Genotoxicity estimation by calculating Mitotic index with Chromosomal aberrations induced by aqua-alcoholic extract of *Annona muricata* leave

Jigyasa Chandravanshi[1], Ankur Chhari[2], Sameena Akhter[3], Shazia Khan[4], Divya Patel[5], Sarfaraz Hanfi[6]
[1,4,5]*Department of Biotechnology, Sant Hirdaram Girl's College, Bhopal, M.P, India*
[2,3,6]*Department of Research & Clinical Genetics, Jawaharlal Nehru Cancer Hospital & Research Centre, Idgah, Hills, Bhopal, 462001, Madhya Pradesh India.*

Abstract- **Genotoxins are chemicals that causes DNA damage, potentially leading to heritable mutations in germ cells or cancer in somatic cells, with test available to assess their effects and safety, which is basically termed as genotoxicity, which can lead to chromosomal disorders which results in changes in chromosomes number or structure leading to growth and development issues. Nowadays, medicinal plants are preferred for the solution of such kind of diseases, many scientists and researchers around the world are working for appropriate solution. *Annona muricata L.*, known as Soursop, is a tropical plant used in traditional medicine for its anti-inflammatory, anti-diabetic, anti-cancer properties and other activities. It's main components are Annonaceous, Acetogenins and other chemical compounds. Microscopic observation of different types of chromosomal aberrations induced by aqua-alcoholic extraction of *Annona muricata* on cultured lymphocytes and the Silver and Chitosan based nanoparticles of its leaves extract was used in this study for the calculation of mitotic index, performed Chromosomal aberration assay, Trypsin binding or G-binding of chromosomes for their clear identification and Trypan Blue Exclusion assay for determining cell viability by using healthy lymphocytes exhibits good results with satisfactory antioxidant activity by using DPPH method.**

***Index Terms- Annona muricata L.*, Genotoxicity, Trypan Blue Exclusion assay, anti-cancer.**

I. INTRODUCTION

Cytogenetic analysis of chromosomes in blood lymphocytes can be used to reveal biomarkers of tumor risk. The frequency of chromosomal aberrations (CAs) appears to correlate with the later incidence of cancer. A chromosomal disorder occurs when there is a change in the number or structure of the chromosomes. The mitotic indicator is a measure of cellular proliferation. It's defined as the chance of cells witnessing mitosis in a given population of cells. This change in the amount or arrangement of the genetic information in the cells may result in problems in growth, development and/or functioning of the body systems. The potential exposure to carcinogens did not modify the effect of CAs on cancer risk but tobacco smoking did increase risk. (**Gyöngyi Farkas, et al., 2022**). Nonspecific structural chromosomal aberrations (CAs) are found in around 1% of circulating lymphocytes from healthy individuals but the frequency may be higher after exposure to carcinogenic chemicals or radiation. CAs have been used in the monitoring of persons exposed to genotoxic agents and radiation. (**Kari. H, et al., 2025**). Dye exclusion is a simple and rapid technique measuring cell viability but it is subject to the problem that viability is being determined indirectly from cell membrane integrity. Many investigations have been carried out to study the anti-cancer properties *of A. muricata*. 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.* Based on a review on the pharmacological activities of *A. muricata*, it has long been used to treat a variety of conditions, including cancer, diabetes, hypertension, respiratory illnesses, fever, and bacterial infections (**Kasole R. et al, 2019**) (**Mutakin M. et al, 2022**). Moreover, *A. muricata* leaves are now utilized to control and treat diabetes, cancer, and hypertension (**Coria-Téllez A.V.et al, 2018**). The flavonoids, such as gallocatechin, kaempferol, quercetin, rutin, and argentinine, that are abundant in the leaf part may contribute to its potent antioxidant effect and improve

other conditions caused by high ROS levels by donating hydrogen (**Mutakin M et al, 2022**).

II. MATERIAL & METHODS

Approvals: Study started with selected individuals belongs to normal healthy individuals. A mixed group of male & female with age group of 18 to 50 years belongs to Bhopal and its peripheral regions, (M.P) India, after final approval received by 'Institution's Ethical Committees' (Approval. No: 1130 d/JNCH/RES/20/12/2024).

Sample Collection: About (2ml) peripheral blood of healthy individuals was taken, an inform consent was taken from all subjects before participation in the study.

Collection of Plant sample:

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. After collection and washing of the leaf, they were separated from the twigs and dried in shade for further preparation.

