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ANTIMUTAGENIC ACTIVITY OF POMEGRANATE EXTRACT AND 2 - METHOXY-ESTRADIOL IN COMBINATION ON SWISS ALBINO MOUSE - *MUS MUSCULAS*

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ABSTRACT: Pomegranate extracts (PGEs) exhibit antioxidative, antiproliferative, and antineoplastic activities. 2-Methoxyestradiol (2-ME), the end metabolite of 17β estradiol (E2), an antiangiogenic agent, inhibits tumor growth. Our earlier investigations demonstrated that aqueous PG extract (PGE) in combination with 2-ME showed antineoplastic effect on Sarcoma-180 tumor cells. The present paper has been oriented to evaluate the antimutagenic or anticlastogenic effect of PGE in combination with 2-ME on normal Swiss albino mice. A comparative evaluation of the antioxidant potential and free radical scavenging activity of ethanolic and aqueous extracts of PG was evaluated by total phenol, flavonoidand Ferric reducing antioxidant power assay (FRAP) content as well as 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity. Ethanolic extract showed more amounts of phenolic compounds, which is a good sign of high antioxidative potentiality. The aqueous extract has high flavonoid content, which dominates the phenolic activity of the ethanolic extract. The aqueous extract has high Ferric reducing power with more scavenging activity in DPPH assay, which promotes the extract to give a protective property. Present results demonstrated that combination effects of aqueous PGE (400 mg/kg of body weight) and 2-ME (1.5 mg/kg of body weight) significantly protect the mouse from cellular and haematological toxicity. This study introduces a novel combination, where the particular combination of PG and 2-ME (*i.e.* 400 mg/kg of body weight + 1.5 mg/kg of body weight) not only enhances the survivability of mouse synergistically, but also inhibits the cytological and hematological toxicity antagonistically, as analyzed by Chou Talalay method, which could serve as an antimutagenic potential.

INTRODUCTION: The anti-mutagenic, anti-diabetic, anti-carcinogenic, and anti-oxidative potentiality of different plants has been reported by many authors [1-8]. Different types of bioactive compounds (*i.e.,* phenol, indole, selenium, flavonoid, ellagitinin, ellagic acid, ascorbic acid, *etc.*) are present in fruits and vegetables, can inhibit different types of diseases by blocking metabolic activities through detoxification [8-11].

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The whole plant extract (methanolic) of *Cleome gynandra* (cappridaceae), commonly known as 'Hurhur and 'Karaila' in India, is used for the treatment of tumors and antiinflammatory actions [12].

Crude methanolic extract from the pericarp of *Garcinia mangostana* (family: Guttiferae) has shown antiproliferative, apoptotic, and antioxidative properties against some human breast cancer cell lines [13]. Ginger- *Zingiber officinale* (Zingiberaceae) extract has a preventive property against different neoplastic diseases because of the potent activity of polyphenolic and flavonoid compounds [14]. Moreover, the mutagenic potentiality of plant extract has also been reported [15]. Pomegranate, one of the important and oldest

edible fruits of tropical regions, has been used for its medicinal purposes. It is reported that PG extract exhibits antiviral, antioxidant, anticancer activities [16-18]. 2-Methoxyestradiol (2-ME), the end metabolite of 17 β- estradiol (E2), shows significant antiangiogenic property that inhibits tumor growth [19].

Given the importance of extract of PG, our studies have been extended to analyze the anti-mutagenic or anti-clastogenic (*i. e.,* non-toxic) effect of PG and 2-ME in combination on *in-vivo* normal Swiss albino male mice considering mouse survivability, bone marrow, and germ cell toxicity with hematological parameters. The evaluation of micro-nucleated RBC from peripheral blood of control and treated mice have also been performed. Additionally, the present study has also been extended to evaluate the comparative antioxidant potential and free radical scavenging activity of both aqueous and ethanolic pomegranate extracts.

The combination effect of aqueous PG (PGA) and 2-ME on a normal male murine model system will provide valuable information on whether the combined effect of the specific dose is less toxic than the single treatment. Preliminary studies have been done on Sarcoma-180 tumor bearing mice using both aqueous and ethanolic PG extracts (PGEs). But, an aqueous extract of PG had shown remarkable antineoplastic and antiproliferative properties than the ethanolic extract of PG [20]. So, the present study aims to investigate the mutagenic and clastogenic properties of the combined effect of aqueous PG and 2-ME on a normal male mouse model system.

MATERIALS AND METHODS:

Preparation of Pomegranate Extract: Pomegranate extract (both aqueous and ethanolic) was prepared with certain modifications of the technique originally described by Chakrabarti *et al.* 1985 [15] and Banerjee *et al.* 2017 [20]. PG fruit was procured from Kolkata Municipality Corporation affiliated market and authenticated by the Botanical Survey of India (BSI), Howrah, and West Bengal. 100 gm of smashed PG seeds were separately allowed to stand cool with 150 ml of distilled water and 150 ml 80% ethanol respectively overnight for aqueous and ethanolic extract preparation, respectively. Then Soxhlet extraction was done for two days. The extracts were poured into a Petri dish and allowed to dry in an incubator at 50ºC until they become sticky dry mass [17].

Antioxidant Potential and Free Radical Scavenging Activity: The antioxidant and free radical scavenging activity of both aqueous and ethanolic PG extracts were estimated on the basis of the following parameters:

a) **Estimation of Total Phenol:** The total phenol of the extracts was measured at 765 nm by Folin Ciocalteu reagent with Gallic acid standard [21].

b) **Estimation of Total Flavonoid:** Estimation of total flavonoid was done by Aluminum chloride colorimetric assay of S. Kumar *et al.,* 2008 [22].

Ferric Reducing Antioxidant Power Assay (FRAP): Ferric reducing power of the extracts was standardized and measured according to the original method of M. Oyaizu, 1986 [23].

c) **2, 2 - Diphenyl - 1 - Picrylhydrazyl (DPPH) Scavenging Assay:** DPPH radical scavenging activity was performed according to the method of Sreejayan and Rao, 1996 [24]. Ascorbic acid was standard.

Preparation of 2-ME Solution: 2-ME solution was prepared according to the method already in practice in laboratory [25]. 5 mg of 2- ME was dissolved in 5 ml absolute alcohol. Then the solution was diluted with normal saline in 1:1 (v/v) ratio. A parallel positive control was made by mixing absolute alcohol and normal saline in 1:1 (v/v) ratio.

Standardization of Dose and Acute Toxicity Study: Three different concentrations of aqueous PG extract (*i.e.* 100 mg/kg, 200 mg/kg and 400 mg/kg of body weight) and suitable doses of 2-ME (*i.e.* 1.5 mg/kg, 2.5 mg/kg, 5 mg/kg of body weight) were prepared.

