Thakkar Jalaram H. et al. / International Journal of Biological & Pharmaceutical Research. 2010; 1(2): 114-123.

ISSN 0976-3651

International Journal of Biological & Pharmaceutical Research
Journal homepage: www.ijbpr.com

IJBPR

EVALUATION OF ANTIMUTAGENIC POTENTIAL OF *ANNONA SQUAMOSA* LEAF EXTRACT

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ABSTRACT

The aim of the current study was to evaluate antimutagenic activity of ethanolic extract of *Annona squamosa* leaf which is belonging to the family *Annonaceae*. Its in vitro and in vivo antioxidant activity of the extracts has already been reported. On the basis of that we have decided to evaluate its antimutagenic activity in vivo in mice using bone marrow chromosomal aberration assay, micronucleus test and sperm abnormality assay. In chromosomal aberration assay, ethanolic extract of *Annona squamosa* (500mg/kg) had shown significant reduction in percentage of cells with aberration. In Micronucleus test, ethanolic extract of *Annona squamosa* (500mg/kg) showed significant reduction in total no. of micronucleus in polychromatic erythrocytes. In sperm abnormality assay, ethanolic extract of *Annona squamosa* (250 and 500mg/kg) had shown significant reduction in total no. of abnormality in sperm shape. In conclusion, these results suggest that ethanolic extract of *Annona squamosa* leaves possessing significant antimutagenic property.

Keywords: *Annona squamosa*, Anticlastogenic activity, Mutagenicity, Chromosomal aberration assay, Micronuclease test, Sperm abnormality assay, Antioxidant activity.

INTRODUCTION:

Reactive oxygen species (ROS) play an important role in process like mutagenesis, carcinogenesis and aging by their ability to damage cellular DNA (Weisburger *et al*., 2001; www.needs.com). Some well-known antioxidants, known for their antimutagenic and anticarcinogenic properties (Gebhart, 1974; Ames, 1983, 1986). Renner (1984) had reported that there exists an association between agents, which show antioxidant activity to act as antimutagens. Naturally occurring antioxidants have been extensively studied for their capacity to protect organisms and cells from oxidation (Khanam *et al*., 2005; Khopde *et al*., 2001; Naik *et al*., 2002; 2003; www.needs.com). The use of plants with medicinal properties for the treatment, cure and prevention of diseases is one of the oldest medicinal methods known in history. At the beginning of the 1990s, the World Health Organization stated that 65–80% of the population of developing countries depended on medicinal plants as their only form of basic health care (Akerele, 1993).

Naturally occurring substances of plant origin and dietary components that have been widely studied for their antimutagenic activity, which includes carotenoids, flavonoids, tea-polyphenols, vitamins, cucuminoids, tannins, coumarins, chlorophyllin, porphyrins and alkylresorcinols from cereal grains (Marina *et al*., 1997). Extensive work has been carried out for demonstrating the antimutagenic potential of some commonly consumed

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spices and vegetables such as turmeric, mustard, green leafy and allium species of vegetables. Anticlastogenic phytochemicals also play an important role in prevention of cancer. Hence there is a need to establish the relations between antioxidants and anticlastogenic agents (Ames *et al*., 1987; Santosh Kumar *et al*., 2008).

Annona squamosa L. (Annonaceae), commonly known as custard apple, is a native of West Indies and is now cultivated throughout India, mainly for its edible fruit. This plant is reputed to possess several medicinal properties (Asolkar *et al*., 1992). The tribals and villagers of Aligarh district (Atique *et al*., 1985) and Chotanagpur division (Topno, 1997) in India extensively use the young leaves of Annona squamosa along with seeds of Piper nigrum for the management of diabetes and its complications. Antidiabetic activity of cold aqueous extract has been reported in STZ-nicotinamide Type 2 diabetic rats (Shirwaikar *et al*., 2004a). they are known to possess insecticidal, anti-ovulatory, abortifacient and anti-implantation properties (Vohora *et al*., 1975; Rao *et al*., 1979; Damasceno *et al*., 2002).

