

Evaluation of Anticlastogenic Activity of Bael Fruit Extract on Cyclophosphamide Induced Genotoxicity in Mice, Using Micronucleus and Chromosomal Aberration Test

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Abstract

Aim: The present articles involves the investigation of anticlastogenic activity of ethanolic extract of bael fruit (*Aegle marmelos*). Acute toxicity study was conducted as per OECD guidelines up to 2000mg/kg body weight. **Methodology:** Anticlastogenic activity was investigated by two models viz, micronucleus test and chromosomal aberration method using mice. In micronucleus assay model the bone marrow was extracted from various groups of animals, staining was performed and the slides were scanned under oil immersion(100X) in LABomed-Digi 2 microscope (90V-260V), for the presence of micronucleus in PCE (Polychromatic Erythrocytes) and NCE (Normochromatic Erythrocytes). **Results:** In chromosomal aberration model the animals has been sacrificed from all the groups and bone marrow was collected, processed and different types of aberrations like chromosome breaks, exchanges, rings and minute were recorded. In micronucleus assay, there was a significant ($P<0.001$) increase in the % MNPCE and decrease in P/N ratio in cyclophosphamide (50 mg/kg, i.p.) treated animals when compared with normal control animals. In chromosomal aberration test, there was a significant increase in total no. of chromosomal aberrations (rings, exchanges, breaks and minute) in cyclophosphamide treated animals when compared with normal control animals and it was time dependent. Bael fruit extract were found to be non mutagenic and significantly ($P<0.001$) decreased the cyclophosphamide induced formation of chromosomal aberrations. **Conclusion:** Based on the present empirical evidences the ethanolic extract of bael fruit was found significant anticlastogenic activity.

Keywords: *Aegle marmelos*, acute toxicity, micronucleus test, chromosomal aberration, genotoxicity.

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INTRODUCTION

Aegle marmelos (Bael) is a well known fruit in India which is present throughout south east Asia as a naturalised species. The fruits are used in traditional medicines and as a food throughout its range. Genotoxicity is a study of agents that interact with DNA or the cellular machinery that regulates fidelity of the genome [1]. Genetic instability involving chromosomal abnormality through minute aberration is closely associated with carcinogenesis and it is well accepted that accumulation of multiple genome wide mutation in multistep fashion leads to tumorigenesis [2]. Clastogen is an agent that induces chromosome breaks and results in gain, loss or rearrangement of chromosomal segments [3]. Cancer is caused by mutations in genes critical for the control of cell growth. Time dependent genetic degeneration, inherited or acquired deficiency in genome maintenance system

forms important factor in carcinogenesis [4]. Many anticancer drugs used in the treatment of various human cancers invariably have cell toxicity and can induce genotoxic effect in non tumor cells that can give rise to secondary tumors [5]. Cyclophosphamide is an important chemotherapeutic agent that interferes with DNA integrity and function and causes the cell cycle to arrest and attempts to repair DNA. Oxidative damage is one of the main mechanisms leading to cancer. Efficient and proficient DNA repair is thus required for the effective maintenance of genome integrity. Epidemiological evidence shows that an inverse relationship exist between the consumption of vegetables and the incidence of cancer which can be attributed to protective components such as B-carotene and vitamin A and ascorbic acid. However, the inverse relationship is observed between the ingestion of green/yellow vegetables and the incidence of human cancers could conceivably be due to many other plant

components [6]. Research has found the essential oil of the Bael fruit to be effective against 21 types of bacteria [7]. It is prescribed for smooth bowel movement to patients suffering from constipation and other gastrointestinal problems. Research also indicates that unripe Bael fruit is effective in combating giardia and rotavirus. While unripe Bael fruit did not show antimicrobial properties, it did inhibit bacteria adherence to and invasion of the gut [8] (i.e. the ability to infect the gut). Aegeline (N-[2-hydroxy-2(4-methoxyphenyl) ethyl]-3-phenyl-2-propenamide) is a known constituent of the bael leaf and consumed as a dietary supplement [9] for a variety of purposes [10-12]. The antioxidant and anti-proliferative activity of bael fruit also has been reported [13]. Anti clastogenic property of bael fruit was not been reported. The scientific investigation is essential to justify the potency and to extend the scope for use. Therefore keeping the above facts in mind the present research work has been

planned to investigate the anti mutagenic property of bael fruit.