The acute toxicity study for the aqueous extracts of the fruit and the drug was also performed using experimental mice. Both aqueous extracts of PG and 2-ME were administered intraperitoneally with increasing doses, to determine the safety limit following OECD guidelines.

Pharmacologic Analysis of Antagonism and Synergism: The interaction between components of PG and 2-ME was analyzed quantitatively with the aid of the Chou Talalay method- a novel multiple drugs effect analysis [25-27]. This method is the most important novel method that establishes the expected effect of a specific drug combination.

Experimental design: Experimental animals were divided into eighteen groups containing 6 mice in each group, as shown in **Table 1.** The mice were given different doses of PG (100mg/kg, 200mg/kg, 400mg/kg body weight) and 2-ME (1.5 mg/kg, 2.5 mg/kg, 5mg/kg body weight) in alternating days in single and combination treatment.

TABLE 1: TREATMENT SCHEDULE, TREATMENT WAS STARTED ON 7th DAY AND WAS CONTINUED FOR 5 CONSECUTIVE DAYS ('-' INDICATES NO TREATMENT) AND STUDY GROUP OF TREATMENT SCHEDULE WITH DOSE

Group no.	Study group and Dose	Day 1	Day 2	Day 3	Day 4	Day 5
1	Negative control	-	-	-	-	-
2	Vehicle for PG (sterile water injected as PG is dissolved in it) 1ml/100 gm. body weight	1 ml	-	1 ml	-	1 ml
3	PG aqueous (single therapy 100mg/kg body weight)	100 mg	-	100 mg	-	100 mg
4	PG aqueous (single therapy) 200mg/kg body weight	200 mg	-	200 mg	-	200 mg
5	PG aqueous (single therapy) 400mg/kg body weight	400 mg	-	400 mg	-	400 mg
6	Vehicle for 2-ME (Sodium chloride and absolute alcohol injected as ME is dissolved in it) 1ml/100 gm. body weight	-	1 ml	-	1 ml	-
7	2-ME (single therapy) 1.5mg/kg body weight	-	1.5 mg	-	1.5 mg	-
8	2-ME (single therapy) 2.5mg/kg body weight	-	2.5 mg	-	2.5 mg	-
9	2-ME (single therapy) 5mg/kg body weight	-	5mg	-	5 mg	-
10	PG aqueous + 2-ME (combination therapy)100mg PG + 1.5 mg ME/kg body weight	100 mg	1.5 mg	100 mg	1.5 mg	100 mg
11	PG aqueous + 2-ME (combination therapy) 200 mg PG + 1.5 mgME/kg body weight	200 mg	1.5 mg	200 mg	1.5 mg	200 mg
12	PG aqueous + 2-ME (combination therapy) 400 mg PG + 1.5 mg ME/kg body weight	400 mg	1.5 mg	400 mg	1.5 mg	400 mg
13	PG aqueous + 2-ME (combination therapy) 100 mg PG + 2.5mgME/kg body weight	100 mg	2.5 mg	100 mg	2.5 mg	100 mg
14	PG aqueous + 2-ME (combination therapy) 200 mg PG + 2.5 mgME/kg body weight	200 mg	2.5 mg	200 mg	2.5 mg	200 mg
15	PG aqueous + 2-ME (combination therapy) 400 mg PG + 2.5mg ME/kg body weight	400 mg	2.5 mg	400 mg	2.5 mg	400 mg
16	PG aqueous + 2-ME (combination therapy) 100 mg PG + 5 mgME/kg body weight	100 mg	5 mg	100 mg	5 mg	100 mg
17	PG aqueous + 2-ME (combination therapy) 200 mg PG + 5 mgME/kg body weight	200 mg	5 mg	200 mg	5 mg	200 mg
18	PG aqueous + 2-ME (combination therapy) 400 mg PG + 5 mgME/kg body weight	400 mg	5 mg	400 mg	5 mg	400 mg

Test Animals: Swiss albino male mice (*Mus musculus*) of 9-10 weeks old and weighing between 18 - 20 gm. were used for the present study. The animals were kept in sterilized, polyvinyl cages at temperature (23°C - 25°C) controlled and well-ventilated rooms of institutional animal house.

A 12:12 h light: dark cycle was also maintained. Mice were provided with standard food pellet and purified water *ad libitum.*

Before experimental work, animals were kept for 10 days under normal healthy laboratory conditions for acclimatization. All experiments were performed in accordance with the guidelines formulated by the Institutional Animal Ethics Committee (Reg No with date: 1795/PO/ERe/S/14 CPCSEA- 31/12/2014) for the care and use of laboratory animals.

Survival Test: The life span of mice in control, vehicle, and treated groups was studied according to the specific protocol [27] and quantitatively analyzed by Chou-Talalay method [26, 27].

Chromosomal Aberration Analysis: Bone marrow toxicity was measured by chromosomal aberration analysis [15]. Chromosome preparation from bone marrow cells of control and treated series was done according to the method as practiced in laboratory [28]. Intraperitoneal injection of Colchicine (0.04%) was given to the experimental animals 1 h 30 min prior to sacrifice. Mice were dissected after cervical dislocation. Then femurs were excised, and bone marrow was aspirated from femur by flashing with hypotonic solution (0.075 M KCl) in the centrifuge tube. The cell suspension was kept for 25 min at 37°C and

centrifuged for 10 min at 1500 rpm to collect the sediment. Then the cell sediment was fixed in aceto-alcohol fixative (3:1 methanol: glacial acetic acid, V/V) and centrifuged for 8 min at 1500 rpm. Then the supernatant was discarded, and the cell sediment was fixed in aceto-alcohol fixative for chromosome slide preparation. The slides were prepared by flame drying technique, and dried slides were stained with 5% Giemsa diluted in phosphate buffer (pH- 6.8). The well-spread metaphases were studied under a binocular research microscope (10×100 magnifications). 300 metaphase plates were studied from three specimens (3 replications) in each treatment.

Hematological Parameter:

Estimation of Haemoglobin: Peripheral blood was collected for estimation of hemoglobin as practiced in the laboratory [29, 30].

Estimation of Total RBC and WBC Count: Total RBC and WBC count were done as practiced in the laboratory [29, 30].

Differential Count of WBC: Total number of neutrophil and lymphocyte were counted according to the method of Sood [30]. Leishman stained smeared slides were observed under a binocular research microscope to study the number of neutrophil and lymphocytes.

