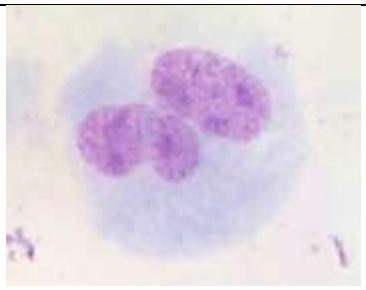
Normal Binucleated Cells



Mononucleated Bud

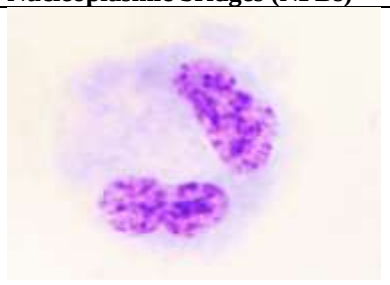


Nucleoplasmic bridges (NPBs)

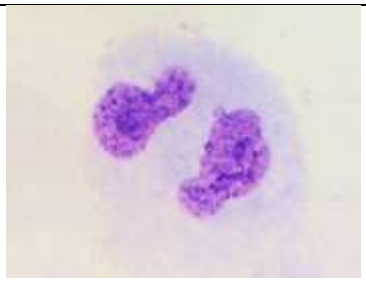


Tri-nucleated Cell

Normal Mononucleated Cells



Qudra-Nucleated Cell



Binucleated Bud

Photograph: 01
Showing different cellular alterations after (*in vitro*) CBMN assay of treated blood samples. Giemsa stain images taken by Motic- BA210, 100X Microscope.

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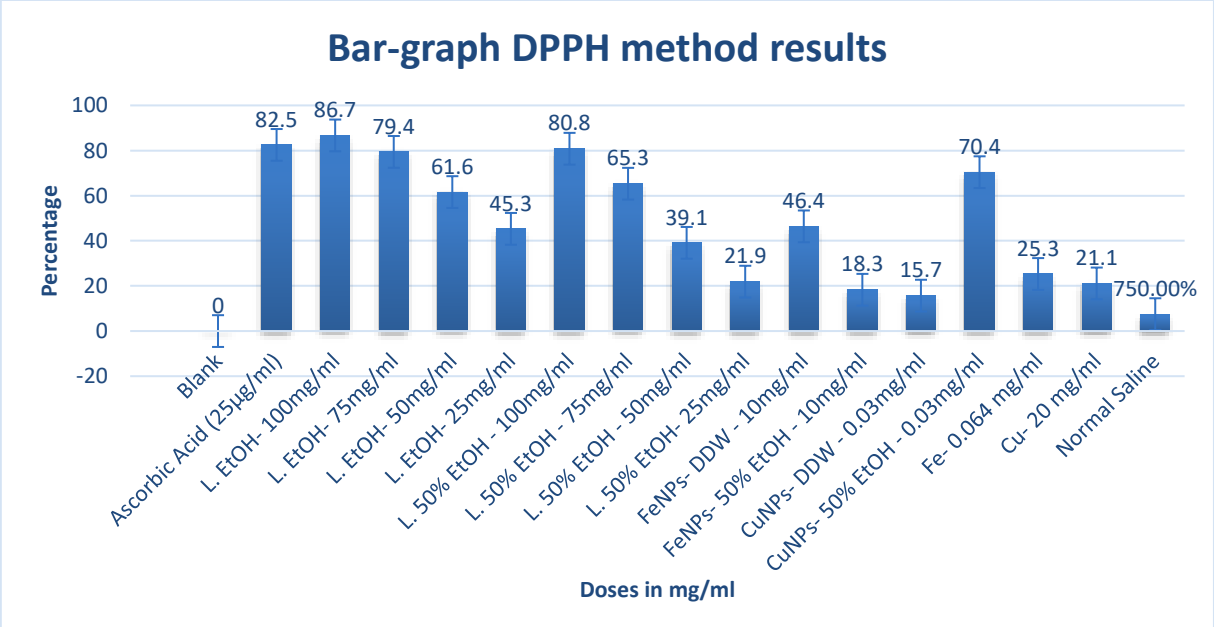
Table: 01 Showing Percent inhibition (Antioxidant activity) by DPPH method

