Volume-4, Issue-10, Oct-2015 • ISSN No 2277 - 8160

Research Paper Zoology

Protective Efects of Aegle Marma Los on Doxyrubicin Induced Genotoxicity in Germ Cells of Mice

Prabhakar Reddy Ch Department of Zoology, Osmnaia University, Hyderabad.

Rudrama Devi. K Department of Zoology, Osmnaia University, Hyderabad.

K. Pratap Reddy Department of Zoology, Osmnaia University, Hyderabad.

ABSTRACT

Adriamycin (ADR) (doxorubicin) is one of most effective chemotherapeutic agents and is the most commonly used anthracyclin antibiotic effective in treatment of various cancers. Adriamycin induces mutations and chromosomal aberrations in normal and tumor cells. Adriamycin has high affinity for cell nuclei and about 60% of total intracellular adriamycin is found in cell nucleus and binds to DNA polymerase and inhibits synthesis of nucleic acid and protein, results in DNA damage and free radical formation. As Adriamycin is widely used, it is important to reduce its toxicity to normal cells which can be achieved by concurrent administration of antioxidants. Herbs are gaining additional focus because of their less toxicity and high efficacy against a number of ailments. Aegle marmalos fruit extract, has been used for broad spectrum of diseases and its isolated compound Aegle marmalos fruit extract,(AMFE) found to be anticarcinogenic and potential antioxidants. In the present investigation studies were carried out to observe the efficacy of Aegle marmalos extract, against Adriamycin induced cytogenetic damage in germ cellsof mice. The animals treated with 200mg/kg, 400mg/kg and 400mg/kg of Aegle marmalos extract, showed to be non mutagenic. Aegle marmalos extract, shows protective effects against the adriamycin induced genotoxicity in germ cells of mice. Hence Aegle marmalos fruit supplementation is safer in chemotherapeutic strategy.

KEYWORDS : Agele marmalos extract, Adriamycin, chromosomal aberrations germ cells.

INTRODUCTION:

Adriamycin (Doxorubicin) is an anthracycline antibiotic used as an antitumor agent against human malignancies such as leukemia, lymphomas and many solid tumors but which also has a wide variety of toxic side effects, including cardio toxicity, cytotoxicity and the induction of chromosomal aberrations. The majority of antineoplastic drugs, besides their generic growth property, display Genotoxic effects which in turn contribute to growth inhibition (Buschini et al, 2003). These genotoxic effects may lead to initiation of unrelated tumours years after cessation of chemotherapy (Beretta et al, 1991). Free radical mediated reactions are responsible for a wide range of chemotherapy-induced side effects and antioxidants are able to protect non-malignant cells and organs against damage caused by cytostatic agents (Weijil et al, 1997). Most cancers can be controlled by adopting appropriate conventional treatments such as surgery, radiation and chemotherapy. However these treatments cause side effects. Hence the important of conventional therapies may decline. Alternative treatments founded in a back to nature approach might yield improved treatment avenues with fewer or no undesirable side effects. In the search of this new treatment, natural products are carving a path as prospective anticancer agents. Induction of chromosomal aberrations in somatic and germ cells in swiss albino mice has been reported (Rudrama Devi, 2014).

Herbs are gaining additional focus because of their less toxicity and high efficacy against a number of ailments. Epidemiological studies have shown that fruits, vegetables, spices, tea and medicinal herbs rich in antioxidants and other micronutrients protect against diverse forms of chemically induced carcinogenesis, inhibit DNA-damage, mutagenesis and lipid peroxidation (Ziech et al, 2011; Birt et al, 2001). Aegle marmelos, known as bael grows in tropical and subtropical parts of the world. Various parts of the AM are used in Indian system of medicine for treatment of many diseases, including diarrhoea, dysentery and dyspeptic symptoms (Shoba et al, 2001; Sharma et al, 2007). Marmelosin, isolated from the AM, has been reported to have anti-helminthic, anti-bacterial, antioxidant activity and anticarcinogenic (Khan et al, 2009; Patil et al, 2010; Khan and Sultana, 2011). Hence in the present investigation a study was undertaken to observe the efficacy of AMF extract against drug induced micronuclei in bone marrow erythrocytes of mice.