Micronuclei Study: Leishman stained micro nucleated RBC from peripheral blood of both control and treated series were studied to find the clastogenic or mutagenic property of the single and combination treatment [31]. Total 3000 RBC studied from three specimens (3 replications) in each treatment.

Germ Cell Toxicity Study:

Sperm Head Abnormality Test: Sperm head morphology was studied by using the Haematoxylin-Eosin double staining method. Sperms were collected from the caudal part of the epididymis and kept in 1 ml 0.9% normal saline for 30 min. The fluid containing sperms was smeared, dried, and stained in the hematoxylin-eosin double stain. The stained slides were studied under binocular research microscope in 10×100 magnifications. Different types of sperm heads such as normal head, big head, amorphous head sperm, *etc*., were observed. Total 3000 sperm heads studied from three specimens (3 replications) in each treatment [32, 33].

Sperm Motility Test: Sperm motility test was done after the slight modification of the original technique of Vega *et al.*, 1988 [33, 34]. Sperms were collected from the epididymis of control and treated mice and kept in 0.2 ml of normal saline (0.9% NaCl) for 15 min at room temperature. Then 0.02 ml diluted sperm containing fluid was transferred to a Neubauer chamber of Haemocytometer for counting. The number of motile and immotile spermatozoa was observed under a binocular research microscope at 10×40 magnifications and studied from 64 chambers (3 replications) of the WBC counting chamber.

Sperm Viability Test: Viable and non-viable spermatozoa were studied by Nigrosin-Eosin staining method after the slight modification of Vega *et al.,* 1988 and by following the WHO protocol [33-35].

The no. of viable and non-viable spermatozoa were observed under a binocular research microscope at 10×40 magnifications and studied from 64 chambers (3 replications) of the WBC counting chamber.

Statistical Analysis: All data of both control and treated series were analyzed by Student's t-test [36] and Chou Talalay method [26, 27].

RESULTS:

Antioxidant Potential and Free Radical Scavenging Activity:

Total Phenol and Flavonoid Content: It is well known that pomegranate has a high antioxidant property. The quantitative determination of total phenol content is expressed as mg Gallic acid equivalence/gm dry weight of PG.

The total phenol content in aqueous extract of PG was 217.71 ± 3.75 mg w/w, and that of ethanolic was 220.54 ± 3.2 mg w/w. The quantitative determination of total flavonoid content is expressed as mg Rutin equivalence/gm. dry weight of PG. The total flavonoid content of the aqueous extract was 381.276 ± 1.12μg w/w and ethanolic extract was 301.305 ± 3.33 μg w/w. The total phenol and flavonoid content in aqueous extract and ethanolic extract of PG are shown in **Fig 1.**

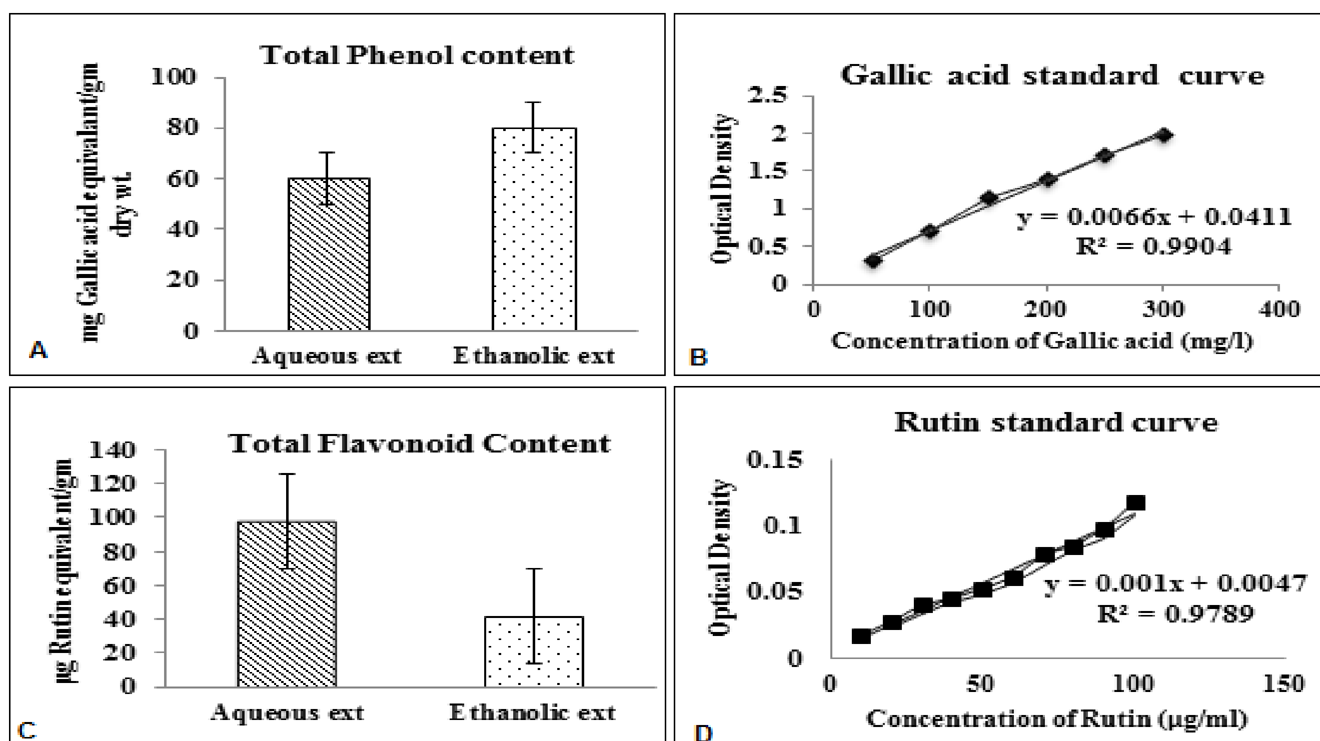


FIG. 1: TOTAL PHENOL AND TOTAL FLAVONOID CONTENT OF PG EXTRACTS. A. HISTOGRAM OF TOTAL PHENOL CONTENT OF AQUEOUS AND ETHANOLIC PG EXTRACT. B. GRAPHICAL REPRESENTATION OF TOTAL PHENOL CONTENT WITH GALLIC ACID STANDARD. C. HISTOGRAM OF TOTAL FLAVONOID CONTENT OF AQUEOUS AND ETHANOLIC PG EXTRACT. D. GRAPHICAL REPRESENTATION OF TOTAL FLAVONOID CONTENT WITH RUTIN STANDARD. VALUES ARE EXPRESSED AS MEAN ± SE (n=3).