Preparation of extracts:

Aqueous & Methanolic extracts of the *A. muricata* (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 inexpensive, and favourable technique, especially in the case of small-scale extraction, such as that at laboratory scale.

Silver Conjugation Synthesis: Biosynthesis of Silver Nanoparticles with extracts of *A. muricata,* a modified method adopted from (**Asif M. et al., 2022**) was followed.

Chitosan based NPs preparation: A modified method adopted from (**B. Ozdamar. et al., 2023**).

Lymphocyte separation Method: Density gradient medium for the isolation of mononuclear cells, Modified method adopted from (**Riedhammer, C., et al., 2014**).

Chromosomal Aberrations steps:

2ml peripheral blood was drawn in heparinized blood collection tubes with the help of trained professionals (phlebotomist) after ICF signing, RPMI 1640 media was used in falcon centrifuge tubes, distribute the 1 ml of blood sample in each tubes with test groups were kept in the centrifuge tubes for 72 hours at 37°C in 5% CO_2 incubator, shake the tubes every 24 hours (2 to 3 time). After 70 hours of incubation 50µl Colchicine was added to the sample and again incubate 35 minutes. Further harvesting of cells, washing of Cells, preparation of slide and Giemsa staining was performed

Microscopic analysis: Motic BA-210 microscope with imagining system with 40 to 100 X of magnification was used.

Free Radical Scavenging Activity by DPPH Method: DPPH- 0.3 mM (5.95mg/50 ml in ethanol) ,Hi-media Laboratories Ltd., Mumbai, Ascorbic acid, Merck India Ltd, Mumbai, India' Uv-Vis double beam spectrophotometer, test tubes, micropipette, tips. Potential of *Annona muricata L.* leaves extracts to scavenge DPPH radical was determined as per (**Mensor *et al.*, 2001)** and (**Manigauha *et al.*, 2009)**. Stock solution of all the extracts (1.0 mg/ml) was diluted to 25, 50, 75, and 100 mg/ml. Two ml samples solution of different concentrations was added to 1.0 ml of 0.3 mM DPPH in ethanol and 2.0 ml of Phosphate buffer (0.2 M, pH 7.4). The reaction mixture was incubated for 30 min in dark at room temperature. After 30 minutes the absorbance was measured by UV-Vis double beam spectrophotometer (Systronics) at **517 nm.**

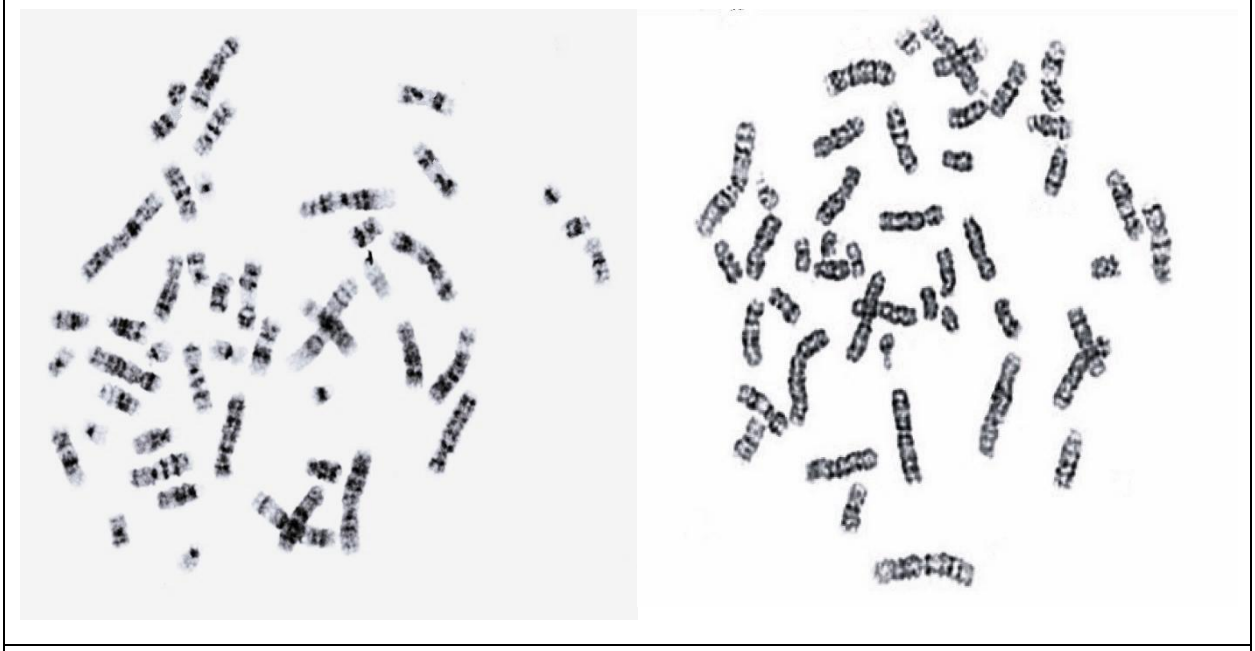
III. OBSERVATION

Table 1: Showing Chromosomal aberrations after (***in vitro***) treatment with different test groups.