S.NO	GROUP	Mean ± SE Absorbance (517 nm)	Antioxidant %
1	Blank	0.355 ±0.0347	0
2	Ascorbic Acid (0.04mg/ml)	0.062 ±0.0255	82.5
3	L. EtOH- 100mg/ml	0.047 ±0.0246	86.7
4	L. EtOH- 75mg/ml	0.073 ±0.0384	79.4
5	L. EtOH- 50mg/ml	0.136 ±0.112	61.6
6	L. EtOH- 25mg/ml	0.194 ±0.0664	45.3
7	L. 50% EtOH - 100mg/ml	0.642 ±0.108	80.8
8	L. 50% EtOH - 75mg/ml	0.587 ±0.00195	65.3
9	L. 50% EtOH - 50mg/ml	0.216 ±0.0371	39.1
10	L. 50% EtOH- 25mg/ml	0.433 ±0.0536	21.9
11	FeNPs- DDW - 10mg/ml	0.520 ±0.00161	46.4
12	FeNPs- 50% EtOH - 10mg/ml	0.420 ±0.00195	18.3
13	CuNPs- DDW - 0.03mg/ml	0.411 ±0.00237	15.7
14	CuNPs- 50% EtOH - 0.03mg/ml	0.105 ±0.0327	70.4
15	Fe-	0.265 ±0.107	25.3
16	Cu-	0.280 ±0.0915	21.1
17	Normal Saline	0.306 ±0.022	7.55

P Value= 0.035, Significant level (<0.05)

Note: L.EtOH: Leaf Ethanolic extract, **L. 50% EtOH**: Leaf extract 50% Ethanol, **FeNPs**: Iron based conjugates, **DDW**: Double distilled water, **CuNPs**: Copper based conjugates, **Fe**: Iron oxide, **Cu**: Copper sulphate.

Graph:01 Showing Percent (Antioxidant activity) by **DPPH** method.



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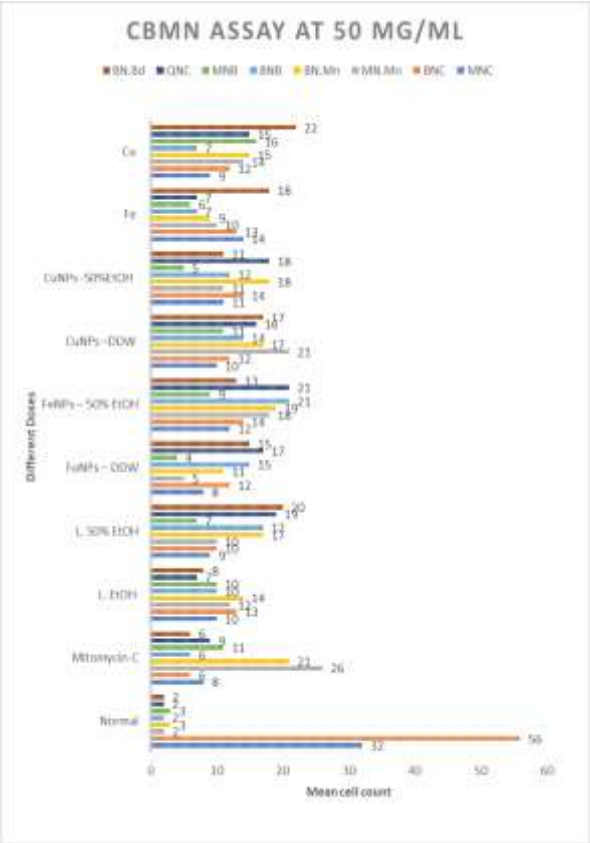