MATERIALS AND METHODS

Collection of Bael Fruit

Bael fruit (*Aegle marmelos*), shown in Figure-1, was collected in the month of June from the local area of Dharwad district, Karnataka, India, The collected fruits were cleaned and fruit pulp was collected and dried.

Extraction

Extraction of Bael fruit was carried out by cold maceration method. These dried pulp were mechanically powdered fruit [125gm]. These powdered materials are extracted with 90% ethanol (200 ml, 72 hours, cold maceration using magnetic stirrer), filtered using suction pump. The extract is stored in a refrigerator at 4°C. The obtained light chocolate brown colour extract was used for further studies [14]. Photochemical analysis was performed.



Fig-1: Image of Bael fruit

Animals

Swiss albino mice weighing 25 ± 5 g, 6-7 weeks old were used in the study. The inbred colonies of mice were purchased from Venkateshwara enterprises, Bangalore. They were acclimatized to controlled conditions of temperature ($23 \pm 20^\circ\text{C}$), 30-70% humidity and 12 hr light-dark cycles. The animals were randomized into experimental and control groups and housed four each in sanitized polypropylene cages containing sterile paddy husk as bedding. They had free access to standard pellets as basal diet and water ad libitum. All the studies conducted were approved by Institutional Animal Ethical Committee (IAEC), SET's College of pharmacy, Dharwad, Karnataka (REG.No.112/1999/CPCSEA). According to prescribed guidelines of committee for the purpose of Control and Supervision of Experiments in Animals (CPCSEA), Government of India.

Acute toxicity Study

Acute toxicity study was carried out using Swiss albino mice (25 ± 5 g) by up and down/staircase method as per OECD guidelines. Both the drugs were orally administered to different groups of mice at the doses of 50 mg, 300 mg, 1000mg and 2000 mg/kg body

weight respectively. Animals were observed for 48 hr to study the general behaviour of animals, sign of discomfort, nervous manifestation and mortality.

Anticlastogenic activity (Micronucleus Test)

Group Classification

Swiss Albino mice of either sex were taken for test provided with food and water. Twenty healthy mice were divided into 5 groups. Group 1 consist of vehicles treated. Animal receive vehicle (10ml/kg) orally for 7 consecutive days. Group 2 consist of clastogenic control challenge group received Cyclophosphamide (i.p. 50mg/kg). Bone marrow extraction from these animals was done at 24 hrs after cyclophosphamide injection. Group 3 consist of extract control received Bael fruit extract (200mg/kg) orally for 7 days. Bone marrow extraction was done from these animals. Group 4 consist of treatment group received Bael fruit extract (100mg/kg) orally for 7 consecutive days, followed by cyclophosphamide (i.p.50mg/kg) as challenge on 7th day. After 24 hrs of cyclophosphamide injection Bone marrow extraction was performed. Group 5 consist of treatment group which received Bael fruit extract (200mg/kg) orally for 7 consecutive days, followed by cyclophosphamide (i.p.50mg/kg) as challenge on 7th

day. After 48 hrs of cyclophosphamide injection Bone marrow extraction was performed [15].