Ferric Reducing Antioxidant Power Assay (FRAP): The reducing power is a measure of the transformation of Fe^{3+} to Fe^{2+} in the presence of sample extract. The reducing nature of a compound may serve as a significant marker of its potential antioxidant activity.

The reducing power of synthetic antioxidant BHT, ethanolic and aqueous extracts of PG is depicted in **Fig. 2**. It is interesting to note that the antioxidant activity of FRAP in aqueous PG extracts is high in comparison to ethanolic extract.

2, 2 - Diphenyl - 1 - picrylhydrazyl (DPPH) Scavenging Assay: In the DPPH scavenging assay, both the ethanolic and aqueous extracts of PG exhibits prominent DPPH free radical scavenging activity in a concentration-dependent manner. Lower absorbance of the reaction mixture shows higher free radical scavenging activity. The percentage inhibition decreases the concentration of DPPH radical due to the scavenging ability of extract and standard ascorbic acid as a reference compound. The aqueous extract exhibits high scavenging activity at all concentrations when compared to the ethanolic extract.

The IC_{50} value for aqueous extract of PG is found to be 986.467 µg/ml indicates high scavenging power, while the ethanolic extract shows 1055.76 µg/ml with low scavenging activity. The IC_{50} value for standard Ascorbic acid is 81.025 µg/ml (**Fig. 2**, **Table 2A, 2B**).

TABLE 2A: PERCENTAGE OF INHIBITION OF DIFFERENT CONCENTRATIONS OF PGA AND PGE

Concentration (µg/ml)	Inhibition of PGA (%)	Inhibition of PGE (%)
1000	82.4	75.12
800	77.52	71.55
600	75.42	69.2
400	71.1	63.45
200	67.22	59.12

TABLE 2B: PERCENTAGE OF INHIBITION OF PGA AND PGE SHOWING EQUATION GENERATED BY GRAPH, R^2 VALUE AND IC50 VALUE. VALUES ARE EXPRESSED AS MEAN ± SE (n=3)

Samples	Equation	R^2 Value	IC_{50} Value (µg/ml)
Standard ascorbic acid	0.0242x+60.784	0.9538	81.025
PGA	0.0184x+63.698	0.9898	986.467
PGE	0.0201x+55.658	0.9816	1055.76

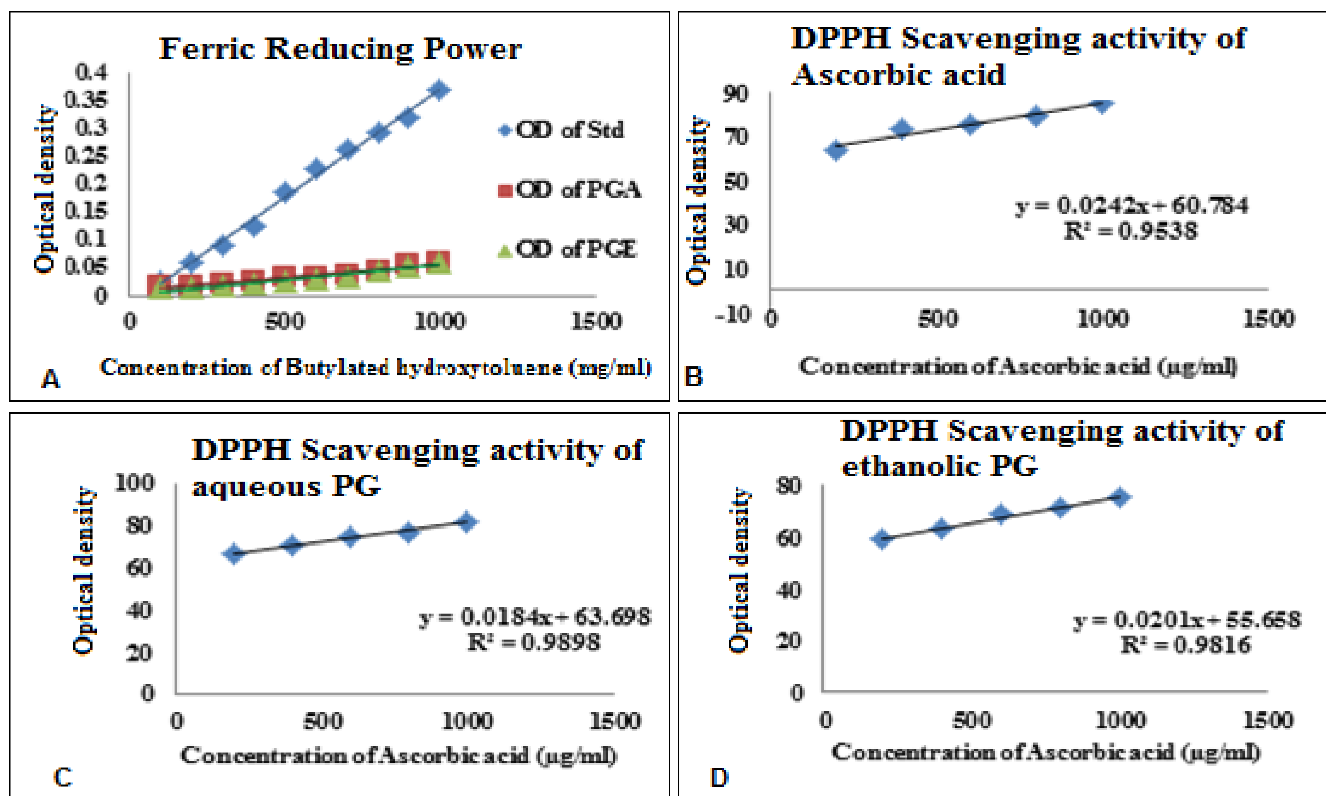


FIG. 2: A. REDUCING POWER OF IRON (Fe^{3+} TO Fe^{2+}) OF AQUEOUS, ETHANOLIC EXTRACTS OF PG AND STANDARD WITH BHT (BUTYLATED HYDROXYTOLUENE). VALUES ARE EXPRESSED AS MEAN ± SE (N=3). B. DPPH SCAVENGING ACTIVITY BY ASCORBIC ACID. C, D. DPPH SCAVENGING ACTIVITY BY AQUEOUS AND ETHANOLIC EXTRACTS OF PG RESPECTIVELY. VALUES ARE EXPRESSED AS MEAN ± SE (n=3)

Mouse Survivability: The survival rate was higher in A400 mg PG + 1.5 mg 2-ME treated group that are similar to a negative control (normal mouse) and vehicle group. But in case of 2.5 mg ME, 5 mg ME single therapy and A400 mg PG + 2.5 mg ME, A200 mg PG + 5 mg ME, A400 mg + 5 mg ME combined treated groups, the survivability rate was significantly (**p>0.001) decreased. The mortality rate is very high in the case of A100 mg PG + 5 mg 2ME, A200 mg PG + 5 mg 2ME, and A400 mg PG + 5 mg 2ME combined treated groups than the negative control (normal) and other treated groups. So, these data are excluded from our experiments of combination index (**Table 3**, **Fig 3**).