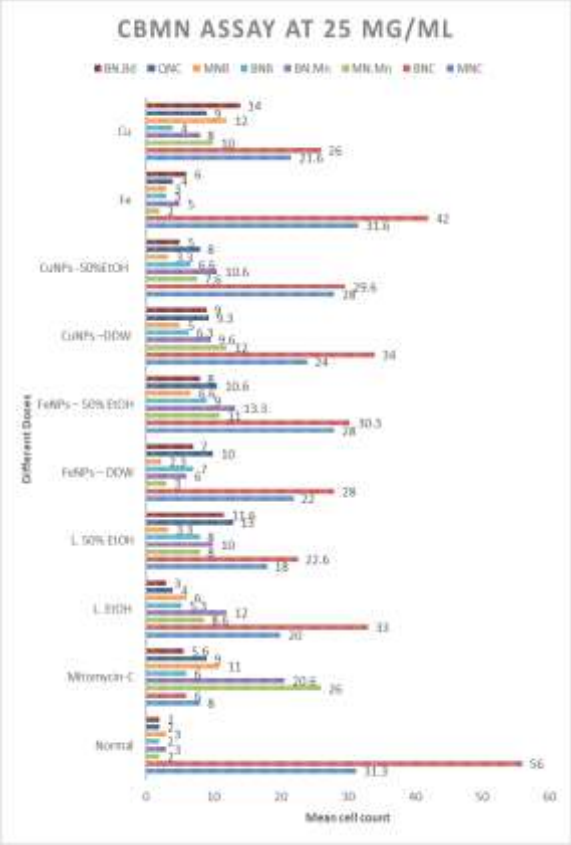

Graph: 02 Showing Test result of CBMN assay against cultured PBMC at Higher Concentration of *in vitro* doses.	**Graph: 03** Showing Test result of CBMN assay against cultured PBMC at Low Concentration of *in vitro* doses.

Table: 02 Showing difference in Micronucleus frequency (MF) among selected test groups results by CBMN assay.

Groups	Low Concentration	Higher Concentration
Normal (Untreated)	1.588	1.621
Mitomycin-C	1.354	1.841
L. EtOH	1.404	1.688
L. 50% EtOH	1.614	1.705
FeNPs – DDW	1.664	1.781
FeNPs – 50% EtOH	1.651	1.755
CuNPs –DDW	1.542	1.666
CuNPs -50%EtOH	1.69	1.683
Fe	1.297	1.559
Cu	1.29	1.468

P value: **0.0017**, significant level (<0.05)

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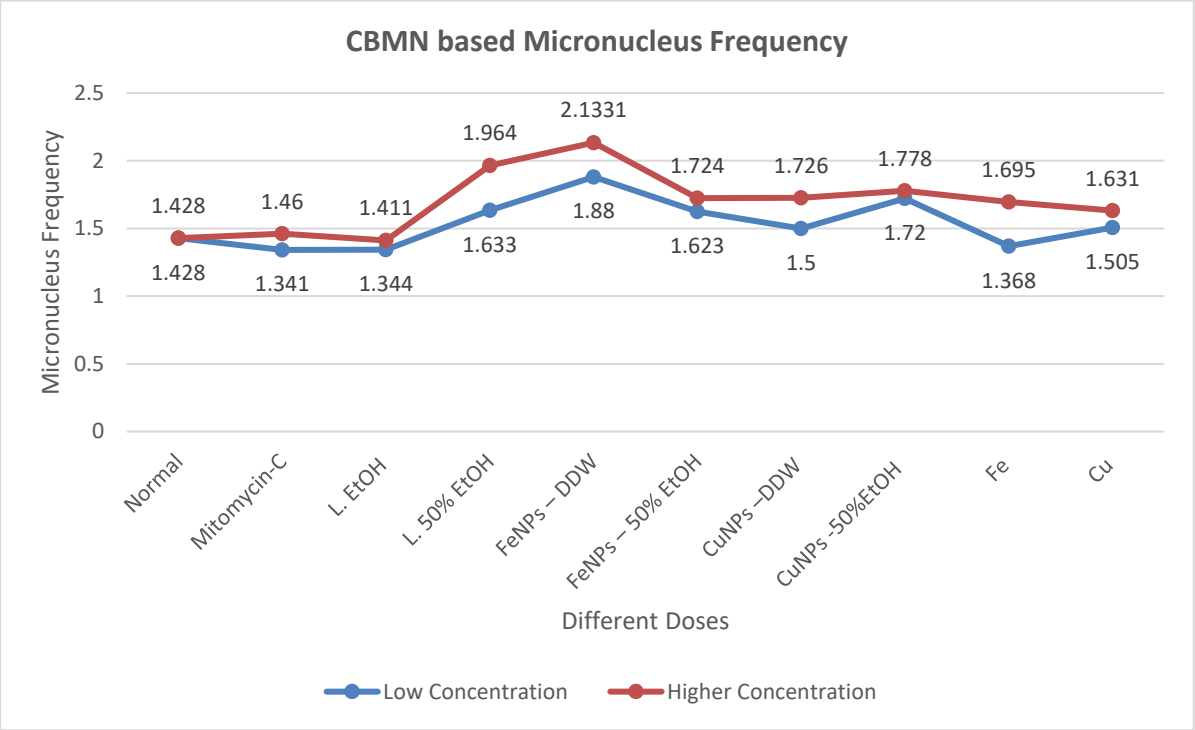
Graph: 04 Showing liner presentation of'Micronucleus Frequency' (**MF**) pattern after *in vitro* treatment with high & low concentration of test samples against cultured lymphocytes.