Extraction of Bone Marrow

The experimental animals were sacrificed by cervical dislocation. Animals were cut open and femur and tibia were removed. For collection of marrow, the upper end of the femur was cut open, till a small opening was visible. A needle was inserted to ensure that the upper end was open. About 0.5 ml of the suspending medium was taken in a syringe and the needle was inserted at the lower epiphysial end. The marrow was flushed into a clean cavity block. If the marrow collected was solid, it was dispersed by repeated aspiration and flushing with the help of the syringe. Similarly tibia marrow was also collected. Altogether 2.0 ml of the suspending medium was sufficient to collect the marrow from femur and tibia. A fine marrow suspension was then transferred to a centrifuge tube and centrifuged at 1000 rpm for 8 min. The supernatant was drawn off and marrow films made by smearing a small drop on a clean glass. Then the slides were air-dried.

Staining Procedure

The smears were fixed in absolute methanol for 10 min. (The methanol should be absolutely pure, so as to avoid artifacts in the slides). The slides were kept in coupling jars containing May Grunewald's stain freshly diluted with equal volume of phosphate buffer (PH=6.8) for 15 min. The slides were transferred to Giemsa, freshly diluted with phosphate buffer (1:6) and kept for 10 min. Washed the slides rapidly in 3 or 4 changes of buffered water. The slides were then allowed to stand undisturbed in buffered water for 5 min., so that differentiation could take place. Slides were air-dried.

Scoring

The slides were scanned under oil immersion (100X) in Labomed – Model Digi 2 microscope (90V-260V) for the presence of MN in PCE and NCE. P/N ratio was determined by counting a total of about 2000 erythrocytes per animal.

Anticlastogenic activity (Chromosomal Aberration Test)

Group Classification

Swiss Albino mice of either sex were taken for test provided with food and water. Twenty healthy mice were divided into 5 groups. Group 1 consist of four vehicles treated, animal receive vehicle (10ml/kg) orally for 7 consecutive days. Group 2 consist of clastogenic control. Challenge group received Cyclophosphamide (i.p. 50mg/kg). Bone marrow extraction from these animals was done at 24 hrs after cyclophosphamide injection. Group 03 consist of extract control, received Bael fruit extract (200mg/kg) orally for 7 days. Bone marrow extraction was done from these animals. Group 4 consist of treatment group,

received Bael fruit extract (100mg/kg) orally for 7 consecutive days. Followed by cyclophosphamide (i.p.50mg/kg) as challenge on 7th day. After 24 hrs of cyclophosphamide injection Bone marrow extraction was performed. Group 5 consist of treatment group received Bael fruit extract (200mg/kg) orally for 7 consecutive days, followed by cyclophosphamide (i.p.50mg/kg) as challenge on 7th day. After 48 hrs of cyclophosphamide injection Bone marrow extraction was performed [16].

Sacrifice and Harvest

Animals were sacrificed by cervical dislocation after 24h of administration of the clastogen. 90 min. prior to death, each animal was injected with 0.04% colchicines in a dose of 4 mg/kg i.p for mitotic arrest. Colchicines solution was prepared in distilled water. Animals were cut open and femur and tibia from both the legs were quickly removed and muscle mass cleaned away from the bones. For collection of bone marrow, the upper end of femur was cut open, till a small opening was visible. A 22 gauge needle was inserted to ensure that the upper end was open. About 0.5 ml of 0.56% (or 0.075 M) hypotonic potassium chloride solution was taken in a syringe and the needle was inserted at the lower epiphysial end. The bone marrow was flushed into a clean cavity block. If the marrow collected was solid, it was dispersed by repeated aspiration and flushing with the help of the syringe. Similarly tibial marrow was also collected. Altogether 2 ml of hypotonic potassium chloride solution was used to collect the marrow from both femur and tibia

Hypotonic Treatment

A fine marrow suspension was made by gently mixing the contents with 3 ml of hypotonic potassium chloride solution in the cavity block. Suspension was transferred to a clean graduated centrifuge tube with the help of a hypodermic syringe and allowed to stand for 20 min.