Keeping in mind the great medicinal value of *Annona squamosa* and high content of polyphenols, flavanoids, present investigation was planned to study the anticlastogenic effect of ethanolic leaf extract of *Annona squamosa.*

MATERIALS AND METHOD

Experimental animals:

Adult Swiss albino mice (8-12 weeks old, weighing 25±5g) were procured from institutional animal housed of Shree S.K. Patel College of Pharmaceutical Research & Education, Kherva, Gujarat. The animals were maintained on standard laboratory condition i.e.; room temperature of 25 ± 10 C; relative humidity 45-55%, 12:12h light/ dark cycle.and given food and water *ad libitum*. The whole experiment was approved from Institutional Animal Ethical Committee. All the protocols and the experiments were conducted in strict compliance according to ethical principles and guidelines provided by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

Collection of plant material:

The leaves powder was collected from Sanjivani Aushdhalay at Bhavanagar. Leaf was identified and authenticated by Dr. Ritesh vaidya, senior lecturer, Mehsana Urban Bank Institute of Biosciences, Ganpat University, Kherva. The dried leaves were finely powdered and were stored in polythene bags at room temperature. Powder was extracted with ethanol according to preliminary phytochemical analysis reported in literature.

Extraction of plant material:

The leaves powder of A*nnona squamosa* was subjected to extraction using soxhlet apparatus. The powdered material was exhaustively extracted with ethanol in soxhlet apparatus by continuous hot extraction. After each extraction, the solvent was recovered using distillation assembly, and the extract was concentrated under reduced pressure. The final yield of extract was calculated and stored in air tight container for experiment.

Experimental plan:

Five groups (n=5) of animals were randomly divided into following groups. Group 1 was Negative control which has received vehicle tween-80 (1-2%, 0.5ml), Group 2 was Positive control, Challenged with cyclophosphamide (i.p.25mg/kg) and bone marrow extraction from these animals was done at 24 h after cyclophosphamide injection. Group 3 was extract control, the animals in this group received *Annona squamosa* extract with (500mg/kg) orally for 14 consecutive days. Group 4 was also Treatment group received *Annona squamosa* extract with vehicle (250mg/kg) orally for 14 consecutive days followed by cyclophosphamide (i.p.25mg/kg) as a challenge on 14th day. After 24 h of cyclophosphamide injection bone marrow extraction was performed. Group 5 was treated with *Annona squamosa* extract (500mg/kg) orally for 14 consecutive days followed by cyclophosphamide (i.p.25mg/kg) as a challenge on 14th day. After 24 h of cyclophosphamide injection bone marrow extraction was performed.

Mouse bone marrow chromosomal aberration assay:

After the treatment, the animals were treated for 90 min with colchicine (0.4ml, 0.05%) through intraperitoneal injection and were sacrificed by cervical dislocation. Bone marrow from the femur bone was collected into hypotonic solution (0.056 M % KCl) and incubated at 37 ºC for 25 min and fixed in fixative solution (Methanol:Acetic Acid; 3:1). The permanent slides were prepared by the drop method that included the chilled blank slides and these slides were gently heated on a spirit lamp to fix cells permanently on the slides (R.J. Preston *et al*., 1987). The prepared slides were stained with Geimsa stain; the slides were dipped into the Geimsa stain (10%) for 20 min. After that washed with PBS (three times) for 5 min.

Evaluation:

At least 20 metaphases per slide were counted and 100 metaphases per animal were scored to assess the frequency of cells with chromosomal aberration. Additionally, observed different chromosomal aberrations like gap, break, exchange, deletion, fragmentation, stickiness, pulverization, ring and dicentric chromosome as a measure of toxicity. (Tijio *et al*., 1962)

Mitotic Index

Stages of mitotic index among 500 cells were calculated per animal. MI was calculated by using the formula. **(**Perez Martın *et al*., 2008; Burbano *et al*., 2007)

$$MI = \frac{(A/A+B)}{100}$$

A= No. of dividing cells (Metaphases + Anaphases).

B= No. of non dividing cells.

(Preston *et al*., 1981).

Mouse bone marrow micronucleus test

After respective treatment all the animals were sacrificed by cervical dislocation and the bone marrow was aspirated from femurs into 1 ml of 5% bovine albumin in phosphate buffered saline (pH 7.2). The cell suspension was centrifuged (800-900 rpm for 8 min) and the smears were prepared on chemically cleaned glass slides and stained with May-Gruenwald(0.02%)–Giemsa(15%). The smears were analyzed under the oil immersion objective and 1000 polychromatic erythrocytes (PCEs) /animal were counted .

Evaluation/Analysis:

At least 1000 micronucleated cells were scored per cell culture to assess the frequency of cells with one, two, or more than two micronuclei.