Note: L.EtOH: Leaf Ethanolic extract, **L. 50% EtOH**: Leaf extract 50% Ethanol, **FeNPs**: Iron based conjugates, **DDW**: Double distilled water, **CuNPs**: Copper based conjugates, **Fe**: Iron oxide, **Cu**: Copper sulphate.

Table: 03 Showing difference in Nuclear Division Index (NDI) among different test groups *in vitro* treatment with Low & Higher concentration of test groups results by CBMN assay.

Groups	Low Concentration	Higher Concentration
Normal (Untreated)	1.588	1.621
Mitomycin-C	1.354	1.841
L. EtOH	1.404	1.688
L. 50% EtOH	1.614	1.705
FeNPs – DDW	1.664	1.781
FeNPs – 50% EtOH	1.651	1.755
CuNPs –DDW	1.542	1.666
CuNPs -50%EtOH	1.69	1.683
Fe	1.297	1.559
Cu	1.29	1.468

P value: **0.012** significant level (<0.05)

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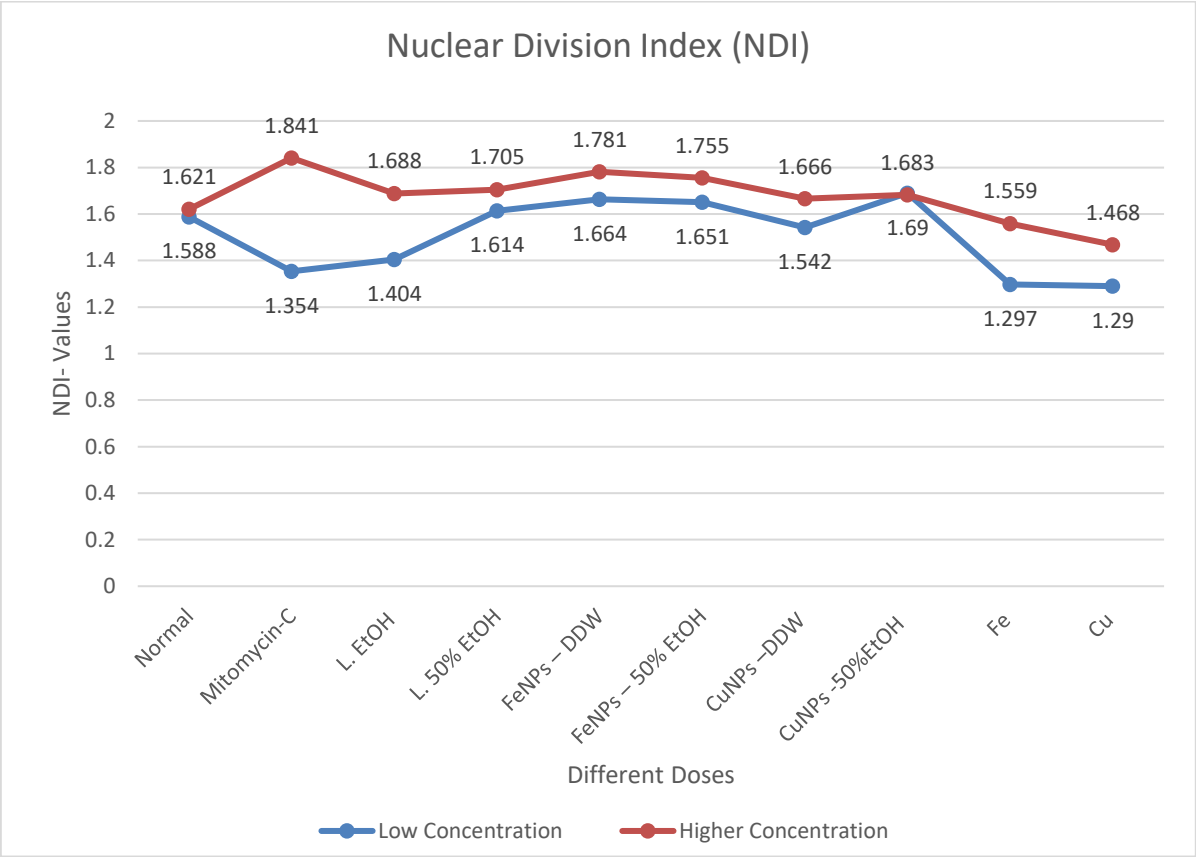
Graph: 05 Showing liner presentation of 'Nuclear Division Index' (**NDI**) pattern after *in vitro* treatment with high & low concentration of test samples against cultured lymphocytes.