Fixation and Observation

After 20 min., the cells were centrifuged at 800 rpm for 8 min. Supernatant was removed by gentle aspiration until a small volume (0.1-0.2 ml) remained above the pellet. The pellet was resuspended in the remaining volume on a cyclo (vortex) mixer. The cells were then agitated on the cyclomixer with drop wise addition of 0.5 ml of freshly prepared cold corony's fixative (Methanol: Glacial acetic acid, 3:1). The resulting suspension was allowed to stand at room temperature for 15-20 min. Then it was centrifuged at 800 rpm for 8 min. Supernatant was gently aspirated leaving a small volume over the pellet. The cells were resuspended in the remaining volume. About 2 ml of fresh, cold fixative was added drop wise with agitation. Steps 6-9 were repeated thrice with the last-but-one fixation lasting at least 1h under refrigeration. Fixative was changed for the last time just prior to the

preparation of the slides. The cell suspension was divided into two parts- one part stored (in about 2ml of fixative) tightly capped at 4°C in a refrigerator; whereas the other part was used for immediate preparation of slides. For overnight stored cells, fixative was changed two more times by repeating steps 6-9. Slides used for smearing were free from any scratches, greasy or particulate matter. Two separate drops were dropped from a height of 30-40 cm on to the chilled slides kept in a refrigerator for at least 1hr before use in a coupling chamber. The slides were quickly dried by passing twice or thrice over a low intensity flame. It is important that all fluid dries up quickly and completely to stick the cell firmly to the glass surface. Adequate numbers of slides were prepared depending upon the frequency of scorable metaphase cells, with a minimum of two slides per animals.

Slides were then stained with Giemsa's stain diluted with phosphate buffer PH=6.8 (1 ml of 5% Giemsa solution in 40 ml of buffer for 5-10 minutes). Quality of staining was checked on test slides before all slides were stained. After staining, the slides were washed rapidly in 3 or 4 changes of buffered water so that differentiation could take place. The lower surface of each slide was wiped with acetone and the slides were then air-dried. Slides were scanned under higher magnification (100 X oil immersion) for examination of each individual metaphase. At least 50 well-spread metaphase cells were screened per animal. Only those cells that were well spread, where nuclei were free of cytoplasm and chromosomes did not overlap were selected of aberrations.

Scoring Criteria

Different types of aberrations (breaks, exchanges, rings and minute) were recorded Separately. Chromosomal breaks are true discontinuities with clearly dislocated fragments and also include fragments without obvious origin. Exchanges also called as translocations. Here one portion of chromosome is transferred to another chromosome. A portion of a chromosome has broken off and formed a circle or ring. This can happen with or without loss of genetic material [17]. Minute was considered as the chromosomes are

broken into small particles like structure. The data were expressed as mean \pm S.E.M. Statistical comparison were performed by one-way ANOVA followed by Tukey's post-test chromosomal aberrations.

RESULTS AND DISCUSSION

Extraction of Bael fruit was performed by using cold maceration method. These dried pulp were mechanically powdered and extracted with 90% ethanol. Phytochemical tests confirmed the presence of flavonoids, steroids, tannins, triterpenoids and saponins, in ethanolic bael fruit extract.

Acute Toxicity Studies

Acute toxicity study was carried out using swiss albino mice (25-30 g) by up and down/staircase method as per OECD guidelines. Bael fruit extract were orally administered to different groups of mice three at the doses of 50, 300, 1000, and 2000 mg/kg body weight respectively. Animals were observed for 48 hrs to study the general behaviour of animals, signs of discomfort and nervous manifestation Bael fruit extract was found devoid of mortality of animals at the dose of 2000 mg/kg body weight. Hence the 1/10th (200mg/kg, p.o.) of the dose selected.