Sperm abnormality assay

The animals were sacrificed by cervical dislocation and dissected out. Both the cauda epididymis were removed and placed in a watch glass containing 1ml PBS (pH 7.2). The cauda epididymis was mixed thoroughly and suspension obtained was filtered through two layers of muslin cloth to remove the tissue debris. The filtered suspension was mixed with 1% aqueous eosin Y (10:1) kept for 30 min. After this period a drop of the sperm suspension was taken on a clear slide and uniform smear was made. About 1000 sperms per animal were examined from each treatment and control groups for the presence of sperm shape abnormalities. Sperm were examined at 400 fold magnifications with blue-green filters.

Evaluation:

If test substance is mutagenic it induced statistically significant abnormal sperms. The different types of abnormal sperms observed like amorphous shape, hook less, banana shaped, folded and double tailed.

RESULTS:

Chromosomal aberration assay:

Evaluation of chromosomal aberration was conducted at two dose levels. As it can be seen in the Table 1 and 2. Prior to statistic verification the ANOVA test was applied to detect the clastogenic effect of the compound tested. According to ANOVA test the protective effect for *Annona squamosa* against CP induced mutagenicity has been verified at the significance level of $p < 0.001$**.**

All the data statistically calculated using student t-test. In this assay chromatid gap and break, chromosomal gap and break, exchange, deletion, fragmentation, pulverization, stickiness, ring, and dicentric chromosomes were taken as a parameter to score the % of cells with aberration.

Table 2 Frequencies of Chromosomal aberration in Bone marrow erythrocytes induced by control Extract treated (without Cyclophosphamide), CP treated, AS-1+ CP, and AS-2 + CP Groups.

Group	Dose	Total no. of cells analyzed	Total no. of aberration	Percentage of cells with aberration
Control	Tween-80 (1-2%)	500	4.40±0.67	0.04±0.01
AS-2	500 mg/kg	500	2.60±0.67	0.02±0.01^{ns}
CP	20 mg/kg	500	80.80±3.05	0.80±0.03$^{###}$
AS -1 + CP	250 + 20 mg/kg	500	77.80±4.39	0.77±0.04^{ns}
AS -2 + CP	500 + 20 mg/kg	500	46.80±3.10	0.46±0.03***

Values are expressed in mean ± SEM. n=5, $^{###}$P< 0.001 vs. normal control. ***P< 0.001 Vs. Cyclophosphamide treated.

Group	No. of Metaphases Analysed	Cromatid		Chromosomal		Deletion	Exchange	Fragment-ation	Pulverizati-on and stickiness	Ring	Dicentric
		Gap	Break	Gap	Break						
Control	500	0.25±0.25	0.50±0.29	0.00	0.00	0.50±0.29	2.00±0.71	0.00	0.00	0.50±0.29	0.75±0.48
AS-2	500	0.20±0.20	0.40±0.24	0.20±0.20	0.00	0.00	1.20±0.20	0.00	0.00	0.40±0.24	0.20±0.20
CP	500	2.75±0.85	3.75±0.94	3.50±1.32	3.50±1.32	9.25±0.62	17.0±1.35	5.50±2.10	3.25±0.62	18.0±1.47	15.0±1.68
AS-1 + CP	500	4.25±0.25	3.25±1.31	3.00±0.40	3.00±0.40	10.75±1.54	16.75±1.65	9.00±0.70	3.50±0.64	19.50±0.64	7.00±0.40
AS-2 + CP	500	2.50±0.64	1.50±0.28	0.25±0.25	0.25±0.25	8.00±0.91	11.25±1.70	0.50±0.28	0.00	17.75±1.88	5.00±0.91

Table 1 Effect of *Annona squamosa L.* pre-treatment on the frequency of chromosomal aberrations induced by cyclophosphamide (CP), in bone marrow cells of mice. The results are expressed as mean ± SEM for five mice in each group. CP - Cyclophosphamide treated Group, AS-1- *Annona squamosa* treated (250 mg/kg), AS-2 – *Annona squamosa* treated (500 mg/kg),.

With reference to results obtained through the experiment, the percentage of aberration 0.04±0.01, was seen in negative control group. There is no significant increased frequency of aberration (0.02±0.01) was found in the group treated with *Annona squamosa* alone (500 mg/kg) when compared with negative control group. Thus, *Annona squamosa* exhibited no signs of genotoxicity in chromosomes of mice bone marrow cells. The results indicates that there is significant ($p<0.001$) increase in frequency of chromosomal aberration in positive control group (0.80±0.03), compare to the results of negative control group (0.04±0.01). Further results indicates that two weeks treatment with *Annona squamosa* 250 mg/kg (AS-1 + CP) produce no significant reduction in the percentage of aberration (0.77±0.04) as compare to positive control group. While in the group, treated with the higher the dose of *Annona squamosa* [500mg/kg or (AS-2 + CP)], shows great reduction in the percentage of aberration (0.46±0.03) compare to positive control group which is statistically significant ($p<0.001$).