Note: L.EtOH: Leaf Ethanolic extract, **L. 50% EtOH**: Leaf extract 50% Ethanol, **FeNPs**: Iron based conjugates, **DDW**: Double distilled water, **CuNPs**: Copper based conjugates, **Fe**: Iron oxide, **Cu**: Copper sulphate.

RESULT

DPPH method was performed as outlined by (Celep *et al*., 2015), with minor adjustments. Ascorbic Acid (0.04mg/ml) was used as a standard solution. Absorbance of 50 µg/ml DPPH solution was determined using a UV-vis spectrophotometer at a wavelength of 517 nm to obtain the absorption value of all test solutions. The stock solution of ascorbic acid standard was prepared by dissolving it in ethanol analytical grade. The DPPH radical scavenging activity was recorded in terms of % Inhibition as shown in (Table: 01) with P value: 0.035. As per our results it was observed that 'L. EtOH- 100mg/ml' concentration shows (Mean ± SD; 0.047 ±0.0246) with higher **86.7%** antioxidant activity, another test group 'L. 50% EtOH - 100mg/ml' (Mean ± SD; 0.642 ±0.108) with good **80.8%** antioxidant activity. Alone metals have minimum DPPH scavenging activity (lesser than 26%) and its conjugate with extract 'CuNPs- 50% EtOH - 0.03mg/ml' (0.105 ±0.0327) shows **70.4%** activity all the results obtained in triplicate sand statistically significant with $p<0.05$ shown in (Graph: 01) Extraction solvent significantly affected antioxidant activity, assessed by DPPH.

Data were expressed as Mean ± SD of triplicate results. Cytotoxicity was monitored by determining proportion of binucleated cells with cellular abnormalities in the (PBMC) cultured cells with lower & higher doses shown in (Table: 03) with P value of 0.040 & 0.0135 respectively with significant level (<0.05). In this experiment negative control was used as untreated normal blood and Mitomycin-C 50µl of (0.04µg/ml) was used as positive control. Different combinations (higher doses/ Lower doses) of test groups of ethanolic & aqueous leaf extracts with metal conjugates are used to analyze the cytotoxic effect. Metals alone did not show any significant changes in the proportion of PBMC, concerning their baseline values compared with untreated negative control (Normal). But in combination with extracts like 'FeNPs, 50% EtOH' shows increase level of micronucleus both in higher and lower dose results. Equally CuNPs-DDW, CuNPs-50% EtOH and FeNPs-DDW shows micronucleus formation in triplicate experiments and

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there (percent cell counts) are graphically represented in (Graph: 2& 3). In contrast, the ethanolic extract group showed significant increase in micronucleus formation. Our results regarding '**Micronucleus Frequency'** & '**Nuclear Division Index**' shows the cytotoxic effect of *Annona muricata L* ethanolic & aqueous leaf extract have good anticancer property.
Likewise, the higher doses of ethanolic leaf extract and the DDW conjugate FeNPs-DDW shows **1.68** and **1.781** NDI values close to positive control with **1.841** respectively increases the micronucleus frequency (P value: 0.0017) significantly after 72hrs of incubation with higher dose NDI with (P value: 0.012) significant level (<0.05) shown in (Table: 02 & 03) and the graphical representation of data in (Graph: 4 & 5).