Micronucleus Test

Table-1 shows that the I.P route with 50 mg/kg body weight dose of cyclophosphamide significantly induced the formation of micronuclei in bone marrow cells of swiss mice after 24 and 48 hr time periods as compared with the solvent control group. The induction of micronuclei formation by cyclophosphamide was significantly inhibited. When the animals were treated with Bael fruit extract (200mg/kg) for 7 consecutive days orally in mice 24 hrs before cyclophosphamide treatment. Experimental group 4 and 5 was observed 2.24 ± 0.100 and 4.32 ± 0.0983 micronuclei in per 1000 polychromatic erythrocytes in mice. Bael fruit alone did not induce significant micronuclei as shown in Figure-2 and Table-1 It has been also observed that there is significant difference in PCE/NCE ratio between Bael fruit experimental group and cyclophosphamide control group, shown in Figure-3.

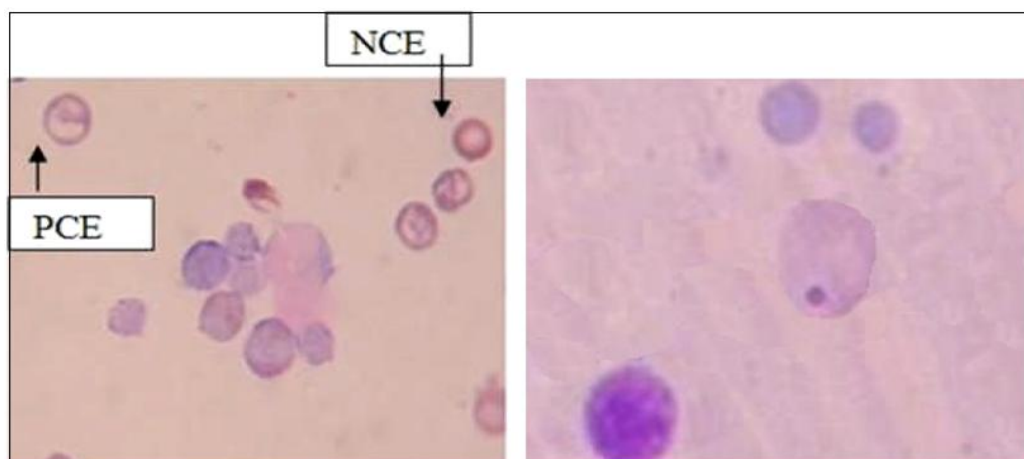


Fig-2: PCE and NCE cells in micronucleus test

Table-1: Effect of *Bael* fruit extract in Cyclophosphamide induced micronucleus test

SL no	Groups	Time of harvesting	No. Of MNPCE On the basis of 1000 PCE cells	PCE/NCE
1	Control	24hr	0.76 ± 0.005	0.915 ± 0.009
2	Cyclophosphamide alone (50mg/kg)	24hr	$4.3 \pm 0.043^*$	$0.620 \pm 0.009^*$
3	<i>Bael</i> fruit extract alone (200mg/kg)	24hr	$1.24 \pm 0.014^*$	0.922 ± 0.011
4	Cyclophosphamide (50mg/kg) + <i>Bael</i> fruit extract (100mg/kg)	24hr	2.24 ± 0.100	$0.798 \pm 0.006^*$
5	Cyclophosphamide(50mg/kg) + <i>Bael</i> fruit extract (200mg/kg)	48 hr	4.32 ± 0.0983	$0.746 \pm 0.006^*$

Values are expressed in Mean \pm SEM, one way ANOVA followed by Tukey's multiple comparison test. Control Vs. Cyclophosphamide