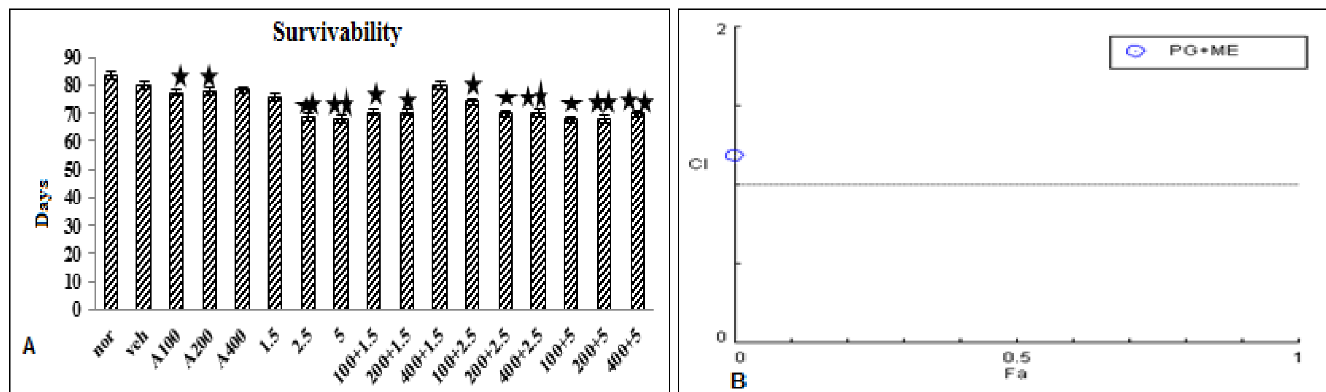


FIG. 3: EFFECT OF DIFFERENT COMBINATIONS OF PG + 2-ME ON SURVIVABILITY OF NORMAL MICE. A. GRAPHICAL REPRESENTATION OF PERCENTAGE INCREASE IN LIFE SPAN (%ILS) OF NEGATIVE CONTROL (NORMAL), VEHICLE AND DIFFERENT TREATED MOUSE. THE %ILS OF A400 + 1.5 ME COMBINATION TREATMENT IS LONGER THAN VEHICLE AND OTHER TREATED GROUPS. VALUES ARE EXPRESSED AS MEAN ± SE; N = 3 (*P > 0.05, **P > 0.001). B. THE COMBINATION INDEX VALUE WAS DETERMINED BY CHOU-TALALAY METHOD. GRAPH SHOWS FA –CI PLOT OF SURVIVABILITY OF DIFFERENT COMBINATIONS OF PG + 2-ME DOSES WHERE ALL VALUES ARE GREATER THAN 1 INDICATING ANTAGONISM, FA = FRACTION AFFECTED

TABLE 3: COMBINATION INDEX (CI) VALUE OF THE COMBINATION THERAPY (PG+2ME) AT DIFFERENT COMBINATIONS. VALUES ARE EXPRESSED AS MEAN ± SE (N=3)

PG+ME doses	CI index			
	survivability	Non affected chromosome	Hemoglobin	RBC
100+1.5	385.071	1.489	1.625	1.732
200+1.5	571.328	0.934	2.063	1.452
400+1.5	1.184	0.122	30966.8	8.584
100+2.5	29.361	0.559	8.231	1.590
200+2.5	2.811	4.180	2.380	1.610
400+2.5	1780.04	17.254	1.982	4.606

CI > 1.3 Indicates Antagonism, CI = 1.1 To 1.3 Moderate Antagonism, CI = 0.9 To 1.1 Additive Effect, CI = 0.8 To 0.9 Slight Synergism, CI = 0.6 To 0.8 Moderate Synergism, CI = 0.4 To 0.6 Synergism, and CI = 0.2 To 0.4 Strong Synergism. The Mortality Rate Is Very High In Case of 100, 200 And 400 Mg Pg + 5mg 2- Me Groups So, These Are Excluded From Our Combination Index

Chromosomal Aberration Analysis: Bone marrow chromosomes were studied from a well-spread metaphase plate. Different types of chromosomal aberrations (*i.e.* chromatid break, deletion, centric fusion, iso-chromatid break, *etc.*) were noticed. Percentage of different concentrations of 2-ME treated affected metaphases were significantly (*p>0.05) higher in comparison to a normal, vehicle, and other treated series **Fig. 4**.