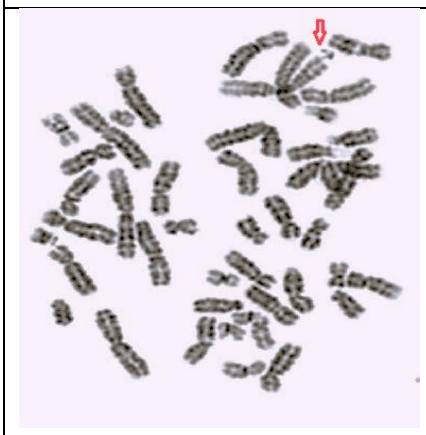
S.NO	GROUP	Metaphase Observed	Mean ± SE Value (1000 cells)			
			Chromosomal aberrations			
			CF	SM	DM	CB
1	Blank (without treatment)	**80**	0	0	0	0
2	L. DDW- 100mg/ml	5	1	2	0	1
3	L. DDW- 50mg/ml	15	2	3	3	5

4	L. DDW-25mg/ml	20	3	5	4	6
5	L. 95% MeOH- 100mg/ml	5	1	3	4	2
6	L. 95% MeOH- 50mg/ml	18	3	2	6	5
7	L. 95% MeOH- 25mg/ml	35	5	4	6	3
8	AgNps-DDW 100mg/ml	25	4	3	2	4
9	AgNps-DDW 50mg/ml	27	2	1	1	2
10	AgNps-DDW-25mg/ml	31	1	1	2	1
11	AgNps-50%-EtOH-100mg/ml	15	5	6	4	5
12	AgNps-50%-EtOH-50mg/ml	17	0	2	5	2
13	AgNps-50%-EtOH-25mg/ml	18	2	3	1	4
14	CSNPs-DDW-100mg/ml	12	3	4	2	3
15	CSNPs-DDW-50mg/ml	13	1	1	0	1
16	CSNPs-DDW-25mg/ml	16	1	2	4	5
17	CSNPs-50% EtOH-100mg/ml	10	4	1	2	3
18	CSNPs-50%EtOH-50mg/ml	11	2	1	1	2
19	CSNPs-50%Etoh-25mg/ml	13	2	1	2	1
20	CSNPs-EtOH- 100mg/ml	15	6	8	7	5
21	CSNPs -EtOH-50mg/ml	15	1	2	3	2
22	CSNPs-EtOH-25mg/ml	17	1	5	2	3
23	CSNPs-95%MeOH-100mg/ml	15	2	1	0	2
24	CSNPs-95%MeOH-50mg/ml	16	3	1	1	1
25	CSNPs-95%MeOH-25mg/ml	17	3	4	3	4
26	Ag 0.17 100mg/ml	10	2	3	1	4
27	Ag 0.17 50mg/ml	11	1	3	2	2
28	Ag 0.17 25mg/ml	13	3	5	2	1
29	CS (20 mg) 100mg/ml	12	0	0	0	0
30	CS (20 mg) 50mg/ml	12	0	1	5	3
31	CS (20 mg) 25mg/ml	15	1	0	3	0

P Value= **0.034**, Significant level (< 0.05)

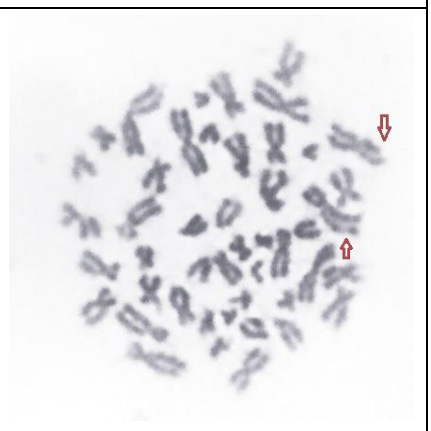


(Normal) Metaphase Chromosomes



Double Minutes

Chromosomal Fragments



Chromatid Breaks

Photograph:1

Showing Normal metaphase chromosome, Chromatid break, Chromosome fragment, Double minutes, Giemsa stain images taken by Motic- BA210 Microscope, 100X magnification.