Mitotic index:

There is significant ($p<0.001$) increase in value of mitotic index (5.92±0.11) was seen with the higher dose of *Annona squamosa* (AS-2 + CP) as compare to positive control group and it is vary close to the value obtained in negative control group. In group treated with lower dose of *Annona squamosa* (AS-1 + CP), remarkable increased value of M.I. (4.43±0.047) was observed but it was not statistically significant compare to positive control group. Group treated with *Annona squamosa* alone did not shown any significant differences compare to negative control group.

The results obtained for M.I is given in table no 6.4 and fig. 13 shows significant increase in M.I with increased dose of *Annona squamosa*, which is statistically significant compare to positive control group.

Micronucleus test

The results obtained for the micronucleus (Mn) is given in Table 4. There is no difference in numbers of micronucleated polychromatic erythrocyte (PCE) was seen in negative control (1.75±0.47) and *Annona squamosa* group treated alone. Effects of the oral prophylactic treatment with 250, 500 mg/kg/day of *Annona squamosa* leaf extract on the frequencies of micronucleated erythrocytes in the bone marrow of normal and exposed mice to CP, are described in Table 4

The data shows that total no of Mn found in control group was 1.75±0.47 and only *Annona squamosa* treated group shows that 1.40±0.24. There was no significant difference observed it means plant *Annona squamosa* is not mutagenic. CP treated group shows 43.25±4.28 total no. of Mn and that shows statistically significant difference ($P< 0.001$) compare to control group. *Annona squamosa* (AS-1 + CP) and (AS-2 + CP) treated group shows 39.25±5.11 and 23.00±2.80 total no of Mn. When these frequencies of Mn PCEs were compared with the frequencies of positive control group, a significant reduction ($p<0.01$) in number of Mn PCEs was found in AS-2 + CP treated group while reduced number of Mn PCEs was also found with the lower dose of *Annona squamosa* but it was not statistically significant (Fig. 3)

Sperm abnormality assay

For sperm abnormality assay parameters selected are hook cut, banana shaped, amorphous shaped, double tailed, and folded on themselves. (Table 5) The data shows that total no of sperm cells found with abnormality in control group was 2.20±0.37 and only *Annona squamosa* treated group shows that 1.80±0.20. There was no significant difference observed it means plant *Annona squamosa* is not mutagenic. CP treated group shows 90.00±0.89 total no. sperm cells with abnormality and that shows statistically significant difference ($P< 0.001$) compare to control group. *Annona squamosa* (AS-1 + CP) and (AS-2 + CP) treated group shows 72.20±3.44 and 32.60±1.72 total no sperm cells with abnormality. When these frequencies of sperm abnormality were compared with the frequencies of positive control group, a significant reduction ($p<0.01$, $p<0.001$ respectively) in number of sperm cells with abnormality was found in AS-1 + CP and AS-2 + CP treated group (Fig.4).

Table 3 Mitotic index of bone marrow cells of Control, Extract treated (AS-2), CP (20 mg/kg), AS-1 + CP, and AS-2 + CP.

Group	Dose	Total no. of cell counted	Mitotic Index (%) (Mean±SEM)
Control	Tween-80 (1-2%)	500	6.71±0.12
AS-2	500 mg/kg	500	$6.75±0.14^{ns}$
CP	20 mg/kg	500	$4.21±0.073^{###}$
AS-1 + CP	250 + 20 mg/kg	500	$4.43±0.047^{ns}$
AS-2 + CP	500 + 20 mg/kg	500	5.92±0.11***

Values are expressed in mean ± SEM. n=5, $^{###}P< 0.001$ vs. normal control. $^{***}P< 0.001$ Vs. Cyclophosphamide treated. (Student t-test), ns= not significant.

Table 4 Data showing comparison of micronucleus found in PCE in control, AS-2, CP, AS-1 + CP, AS-2 + CP treated animals.