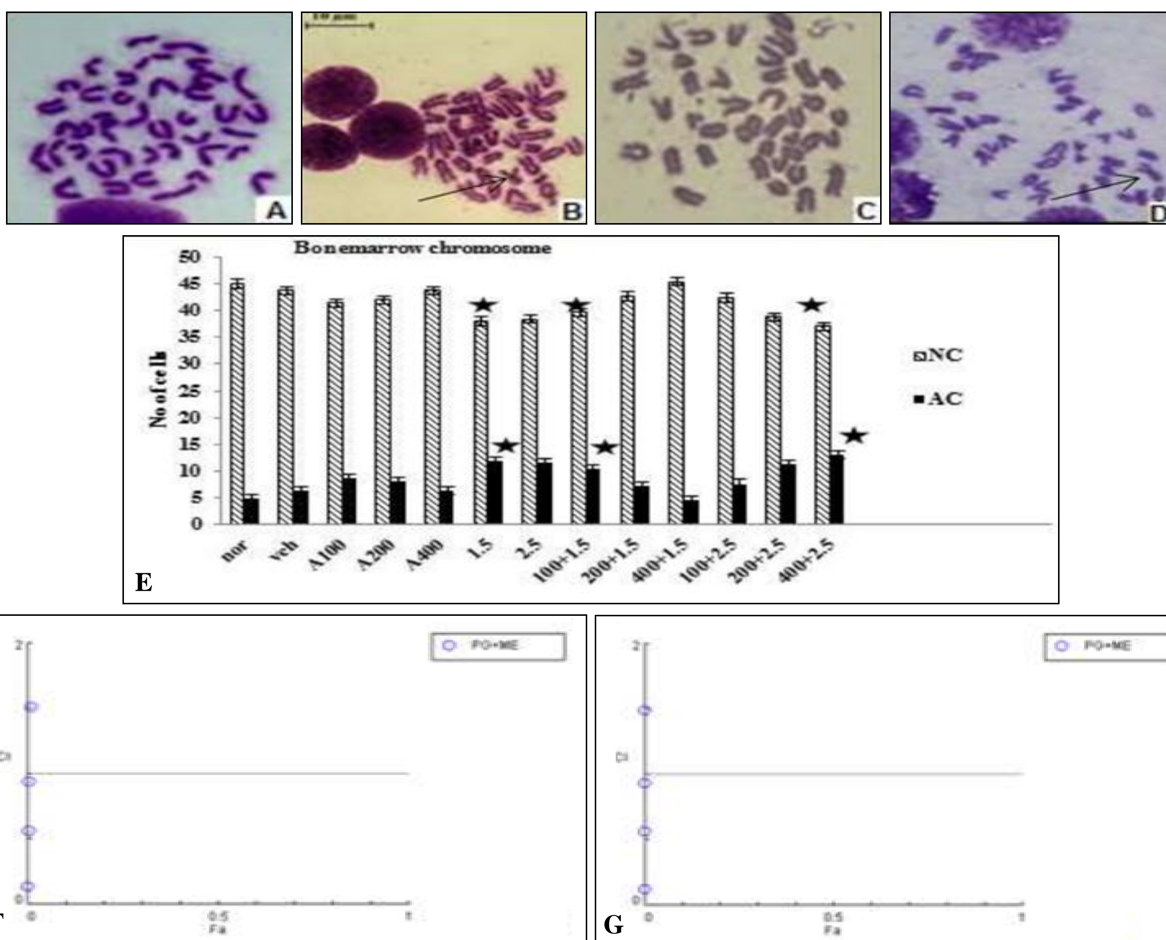


FIG 4: EFFECT OF DIFFERENT COMBINATIONS OF PG + 2-ME ON BONE MARROW CHROMOSOME OF NORMAL MICE. A. METAPHASE WITH NORMAL CHROMOSOME IN NORMAL MOUSE, B. AFFECTED METAPHASE WITH CENTRIC FUSION (METACENTRIC CHROMOSOME) IN 1.5 ME SINGLE THERAPY CONDITION (ARROWED), C. METAPHASE WITH NORMAL CHROMOSOME IN A400 + 1.5 ME COMBINATION TREATED CONDITION, D. METAPHASE WITH CENTRIC FUSION (ARROWED) IN A400 + 2.5 ME COMBINATION TREATED CONDITION, E. GRAPHICAL REPRESENTATION OF AFFECTED CELL (AC) AND NONAFFECTED CELL (NC) OF NEGATIVE CONTROL (NORMAL), VEHICLE AND DIFFERENT TREATED MOUSE. THE AC OF A400 + 1.5 ME COMBINATION TREATMENT IS MINIMUM THAN VEHICLE AND OTHER TREATED GROUPS. VALUES ARE EXPRESSED AS MEAN ± SE; N = 3 (*P > 0.05). F, G. THE COMBINATION INDEX VALUE WAS DETERMINED BY CHOU-TALALAY METHOD. GRAPH SHOWS FA –CI PLOT OF AC AND NC OF DIFFERENT COMBINATIONS OF PG + 2-ME DOSES WHERE ALL VALUES ARE GREATER THAN 1 INDICATING ANTAGONISM, FA = FRACTION AFFECTED

Hematological Parameters:

Estimation of Haemoglobin: Haemoglobin content significantly (*p>0.05) decreased in 2.5 mg ME, 100 mg and 200 mg PGE single and in 200 mg PGE+ 2.5 mg ME, 100 mg PGE+2.5 mg ME combination treatment groups in comparison to the normal, vehicle, and other treated groups as shown in **Table 3.**

Estimation of Total RBC Count: Total RBC count was normal and restored in 400 mg PGE + 1.5 mg ME combination treatment group as shown in **Table 3, Fig 5A**.

Estimation of Total WBC Count: Total WBC count decreased in 1.5 mg ME single therapy, and 100 mg PGE + 1.5 mg ME but increased in Vehicle, 100 mg PGE, ME Vehicle, 5 mg ME and 400 mg PGE + 2.5 mg ME combination therapy treated series, but in other treatments particularly in 400 mg PGE + 1.5 mg ME it was restored **Fig. 5A.** Interestingly, the percentage of hemoglobin along with the total count of RBC increased or restored, and the total count of WBC was restored in the particular concentration of the combined treatment (*i.e.*, 400 mg PGE + 1.5 mg ME /kg body weight).

Differential Count of WBC: Total number of neutrophil and lymphocyte restored in both single and combination treatment groups. No significant reduction in lymphocyte count was noticed. Interestingly, the lymphocyte population slightly increased, and neutrophil population slightly decreased in A 400 mg PG + 1.5 mg 2ME combination treatment group in comparison to normal and treatment groups **Fig. 5B.**

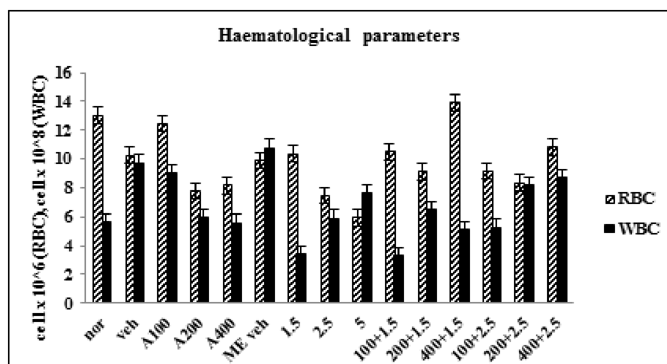


FIG. 5A: HISTOGRAPHICAL REPRESENTATION OF TOTAL RBC AND WBC COUNT. VALUES ARE EXPRESSED AS MEAN ± SE (n=3)