Table 2: Showing Mitotic Index percentage after (***in vitro***) treatment with different test groups.

S.NO	GROUP	Mitotic Index (MI)
1	Blank (without treatment)	**0.08**
2	L. DDW- 100mg/ml	0.005
3	L. DDW- 50mg/ml	0.05
4	L. DDW- 25mg/ml	0.02
5	L. 95% MeOH- 100mg/ml	0.005

6	L. 95% MeOH- 50mg/ml	0.018
7	L. 95% MeOH- 25mg/ml	0.035
8	AgNps-DDW-100mg/ml	0.025
9	AgNps-DDW-50mg/ml	0.027
10	AgNps-DDW-25mg/ml	0.031
11	AgNps-50%EtOH-100mg/ml	0.015
12	AgNps-50%EtOH-50mg/ml	0.017
13	AgNps-50%EtOH-25mg/ml	0.018
14	CSNPs-DDW-100mg/ml	0.012
15	CSNPs-DDW-50mg/ml	0.013
16	CSNPs-DDW-25mg/ml	0.016
17	CSNPs-50%EtOH-100mg/ml	0.01
18	CSNPs-50%EtOH-50mg/ml	0.011
19	CSNPs-50%EtOH-25mg/ml	0.013
20	CSNPs-EtOH-100mg/ml	0.015
21	CSNPs-EtOH-50mg/ml	0.015
22	CSNPs-EtOH-25mg/ml	0.017
23	CSNPs-95%MeOH-100mg/ml	0.015
24	CSNPs-95%MeOH-50mg/ml	0.016
25	CSNPs-95%MeOH-25mg/ml	0.017
26	Ag (0.17) 100mg/ml	0.01
27	Ag (0.17) 50mg/ml	0.011
28	Ag (0.17) 25mg/ml	0.013
29	CS (20 mg) 100mg/ml	0.012
30	CS (20mg) 50mg/ml	0.012
31	CS (20MG) 25mg/ml	0.015

Formula:

$$\text{Mitotic Index (MI)} = \frac{\text{(Number of cells in mitosis)}}{\text{(Total number of cells)}} \times 100$$

Note: Mitomycin- MTC, DDW -Double distil water, MeOH -Methanol, EtOH -Ethanol, AgNP- DDW -Silver nano particle double distil water, AgNP-50% EtOH -Silver 50% Ethanol, CS-DDW -Chitosan double distil water nano particle, CS- 50% EtOH -Chitosan 50% ethanol, CS-EtOH -Chitosan Ethanol, CS-95% MeOH -Chitosan Methanol, Ag- silver ,CS- Chitosan.

Table 3: Showing results of '**Trypan Blue Exclusion assay'** on different concentration of test groups against PBMC (20000 cells/well).

S.No.	GROUPS	(Mean) % Cell Viability	
		Higher Doses	**Lower Doses**
1	Normal	98.2	98.2
2	Mitomycin (MTC)	3.19	12.9
3	100% DDW	34.14	34.14
4	95% MeOH ext.	38.74	52.12
5	AgNp-DDW ext.	37.58	60.14
6	AgNp-50%EtOh ext.	40.91	36.2
7	CSNP-DDW ext.	7.1	22.31
8	CSNP-50% EtOH ext.	31.58	48.5
9	CSNP-EtOH ext.	31.49	52.8

10	CSNP-95%MeOh ext.	11.6	18.6
11	Chitosan	18.13	30.5
12	Ag Metal	58.66	84.7

P-Value= **0.019** Significant level (<0.05)

Graph 1: Showing results of **Trypan Blue Exclusion assay** on different concentrations of test groups against PBMC.