Group	Dose	Total no. of PCE counted	Total no. of MN found in PCE
Control	Tween-80 (1-2%)	1000	1.75±0.47
AS-2	500 mg/kg	1000	1.40 ± 0.24^{ns}
CP	25mg/kg	1000	$43.25\pm4.28^{###}$
AS-1 + CP	250 + 25 mg/kg	1000	39.25 ± 5.11^{ns}
AS-2 + CP	500 + 25 mg/kg	1000	23.00±2.80**

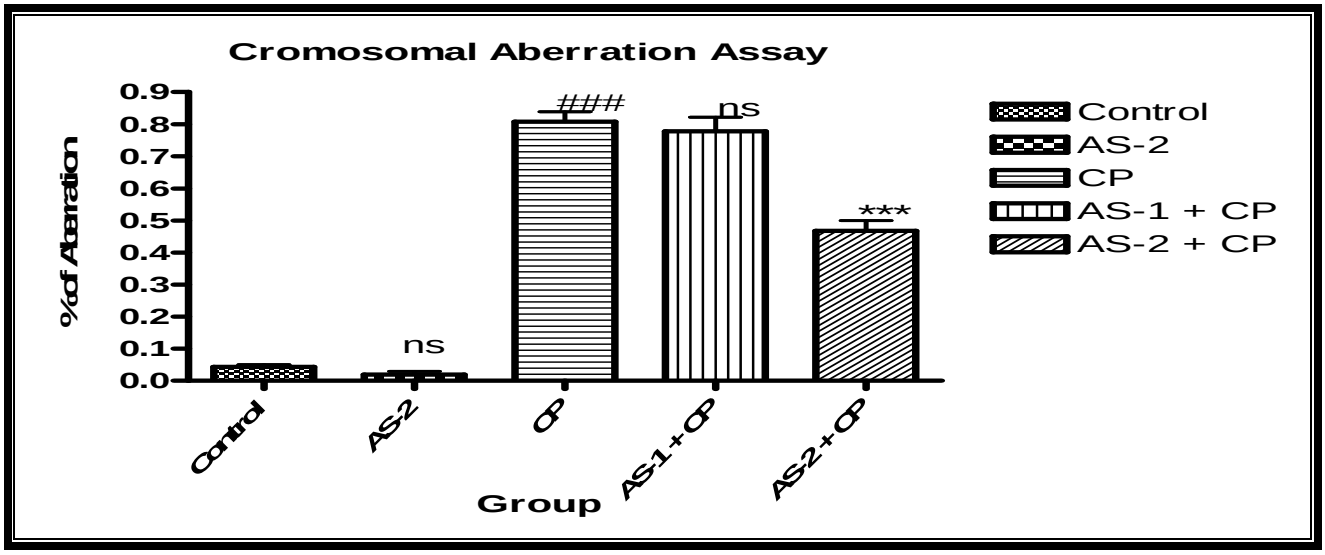
Values are expressed in mean ± SEM. $^{###}$P< 0.001 vs. normal control. **P< 0.01 vs. Cyclophosphamide treated, ns= not significant.

Table 5 Data showing comparison of Total no. of sperm cells with abnormality in control, AS-2, CP, AS-1 + CP, AS-2 + CP treated animals.

Group	Dose	Total no. of cells analysed/animal	Total no. of Sperm cells with abnormality
Control	Tween-80 (1-2%)	1000	2.20±0.37
AS-2	500 mg/kg	1000	1.80 ± 0.20^{ns}
CP	25 mg/kg	1000	$90.00\pm0.89^{###}$
AS-1 + CP	250 + 25 mg/kg	1000	72.20±3.44**
AS-2 + CP	500 + 25 mg/kg	1000	32.60± 1.72***