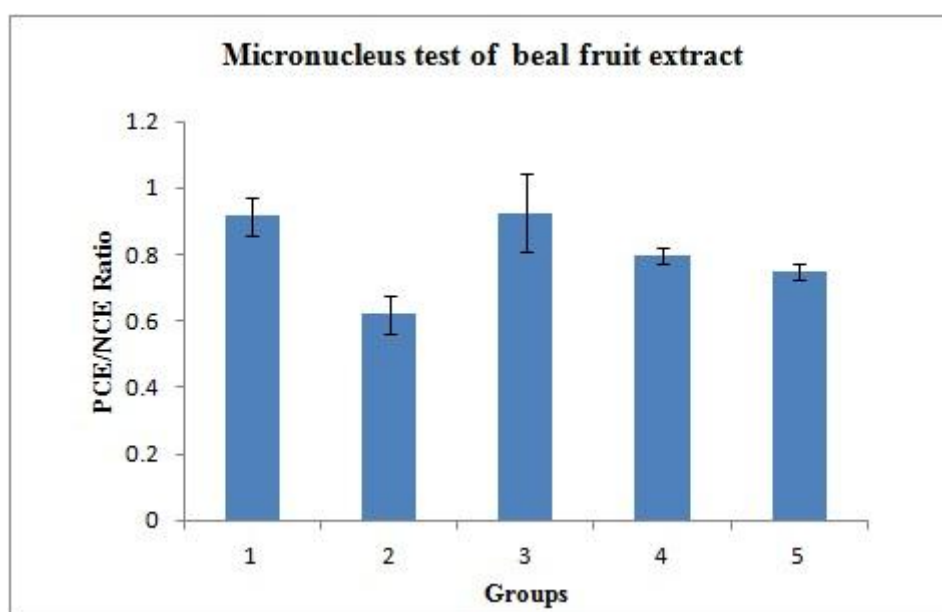


Fig-3: Graph of PC/NC ratio of micronucleus test results

Chromosomal Aberrations Test

The frequencies of different chromosomal aberrations observed in bone marrow cells of control and treated groups are presented in Table-2. A significant increase ($P < 0.05$) in the mean frequency of the total aberrations was noted in cyclophosphamide

treated group (30.3 ± 0.0876) and at different time periods as compared with the solvent control group (6.51 ± 0.012). However, in *Bael* fruit extract group the frequencies of total aberrations are slightly and insignificantly higher (7.5 ± 0.288) than the control. In addition there was a significant ($P < 0.05$) decrease in the

mean percentage of total aberrations of Bael fruit extract experimental groups 4 and 5 (15.25 ± 0.01732) and (16.78 ± 0.01453) at different time periods (24 and 48 hr) as compared with cyclophosphamide groups 2 (30.3 ± 0.0876) was observed. These results suggest that Bael fruit extract had protective effect against cyclophosphamide monitored by decreasing the incidence of aberrations. Total chromosomal aberration against the various groups has been depicted in Figure-4 and Table-2. The evaluation of micronucleus frequencies *in vivo* is one of the primary genotoxicity tests recommended internationally by regulatory agencies for product safety assessment. The antimutagenic activity of Bael fruit extract was evaluated by measuring their inhibitory effect on cyclophosphamide induced mutagenesis. Bael fruit extract administered orally to the animals in the respective treatment groups for 7 days, followed by cyclophosphamide challenge treatment. The bone marrow samples were collected after 24 and 48 hr. frequencies of MN in PCEs and P/N ratio were evaluated. Cyclophosphamide and its metabolites may

be involved in the toxic reactions and cause DNA damage, inducing genotoxic effects in the cells.^{54,55} Upon administration of cyclophosphamide there was significant ($P < 0.001$) rise in % MNPCE and it was time dependent. A significant increase in % MNPCE indicates that cyclophosphamide induced chromosomal damage in mouse bone marrow cells. These fragmented chromosomes were condensed to form micronuclei which are not included in the main nucleus.⁵⁶ Administration of Bael fruit extract alone do not produce any significant variation in % MNPCE and indicating that these does not possess any genotoxicity. Bael fruit extract has significantly ($P < 0.001$) decreased the cyclophosphamide induced formation of micronuclei in PCE and NCE, which may be due to the inhibition of cyclophosphamide induced chromosomal damage. There was a significant ($P < 0.001$) decrease in the P/N ratio of cyclophosphamide treated animals and the decrease was the time dependent. Decrease in P/N ratio was due to increase in NCEs which signals a cytotoxic effect.