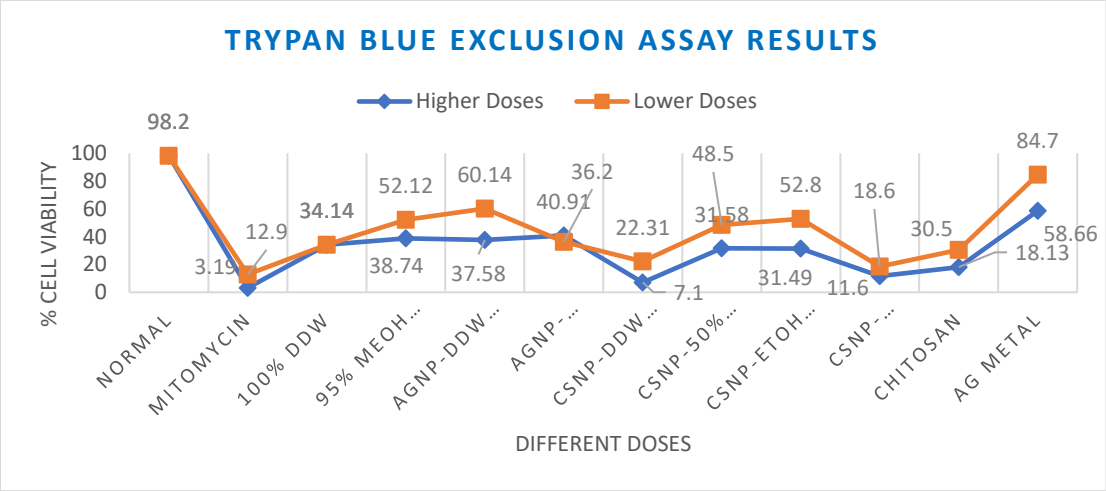


Table: 4 Showing Percent inhibition (Antioxidant activity) by **DPPH** method.

GROUP	MEAN±SE Absorbance (517nm)	Antioxidant %
BLANK	0.331	0.00%
STANDARD	0.189±0.0078	56.70%
100%-DDW (100%)	0.073±0.00711	80.20%
100%-DDW(75%)	0.0766±0.0019	79.29%
100%-DDW(50%)	0.181±0.0039	51.80%
100%-DDW(25%)	0.182±0.00313	50.80%
95%MeOH(100%)	0.0566±0.0035	84.70%
95%MeOH(75%)	0.0603±0.00467	83.70%
95%MeOH(50%)	0.0823±0.00237	77.70%
95%MeOH(25%)	0.186±0.00465	49.70%
AgNps-DDW	0.094±0.00545	74.50%
AgNps-50% EtOH	0.096±0.00592	74.05%
CS-DDW	0.008±0.00237	97.83%
CS-50% EtOH	0.17±0.031	54.05%
CS-EtOH	0.254±0.0345	31.35%
CS-95% MeOH	0.0336±0.00516	90.91%
Ag Metal	0.0033±0.00459	91.08%
CS	0.0096±0.00237	97.40%

Normal Saline	0.306 ±0.022	7.55%
2% DMSO	0.7767±0.0161	2.33%

P-Value= **0.02309** Significant level (<0.05)

Note:

Mitomycin- MTC, DDW -Double distil water, MeOH -Methanol, EtOH -Ethanol, AgNP- DDW -Silver nano particle double distil water, AgNP-50% EtOH -Silver 50% Ethanol, CS-DDW -Chitosan double distil water nano particle, CS- 50% EtOH -Chitosan 50% ethanol, CS-EtOH -Chitosan Ethanol, CS-95% MeOH -Chitosan Methanol, Ag- silver ,CS- Chitosan.

IV. RESULTS

Chromosomes was visualized under microscope Motic; BA-210 at 100X magnification well blocked in cultured lymphocytes by (Colchicine) at metaphase stage of cell division and metaphase preparations in larger numbers were observed (Photograph:1) at lower doses of different test group, though were available, at higher concentrations of test group it shows very low cell division and challenging to obtain good metaphase plates. The Giemsa staining techniques was used to best allow the classification of human metaphase chromosomes and after 'Trypsin banding' it looks well distributed.

The results on the frequency of aberrant cells in this *in vitro* study, the (Negative) control group and (Positive) control (Mitomycin-C) group are summarized with all tested group with different concentration with P= 0.034 at significant level (< 0.05) (table: 1). The percent metaphase observed rate with no chromosomal aberrations in the negative control group was 80% and 5% metaphase seen in positive control group. The test group showed a statistically significant enhancement of the frequency of aberrant cells with increase concentration the metaphase count was decreased in all tested groups mainly in 95% MeOH 100mg/ml, CSNPs 50% EtOH (100mg and 50mg) and Ag metals group. The observed chromosomal aberrations were mainly; Chromosomal Fragment, Chromatid Break, Single & Double minutes with different concentration.

As per Mitotic index calculation given in (Table:2) our result shows 0.08 MI the highest index (Negative control) compared with 0.005 MI (Positive control) and L.95% MeOH 100mg was observed as the lowest index value followed by 0.015, 0.012 MI of AgNP-DDW 100mg, CSNP-DDW 100mg respectively and maximum test groups share the same index value around 0.01, lowest metaphase index is directly proportional to increased damage it was well observed in all higher doses.