MATERIALS AND METHODS MATERIALS AND METHODS

Chemicals

Doxyrubicin kindly provided by Director, MNJ Institute of oncology and Mytomycin from biochem pharma limited. The chemicals used in the study are purchased from Ranboxy Laboratories, Hyderabad, A.P.

Animals

Six to eight weeks old male mice (*Mus Musculus*) of swiss albino mice weighing about 25-27 gms procured from National Institute of Nutrition, Hyderabad, were used in this study. The mice were housed in poly propylene cages in a well ventilated room and were provided with standard pellet diet(M/S Lipton India limited) and water adlabitum.

Plant material

The plant material was procured from wholesale spice and herbs market Hyderabad. Professor Pratiba Devi, Medicinal Plant Division, Department of Environmental Botany, Osmania University, Hyderabad, verified the identity of plant material. The plant material was chopped and coarsely powdered to a mesh size of 1 mm as described by Antonio and Brito[12].

Preparation of extract

Powdered plant material was repeatedly extracted in 4000 mL round bottom flask with 2000 mL methanol. The methanolic extracts were cooled at room temperature, filtered and evaporated to dryness under reduced pressure in a rotatory evaporator (Buchi Rotavapor).

Dosage schedule

Two experiments were conducted. In the first experiment four groups were maintained to study whether the plant extract is toxic or not in bone marrow cells. Hence the group I received control saline where as group II, group III & group IV were orally administered. with doses of 200mg /kg/bw, 400mg/kg and 600mg/kg/wt of AMF extract for seven days. In the secondary experiment Group I -Control,,Group II-200 AMF+16mg/kg DOX, Group III- 400 AMF+16mg/kg DOX, Group IV-600AMF+16mg/kg DOX given interpertanlly 24 hrs prior to the administration of plant extract.

Cytogenetic analysis of chromosomal aberrations in germ cells of mice:

Slightly modified procedure of Evans et al (1964) was used to prepare the slides to evaluate the action of doxyrubicin and aegle marmalos fuirton different stages of spermatogenesis. Both control and treated groups of animals were sacrificed after 28days of exposure to test compounds with a view to cover spermatogenetic cycle of mouse These

sampling time yields valuable data on which stages of spermatogenesis are more sensitive to the test agent and on the persistence and reversibility of the induced damages. In the present study the air drying technique of Evans et al (1964) was employed with slight modifications to study the effect of test compounds on meiotic cells of mice.

Methodology: All the animals were sacrificed by cervical dislocation on 28th day. Animals were dissected out for testis and kept in 0.9% physiological saline. Tunica albugenia, the membrane covering the testes was removed carefully and the tubules were transferred to another Petri dish containing 5ml of 1.2% tri-sodium citrate. The tubules of the testes were teased in hypotonic solution. The cell suspension was collected in clean centrifuge tubes and incubated at 37°C for 45minutes. After incubation the tubes were centrifuged for 10minutes at 1000rpm. The supernatant was discarded and to the pellet 5ml of freshly prepared pre-chilled fixative (3:1 methanol and acetic acid) was added drop wise from the sides of the centrifuge tubes and immediately dispersed the cell suspension by aspirating several times with a Pasteur pipette. The tubes were left undisturbed for 10 minutes at room temperature. This step was repeated 4 to 5 times. In the final change the cells were suspended in 0.5ml of fresh fixative.

Preparation of the Slides: 3 to 4 drops of cell suspension were dropped from a height of 30-35cms on clean grease free, pre chilled slides with a pipette. The slides were stained with 2% giemsa for 8 – 10min Gin inures and later they were rinsed in double distilled water and allowed to dry.

Scoring: The slides were screened and a total of 100 well spread spermatocytes at the diakinesis of metaphase-1 of meioses were observed per animal for the presence of various types of chromosomal aberrations like structural and numerical aberrations in control and treated groups and micro photographed.