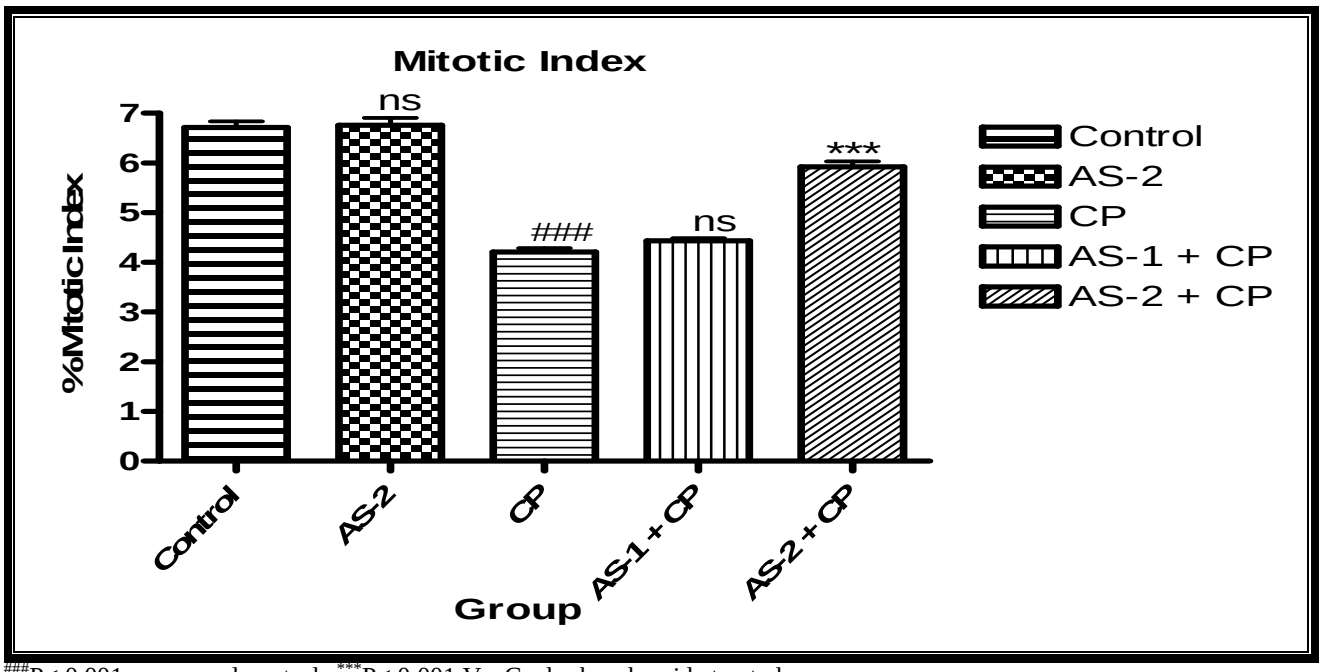
Values are expressed in mean ± SEM. n=5

Figure.1. Data showing comparison of chromosomal aberration in control, CP treated AS-1 + CP, and AS-2 + CP Groups.



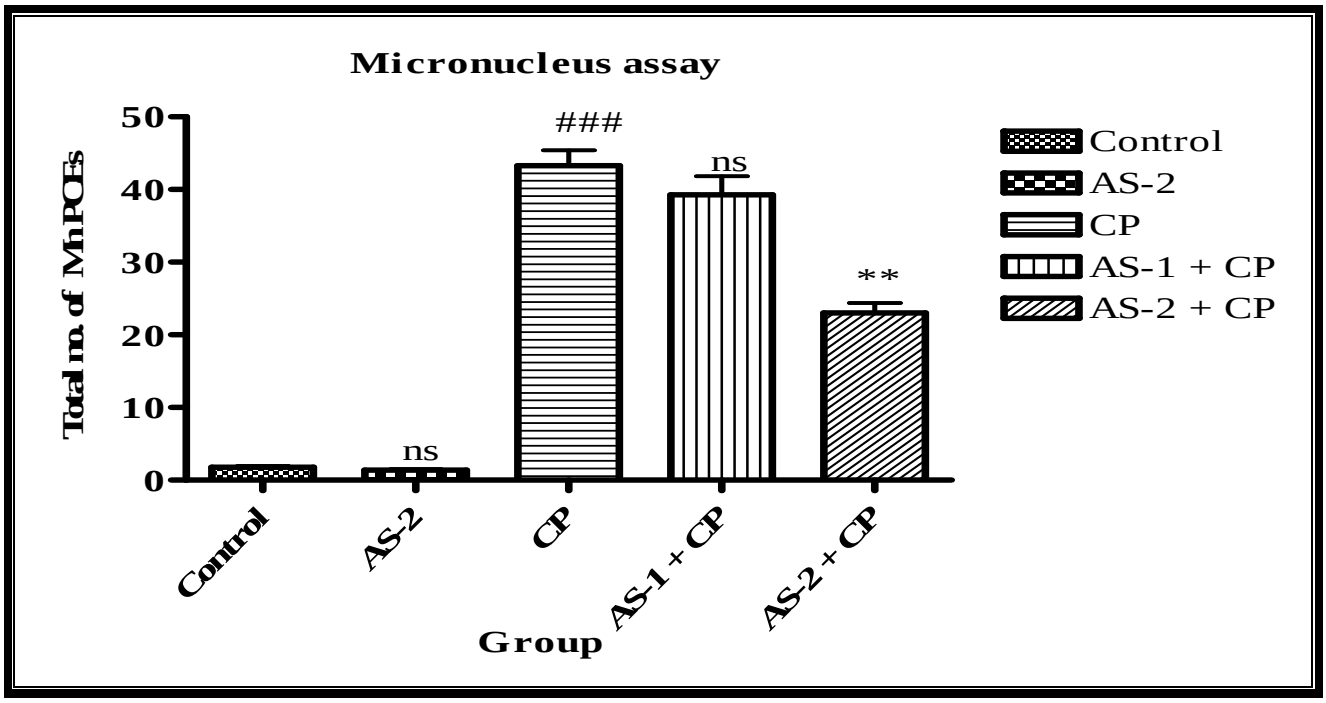
Values are expressed in mean ± SEM n=5, $^{###}$P< 0.001 vs. normal control. ***P< 0.001 Vs. Cyclophosphamide treated. (Student t-test), ns= not significant CP - Cyclophosphamide treated Group, AS-1- *Annona squamosa* treated (250 mg/kg), AS-2 – *Annona squamosa* treated (500 mg/kg).