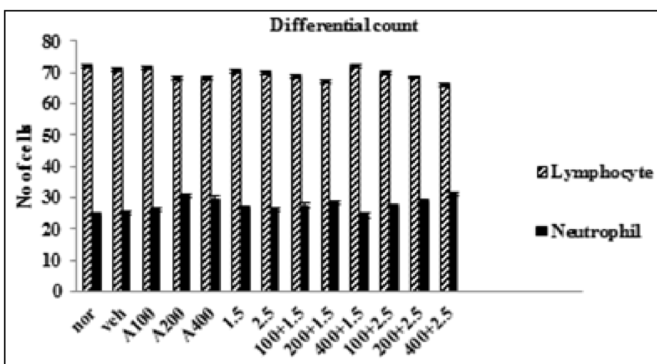
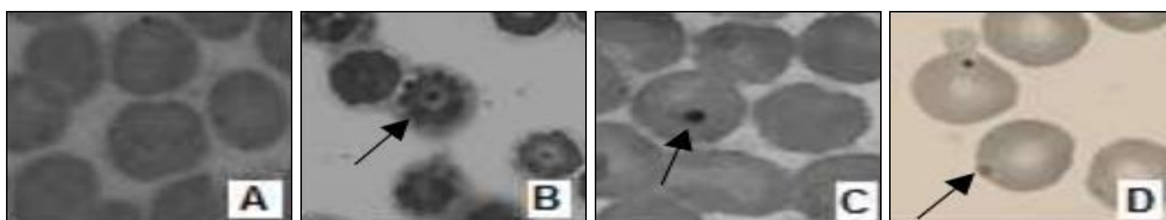


FIG. 5B: GRAPHICAL REPRESENTATION OF DIFFERENTIAL COUNTS OF WBC. VALUES ARE EXPRESSED AS MEAN ± SE (n = 3)



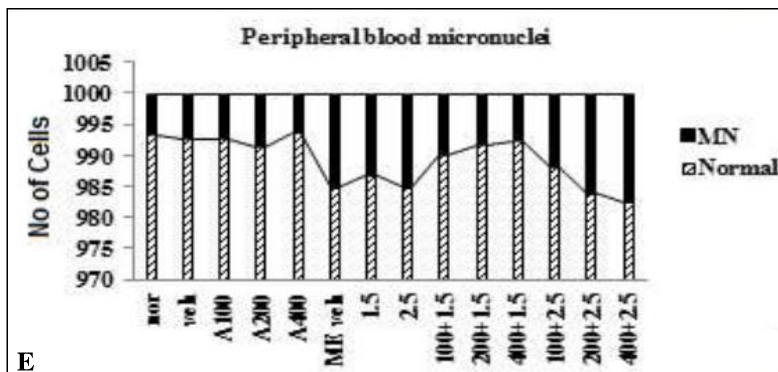


FIG. 6: MICRONUCLEI FROM PERIPHERAL BLOOD. A. NORMAL RBC, B, C, D. MICRONUCLEATED RBC (ARROWED), E. GRAPHICAL REPRESENTATION OF MICRONUCLEATED BLOODS CELLS. VALUES ARE EXPRESSED AS MEAN ± SE (n =3)

Micronuclei Study from Peripheral Blood: Micronuclei (MN) are extra-nuclear bodies that contain damaged chromosomal fragments which are not incorporated into daughter nuclei during cell division [31].

These are also described as remnants of the nucleus of red blood cells found in organs during pathological conditions [37, 38]. Therefore, the micro-nucleus assay is used for genotoxicity screening of drugs and chemicals.

The frequency of micronuclei in the RBC was lower in A400 + 1.5 ME treated series while it was higher in 1.5 mg, 2.5 mg 2-ME, A 100 + 1.5 mg ME, A 200 + 1.5 mg ME, A 100 + 2.5 mg ME, A 200 + 2.5 mg ME, A 400 + 2.5 mg ME/kg body wt. treated series **Fig. 6**.

Germ cell Toxicity Study:

Sperm Head Abnormality Test: Different types of sperm head abnormalities such as amorphous head, big heads, pin head, *etc*., were noticed after the treatment of different concentrations of PG and ME as single therapy and PG+ME as a combination treatment. The frequency of sperm head abnormality in both HE and TB staining methods in normal and treated series, particularly in A 400 mg and 1.5 mg ME /kg body weight group, is almost similar. Interestingly, a steady increase of sperm abnormalities was noted after the administration of higher concentration of ME (2.5 and 5 mg). The frequency of sperm head abnormalities is the maximum in case of 5mg ME/kg body wt. dose because the mortality rate is very high (*i.e.,* lethal) in the case of 5 mg 2 ME/kg body wt. dose **Fig .7**.

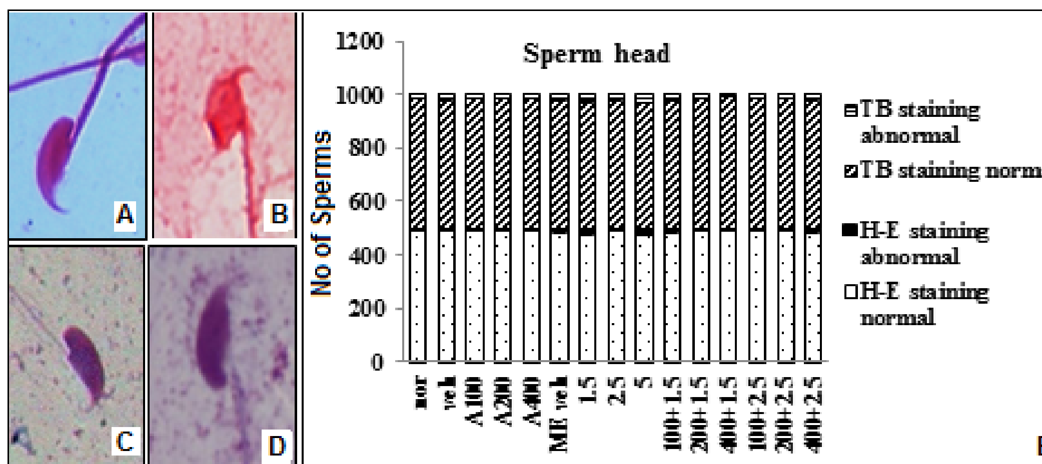


FIG. 7: SPERM HEAD ABNORMALITY. A: NORMAL SPERM OF H-E STAINING, B: AMORPHOUS HEAD SPERM OF H-E STAINING, C: NORMAL HEAD SPERM OF TB STAINING, D: ABNORMAL HEAD SPERM OF TB STAINING, E: GRAPHICAL REPRESENTATION OF SPERM HEADS ABNORMALITIES IN H-E AND TB STAINING. VALUES ARE EXPRESSED AS MEAN ± SE (n = 3)

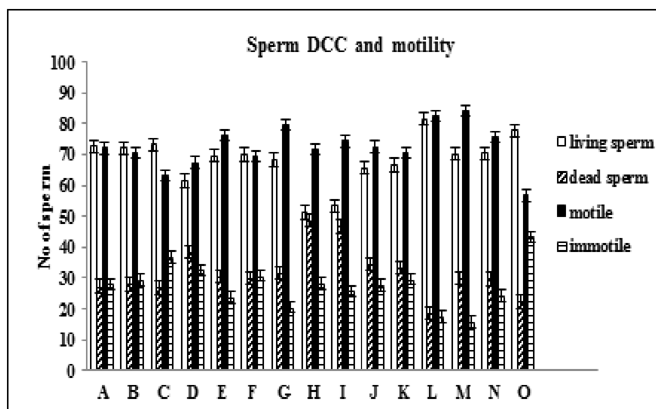


FIG. 8: GRAPHICAL REPRESENTATION OF DEAD AND LIVING SPERMATOZOA AND MOTILE AND IMMOTILE SPERMATOZOA.A: NORMAL, B: VEHICLE, C: A100, D: A200, E: A400, F: ME VEHICLE, G: 1.5 ME, H: 2.5 ME, I: 5 ME, J: 100+1.5, K: 200+1.5, L: 400+1.5, M: 100+2.5, N: 200+2.5, O: 400+2.5. VALUES ARE EXPRESSED AS MEAN ± SE (n=3).