Table 1: Frequency of Chromosomal Aberrations recorded in germ cells of mice with various doses of *Aegle marmelos* fruit extract

Treatment	Normal metaphases scores %	Abnormal metaphases scores
Control	485(97.00)	15(3.00)
200 mg/kg AMFE	484(96.80)	16(3.20)
400 mg/kg AMFE	482(96.40)	18(3.60)
600 mg/kg AMFE	480(96.20)	20(4.00)

The values in the parenthesis are percentages

Table 2: Classification of chromosomal aberrations in germ cells of mice treated with *Aegle marmelos* fruit extract

Treatment dose (mg/kg)	Changes in chromosomal number				Structural changes
	Autosomal univalents	Sex chromosomal univalents	Aneuploids	Polyploids	Translocations
Control	5(1.00)	8(1.60)	2(0.40)	0(0.00)	0(0.00)
200 mg/kg AMFE	6(1.20)	8(1.60)	2(0.40)	0(0.00)	0(0.00)
400 mg/kg AMFE	8(1.60)	7(1.40)	2(0.40)	0(0.00)	1(0.20)
600 mg/kg AMFE	9(1.80)	9(1.80)	1(0.20)	0(0.00)	1(0.20)

The values in the parenthesis are percentages

Table3 Frequency of CA's recorded in CP induced genotoxicity primed with *Aegle marmelos* fruit extract

Group	Dose	Normal metaphases	Abnormal metaphases	% Of Inhibition
Group I	**Control**	481(96.20)	19(3.80)	
Group II	**16 mg/kg Dox**	394(78.80)	106(21.20)	
Group III	**200 AMFE + 16mg/kg Dox**	425(85.00)	75(15.00)	29.24
Group IV	**400 AMFE + 16mg/kg Dox**	431(86.20)	69(13.80)	34.90
Group V	**600 AMFE + 16mg/kg Dox**	454(90.80)	46(9.20)	56.60

The values in the parenthesis are percentages *P<0.05

Table4 Classification of chromosomal aberrations in germ cells of mice treated with doxorubicin and primed with *Aegle marmelos* fruit extract .

Treatment dose (mg/kg)	Changes in chromosomal number				Structural changes
	Autosomal univalents	Sex chromosomal univalents	Polyploids	Aneuploids	Translocations
control	**6(1.20)**	9(1.80)	2(0.40)	2(0.40)	0(0.00)
16 mg/kg Dox	**38(7.80)**	28(5.60)	28(5.60)	8(1.60)	6(1.20)
200 AMFE + 16mg/kg Dox	**30(6.00)**	18(3.60)	19(3.80)	6(1.20)	2(0.40)
400 AMFE + 16mg/kg Dox	**26(5.20)**	16(3.20)	16(3.20)	6(1.20)	5(1.00)
600 AMFE + 16mg/kg Dox	**20(4.00)**	10(2.00)	8(0.60)	4(0.80)	4(0.80)

The values in the parenthesis are percentages

RESULTS AND DISCUSSION

Cytogenetic analysis of germ cells is a valuable method for evaluating the morphological evidence of damage of chromosome in a number of species including man .Identification of chromosomal aberration in germ cells of mammals ,of a particular interest in mutagenic studies because the gametes transmit the effects from one generation to another .

The mutagenic effects of various doses of the AMFE were conducted to study the effects on the germ cells of mice and the results were tabulated in Tables- 1-4depicted graphically in Graph.1-3

The frequencies (%) in the controls recorded were 3.00% of abnormalities when compared to AMFE extract treated mice were 3.20, 3.60 & 4.00 respectively (Table- 1). Change in the chromosomal number were recorded as autosomal univalent in controls was 1.00 when compared to treated mice were 1.20, 1.60 & 1.80. Sex chromosomal univalents in controls were 1.60 with that of AMFE extract treated mice was 1.60, 1.40 and 1.80 with 200, 400 & 600 AMFE mg/kg groups respectively. Among polyploids, aneuploidy results in controls were 0.40 when compared to that of AMFE extract mice was 0.40, 0.40 and 0.20 respectively. Polyploids were not in all the treated groups. Structural changes included on translocations were noted at observed in control and 400 and 600 mg/kg groups only (Table- 2The differences in the frequencies in the chromosomal aberrations between controls and treated mice were analyzed using X^2 test and the results were found to be insignificant (P>0.05, Table- 2)