Figure. 2. Frequencies of Mitotic index in different group in CP treated bone marrow cells for 24 hr.



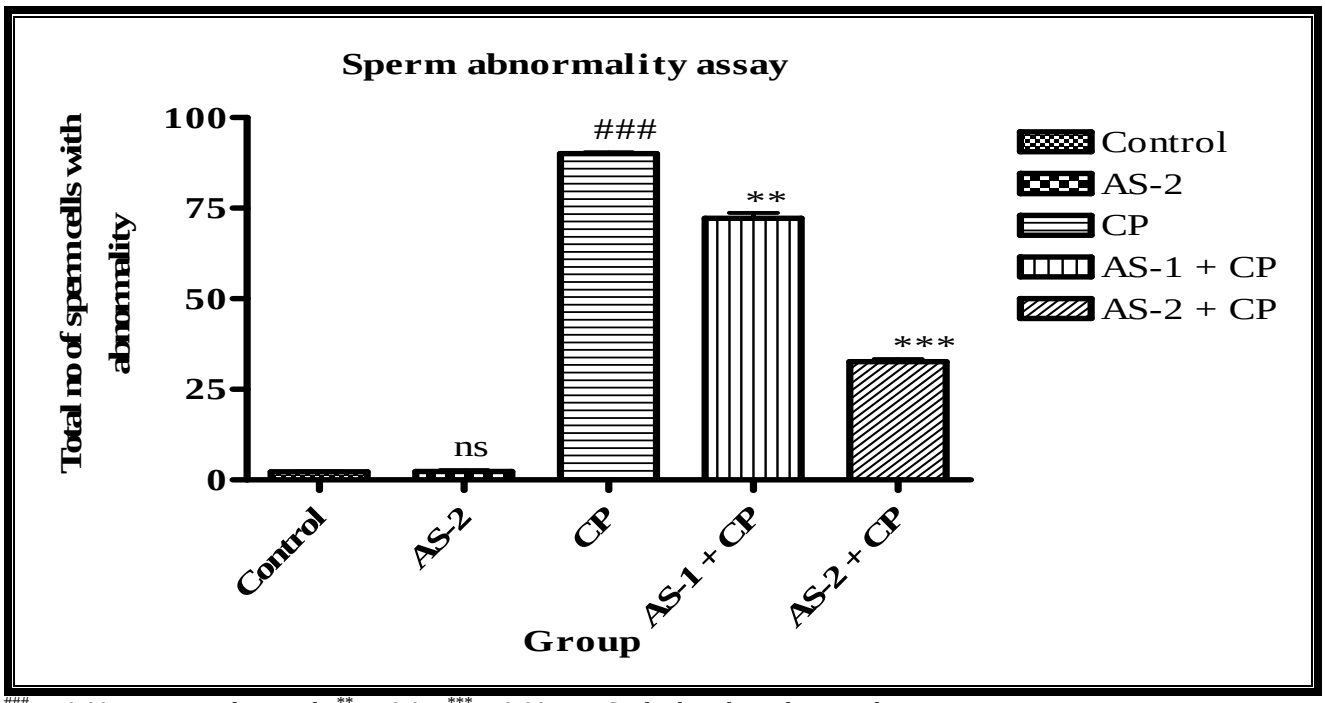
###P< 0.001 vs. normal control. ***P< 0.001 Vs. Cyclophosphamide treated.

Figure. 3. Data showing comparison of micronucleus found in PCE in control, AS-2, CP treated, AS-1 + CP, and AS-2 + CP.



###P< 0.001 vs. normal control. **P< 0.01 vs. CP treated, ns= not significant.

Figure. 4. Data showing comparison of Total no of sperm abnormality in control, AS-2, CP treted, AS-1 + CP, and AS-2 + CP.