CONCLUSION & DISCUSSION

The present study demonstrated cytotoxicity and antioxidant activities of *Annona muricata* extracts. The current findings thus reinforce the scientific evidence of the Cell toxicity and induce DNA damage potential of this plant used in traditional medicine. Our research demonstrated that *A. muricata* aqueous and ethanolic leaf extracts shows cytotoxic effect in higher dose with good antioxidant activity by DPPH method. Several antioxidant screenings have been conducted on *A. muricata*. Natural antioxidants from plant species have gained interest due to their protective effect against oxygen-derived from free radicals involved in the development of many diseases such as cancer, cardiovascular affections, arthritis, as well as degenerative illness such as Parkinson and Alzheimer (M. Almeida, *et al*., 2011). A compilation of studies on the antioxidant activity of *A. muricata* considering different assays, the different plant parts, and the different solvents used has been done (J. Correa-Gordillo, *et al*., 2012). Some of the methods used for determining the total antioxidant capacity included the free radical scavenging capacities using DPPH.

The cytotoxic activity of this plant occurs due to the presence of acetogenin, which is the most-abundant chemical family in various parts of *A. muricata*. In the present study, the DNA damage was measured with the micronucleus assay. Cytokinesis block used in the assay enables accurate evaluation of MN generationafter48hrs of treatment, since cells undergoing mitosis at the time of incubation in presence of cytochalasin-B the cytokinesis blocked in dividing cell and most of the cells shows binucleated appearance(i.e., cells potentially generating micronuclei) are easily identified as binucleated cells. The main advantages of CBMN assay are relative rapidity and simplicity with low cost. Simultaneous use of phase contrast microscopy enables more accurate definition of cell borders, reducing the risk of MN misinterpretation (Photograph: 01). Anti-tumoral activity has been reported for extracts and some isolated acetogenins of *A. muricata*. It was reported by reported (Hamizah *et al*. 2012) that the ethanolic extract of *A. muricata* leaves showed greater anti-tumor activity in murine models than *curcumin*, a known natural chemo preventive.

FUTURE PROSPECTIVE

A. muricata is widely used in traditional medicine to treat a myriad of conditions including hypertension, diabetes and cancer. Decoctions of all parts are widely used in preparations. *In vitro* and *in vivo* studies support the majority of the traditional uses but lack clinical validation, as most of the trials have not been clinically validated. More than 200 phytochemicals have been identified in this plant, mainly acetogenins, alkaloids and phenols. These phytochemicals have shown pharmacological activities such as antimicrobial, antioxidant, insecticide, larvicidal, selective cytotoxicity to tumor cells, anxiolytic, anti-stress, anti ulceric, wound healing, anti-jaundice, hepato protective, hypoglycemic, immune modulatory, and antimalarial among others. Many new phytochemicals are also yet to be identified in *A. muricata*.
Finally, to answer the original question paused in the title, after reviewing all these studies, we can with much certainty affirm that it is true, the natural therapy to most disease conditions including cancer is growing in our back yard, especially in the Tropics. It only requires big steps to be taken for more detailed studies and advocating for pharmaceutical development or local formulations that may not necessarily be a replacement of the current treatment regimens, but where the extracts of *A. muricata* have shown better and promising activity like in cancer and other non-communicable diseases, be used to help save the human race. Using the natural compounds in their proved form, rather than taking years trying to develop a patentable synthetic analog as the human race suffers, may be the best service we might ever give to this world.

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COMPETING INTERESTS

"The authors have no relevant financial or non-financial interests to disclose."

AUTHOR CONTRIBUTIONS

"First authors [Akanksha Patel] contributed to the study experimental part. Material preparation, data collection and analysis were performed by [Sarfaraz Hanfi & Sameena Akhter]. The first draft of the manuscript was written by [Sarfaraz Hanfi & Akanksha Patel] checked by [Ankur Chhari, Sameena Akhter]. [Shazia khan (HOD), & Divya Patel] is dissertation mentor form SHGC, all authors commented on previous versions of the manuscript all authors read and approved the final manuscript."

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