Our results about DPPH based antioxidant test after three repetition and comparison with Standard control (Ascorbic Acid 0.025mg/ml) and different solvents used in the sample preparation with calculated (Mean ± SD) values of inhibition and percentage antioxidant with P-value: 0.023 at significant level (<0.05) with absorbance of 0.189 ±0.0078 (Mean ± SD) at 517nm of Standard control (Ascorbic Acid 0.025mg/ml) shows 56.70% antioxidant activity near to IC_{50} value and the test groups like DDW extract (100mg/ml), 95% MeOH (100mg/ml) gives above 80% antioxidant activity alone Ag very minutely neutralize free radicals but with conjugates it shows increase activity of more than 70%. Chitosan polymer shows good antioxidant activity alone 0.0136 ±0.00237 (73.4%) the Chitosan based nanoparticles with DDW extract shows excellent antioxidant activity 97.83% on 100mg/ml concentration.

Cytotoxicity assay by Trypan Blue Exclusion method shows good result after 24hrs of incubation with 20000 cells/well seeded in 12 well ELISA plates (Himedia Ltd.). As compare to positive control (Mitomycin-C), DDW extract, CSNPs DDW and CSNPs 95% MeOH shows lowest cell viability of 8.65%, 7.1% and 11.6% respectively (Table: 3) and Ag alone and with conjugates shows mild to moderate effect on cell growth graphically showing in (Graph:1).

V. CONCLUSION & DISCUSSION

Crude plant extract may include hundreds of different biologically active phytochemicals in a variety of abundances, making it difficult to identify the bioactive phytochemical responsible for a specific biological action (**Enke and Nagels, 2011**). Indeed,

the combined action of various phytochemicals with synergistic, additive, or antagonistic activity results in the overall activity of medicinal plant extracts (**Junio et al., 2011**). Numerous extracts from plants and animals are essential for treating a variety of ailments (**Keesing and Ostfeld, 2021**). Many ancient and modern societies have relied heavily on medicinal plants for their medical needs. Extensive anticancer investigations have been conducted on *A. muricata* due to its reported ethnomedicinal uses against tumors and cancer (**Adewole and Ojewole, 2009**). In addition, these plants also induce chromatid or chromosome breaks, with double and single minute appearance in metaphase plates. Trypan Blue Exclusion assay results shows the treatment tends to possess the highest anti-cancer activity due to *Annona muricata* extract loaded in the nanoparticles.

Since few decade it has been shown several times by tons of researcher that chromosomal aberrations in human peripheral blood lymphocytes are a sensitive biomarker of genotoxic damage and related studies. A large number of human bio monitoring studies are based on the data from the chromosomal aberration assay in human lymphocytes, either alone or in combination with other biomarkers of cellular damage like Trypan Blue Exclusion test. The chromosomal aberration assay has also been proven to be a convenient test for somatic risk assessment. Thus, there is a need to investigate the antitumor/ cancer activity by determining the best effective doses with more precise concentrations of conjugates required contemporary methods for the characterization of Nanoparticles/ Conjugates by FTIR spectroscopy analysis to identify the functional groups of the extract that are adsorbed onto the NP surface and Dynamic Light Scattering (DLS) can measure the size distribution and zeta potential (surface charge) of the prepared NPs.

FUTURE PROSPECTS: The extracts alone and with conjugates was found to induce cell death in the cultured lymphocytes with increased chromosomal/DNA damage, succeeding to create *Annona muricata* NPs as a potential candidate for targeted therapies against cancer. Future studies must be focused on targeting the conjugated nanoparticles toward specific tumor sites with increased specificity with the use of a newly composed linker and functionalizing agent that improves the overall utilization of nanoparticles as effective therapeutic agents. The potential of *A. muricata* leaves to be used as effective anticancer agent can clearly be enlightened by understanding its mechanisms of action on the human body system and to develop an affordable and inexpensive anti-cancer drug for mankind.

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ETHICS APPROVAL: Approval was granted by the Ethics Committee of Jawaharlal Nehru Cancer Hospital, Idgah Hills, Bhopal, (M.P) India. (Approval No:1130 d/JNCH/RES/20/12/2024)

CONSENT TO PARTICIPATE: To collect (2ml) peripheral blood of healthy individuals an inform consent was taken from all subjects before participation in the study.

CONSENT TO PUBLISH: No photographs and images of any individual used in the manuscript.

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