###P< 0.001 vs. normal control. **P< 0.01, ***P<0.001 vs. Cyclophosphamide treated.

DISCUSSION:

Data accumulated over many years clearly show that oxidative DNA damage plays an important role in a number of disease processes. The accumulation of oxidative DNA damage in non-dividing cells is thought to contribute to age-associated diseases. In Multiple processes such as base- and nucleotide-excision pathways exist to repair the wide range of DNA damages. If left unrepaired, oxidative DNA damage can lead to detrimental biological consequences in organisms, including cell death, mutations and transformation of cells to malignant cells. Therefore, DNA repair is regarded as one of the essential events in all life forms. There is an increasing awareness of the importance of oxidative DNA damage and its repair to human health. Thus, it becomes exceedingly important to understand, at the fundamental level, the mechanisms of oxidative DNA damage, and its processing by DNA repair enzymes as well as how unrepaired DNA lesions may lead to cytotoxicity, mutagenesis and eventually to diseases and aging (Miral *et al*., 2005).

Chromosomal analysis of bone marrow cells in vivo from mammals is a standard method for testing potential mutagenic or anti mutagenic effects of viruses, radiation, drugs and chemicals pollutants. The mammalian bone marrow chromosomal aberration assay can detect clastogenic or aneugenic effects of a test agent. However, in the chromosomal aberration assay, these effects are observed directly by examination of metaphase chromosome spreads. The recommended methodology has been published in OECD Test Guideline 475 (OECD, Test Guideline 475, 1997).

The assay is based on the ability of a test agent to induce chromosome structural or numerical alterations that can be visualized microscopically. The target tissue for the chromosomal aberration assay is the bone marrow because it is a rapidly dividing, well-vascularized tissue. In order to accumulate metaphase cells, cell division is arrested by administration of a mitotic inhibitor, such as colchicine, 1.5 hour prior to sacrifice. Bone marrow cells are obtained immediately, exposed to hypotonic solution, fixed and stained. Using light microscopy, an observer should score a minimum of 1000 cells/animal for mitotic index and a minimum of 100 metaphase cells/animal for chromosomal aberrations (Iain *et al*., 2005).

The results obtained in this study indicate that Annona squamosa leaf extract pre-treatment significantly reduced the frequency of structural chromosomal aberrations induced by CP in bone marrow cells.

The relationship between cell cycle progression and inhibition of cell proliferation was examined by determining the mitotic index at various time points during drug exposure. Decrease in percentage of mitotic index

in CP treated group shows that there was decrease in cell proliferation in bone marrow cells of mice, while pre-treatment with *Annona squamosa* given a significant improvement in mitotic activity of bone marrow cells. The improvement in mitotic activity of bone marrow cells of animals pre-treated with *Annona squamosa* may focus attention on the beneficial effect of *Annona squamosa* to overcome one of the most serious problems in cancer chemotherapy, which is the bone marrow suppression and related immunosuppression. Since most toxic deaths related to chemotherapy are from infection associated with immunosuppression, the use of *Annona squamosa* as known immunopotentiating agent may protect the patients from lethal infection episodes.

So we think on the basis of our results, together with other published similar studies, that *Annona squamosa* leaf extract produce antimutagenic effect against a potent chemical mutagen cyclophosphamide in variety of tests. We recommend that consumption of *Annona squamosa* in routine life can prevent the chances of occurrences of variety of diseases arise due to mutation in gene.

CONCLUSION:

The protective effect of *Annona squamosa* leaf extract was seen against the mutation induced by cyclophosphamide. The tests were performed on mice bone chromosomal aberration assay and mitotic index. All the results find statistically significant. Therefore, from the present study, it can be concluded that *Annona squamosa* leaf ethanolic extract possesses antimutagenic property. However, pre-treatment with *Annona squamosa* leaf extract did not return the frequency of structural chromosomal aberrations to the control level. The observed antimutagenic activity of *Annona squamosa* against Cyclophosphamide might be associated with its antioxidant constituents such as poly-phenolic compound, flavanoids and other micronutrients. Further investigations will be needed to evaluate the same activity of *Annona squamosa* on other test system as well as to characterize the active compounds in detail.

ACKNOWLDGEMENT:

We are thankful to Sanjivani Aushdhalay at Bhavanagar for providing *Annona squamosa* leaf powder for this research study. We are also thankful to S.K.Patel college of Pharmaceutical Education and Research, Ganpat University, Kherva, Mahesana, Gujarat, India for providing financial support and facilities for research study.

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