Table-2: Effect of Bael fruit extract in cyclophosphamide induced chromosomal aberration in mice

SI no	Groups	Time of harvesting	Total ch. AB	Ch. fr	Ch. Break/ gap	R.F	C.A
1	Control	24hr	6.51 ± 0.012	4.7 ± 0.478	2.75 ± 0.478	0	0
2	Cyclophosphamide alone (50mg/kg)	24hr	30.3 ± 0.087	14.25 ± 0.478	10.5 ± 0.750	2.5 ± 0.866	2.5 ± 0.288
3	Bael fruit leaf and stem extract alone (200mg/kg)	24hr	7.6 ± 0.088	5.0 ± 0.408	2.75 ± 0.478	2.5 ± 0.500	0
4	Cyclophosphamide(50mg/kg) + Bael fruit leaf and stem extract(100mg/kg)	24hr	15.25 ± 0.017	6.25 ± 0.478	5.50 ± 0.288	2.0 ± 0.408	2.0 ± 0.577
5	Cyclophosphamide(50mg/kg) + Bael fruit leaf and stem extract(200mg/kg)	48hr	16.78 ± 0.014	7.25 ± 0.250	5.50 ± 0.645	2.5 ± 0.645	2.33 ± 0.333

All values are expressed as Mean \pm SEM. One way ANOVA followed by Tukey's multiple comparison test. control Vs. Cyclophosphamide. Ch.Ab.-chromosomal aberration, Ch.fr-chromatid fragment, Ch.break/Gap-chromosomal break/gap, R.F-ring formation, C.A-centromeric association

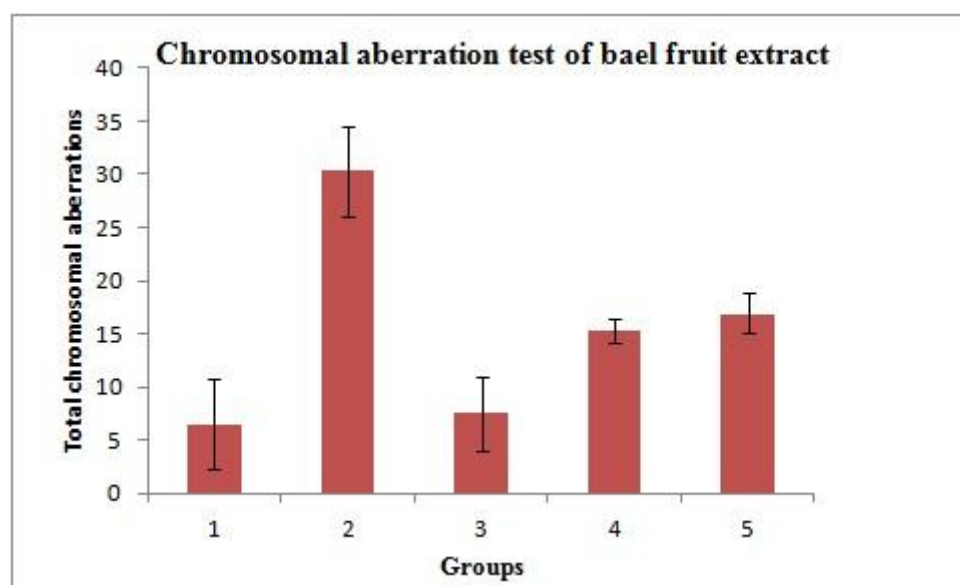


Fig-4: Graph of Chromosomal aberration test results

CONCLUSION

Findings of this study suggest that Bael fruit extract possess anticlastogenic activity. Anticlastogenic activity was studied using invivo micronucleus assay and chromosomal aberration test. The present study of Bael fruit extract was studied for anticancer activity.

Conflict of Interest

The authors have declared that no conflict of interest exists.

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