Sperm Motility Test and Viability Test: The number of motile and viable spermatozoa was slightly decreased in 400 mg PG, 1.5 mg, 2.5 mg 2-ME/kg body wt. single therapy and 100 mg PG + 2.5 mg 2-ME and 200 mg PG + 2.5 mg 2-ME/kg body wt. treatments in comparison to normal, vehicle and A 400 mg PG + 1.5 mg ME / kg body weight treated series **Fig. 8**. The viability and motility of sperms in A 400 mg PG + 1.5 mg ME treated group are restored properly.

On the basis of the above observations in control and all treated series (*i.e.* single and combination treatments), A 400 mg PG + 1.5 mg 2-ME/kg body wt. combination dose shows less toxic and safe for treatment.

DISCUSSION: Toxicological studies have undergone a significant advancement during the last four decades, particularly in the field of mutagenicity as well as carcinogenicity bioassay system. Mutations in somatic cells induce the pathogenesis of different types of degenerative diseases, *i.e.*, cancer, heart diseases, *etc*. Usage of plant products such as leaf, bark, fruits, roots, etc. is a very common practice since ancient time in the field of medicine [9, 39-47].

Moreover, the usage of two or more extracts or drugs together is often more effective in the treatment of different diseases than single therapy or monotherapy. In addition, the application of plant extract or drug at a low dose than the maximum tolerable dose (MTD) can limit the toxic side effects [29, 48- 50]. So, in the present paper, we have evaluated the antimutagenic or anticlastogenic effect of PG in combination with a low concentration of 2-ME on *in-vivo* normal Swiss albino male mice. We have evaluated the antioxidant potential and free radicle scavenging activity of both aqueous and ethanolic extracts of PG. Ethanolic extract showed more amounts of phenolic compounds, which is a good sign of high antioxidative potentiality [51, 52]. The aqueous extract has high flavonoid content, which dominates the phenolic activity of the ethanolic extract.

The aqueous extract has high ferric reducing power with more scavenging activity in DPPH assay, which promotes the extract to give a protective property. In our earlier study [17, 20] the mice were exposed to the ethanolic extract of PG, which showed cellular toxicity with respect to hematological and cytological abnormalities and short life span of treated mice in comparison to aqueous extract. Any drug should elongate the life span of the host and minimize the side effects. Many such drugs are there that reduce the tumor growth but decrease hemoglobin level associated with hematological problems.

The safer compound antineoplastic, as well as anti-proliferative activities, will be of immense clinical importance. Our present study indicated that the combination treatment of A400mg PG/kg body weight + 1.5 mg 2-ME/kg body wt. dose is quite safer and applicable for the safety of the mice. It was observed that the particular dose significantly played a safety role in the normal mouse model system. The survivability of the A400+1.5 mg 2-ME treatment was similar to the normal group, whereas the monotherapy of 2-ME significantly reduced the life span. 5mg 2-ME/kg body dose was found to be lethal as the mortality was high. The life span in the particular treatment (*i.e.*, A400 + 1.5 mg 2- ME) was enhanced synergistically as on the basis of the analysis by Chou Talalay method. The cytotoxic effect of the combination dose on the normal murine system has been studied by chronological analysis of simple and complex chromosomal aberrations. The bone marrow chromosomes of A100 mg PG and 1.5 mg 2-ME and some combination treatments got significantly abnormal. Whereas A400 + 1.5 mg 2- ME dose showed a smaller number of abnormal metaphases just like normal group, which was synergistic. The Hb% along with the total RBC count was restored like normal mice in the particular condition.

The differential count was nothing to be significantly altered. Lymphocyte count was as similar as normal and vehicle groups, which indicated no alteration of immune system of the host with consistence of WBC population. The anti-mutagenic or anti clastogenic effect of the combination dose on the normal murine system has also been studied by analysis of the frequency of micronuclei in the RBC. The most efficient anti-mutagenic effect of combination dose was observed at A 400 mg + 1.5 mg 2- ME.

Comparative analysis of sperm head abnormalities, sperm viability and motility in normal, vehicle and treated series revealed that the sperm head abnormality decreased while the viable as well as motile spermatozoa increased in A400 mg +1.5 mg ME treated series. So, anti-mutagenic and protective role of A 400 mg + 1.5 mg ME in male reproductive system of mouse has been noticed in the present study.

CONCLUSION: On the basis of the above observations in control and all treated series (i.e. single and combination treatments), A 400 mg PG + 1.5 mg 2-ME/kg body wt. combination dose shows less toxic, safe, and effective for treatment. Total phenol, flavonoid, FRAP, DPPH are the major important components of PG that possess antioxidant and free radical scavenging activity.

Interestingly, among the ethanolic and aqueous extracts of PG, the aqueous extract possesses more flavonoid, FRAP, DPPH content than the ethanolic extract.

But the phenol content is higher in ethanolic extract than in aqueous extract of PG. In our previous study[20]we have attempted to evaluate the comparative antioxidant potential of ethanolic and aqueous PG extracts in respect to total phenol content.It was found that ethanolic extract of PG depicted high phenolic content in comparison to aqueous PG extract, which played an important role in the induction of extensive chromosomal damage, cellular toxicity, and low survivability of the mouse. The higher amount of flavonoid, FRAP, DPPH, and a lower amount of phenols in aqueous extract of PG is acting as a major potent antioxidant and has less mutagenic as well as less clastogenic effect. In addition to the effect of PGE, ME showed mutagenic effects on bone marrow chromosome, hematological and germinal cell toxicity in a dose-dependent manner. But the low concentration of ME, *i.e*., 1.5 mg/kg body weight, is an ideal dose in the combination treatment that showed anti-mutagenic effects.

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