Increasing concentrations of AMF extract GE (200, 400 and 600 mg/kg) was primed to mice and they were administered with 16 mg/kg of DOX to evaluate the mutagenic effect of AMFE extract. The results were tabulated in table- 3-4and illustrated in graph- 3,4

Among the non primed groups the controls have shown only 3.80% of abnormal metaphases when compared to 21.20 in DOX alone administered mice. There was a significant decrease in the percentage of abnormal metaphases in mice primed with AMF extract (200, 400 and 600 mg/kg) as 15.00, 13.80 and 9.20 respectively for various concentrations (Table 3).

The inhibitory effects of AMFE extract against doxorubicin induced chromosomal aberration in germ cells of male mice were 29.24, 34.90 and 56.60% in III; IV & V grouped animals Table- 3and graph.3

Cohen and Hirschhorn (1971) have stated the significance of cytogenetic damage caused by the chemical compounds to meiotic cells. This analysis has to be carried out by considering the different stages of spermatogenetic cycle. Such a test on germ cells in spermatogonia was called as "spermatocyte test" in treated males by Leonard (1972). It involved a study of diakinesis to metaphase I for scoring chromosomal aberration caused by the test agents.

The severity of the mutagen depends, to some extent, whether the affected cells is a unicellular organism or one of many cells in a multi-cellular organism. If the affected cellof latter is a germ cell that is involved in the reproductive process,then the resulting offspring will carry in their genetic material the potentially harmful information . If the affected cell in a multicellular organism is a somatic cell, then it may experience impaired function or impaired susceptibility to homeostatic, regulatory controls. In this case ,the cell may have taken a step along the path leading to the emergence of a cancer. Meiotic studies on the pachytene stage of spermatogenesis have demonstrated that infertile men have impaired chromosome synapsis, a significantly decreased frequency of recombination, and an increased frequency of chromosomes completely lacking a recombination site. Such errors make these cells susceptible to meiotic arrest and the production of aneuploid gametes (Renée and Martin et.al.,2006). Results are accordance with Marchetti et al (2007) that tested the hypothesis of parental mutagenic exposure during the late spermatogenesis can induced damage that persists in the fertilizing sperm and risk of embryos with paternally transmitted chromosomal aberrations depends on the efficiency of maternal DNA repair the first cycle after fertilization. Results showed that female mice with defective DNA double-strand break repair had significantly increased frequencies of zygotes with sperm-derived chromosomal aberrations after matings with wild-type males irradiated 7 days earlier with 4 Gy of ionizing radiation. These findings demonstrate that mutagenic exposures during late spermatogenesis can induce damage that persists for at least 7 days in the fertilizing sperm and that maternal genotype plays a major role in determining the risks for pregnancy loss and frequencies of offspring with chromosomal defects of paternal origin.

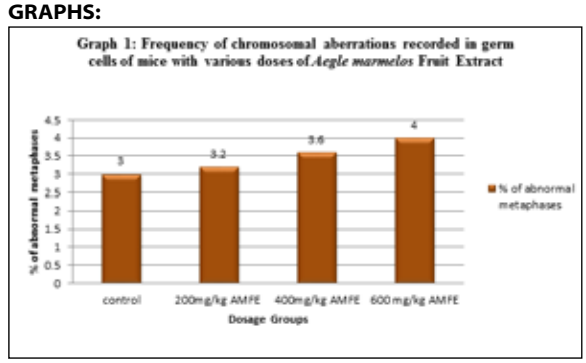
The mutagenic effect of doxorubicin (ADR) on mouse spermatogonial stem cells were examined by analysis of spermatocyte chromosomes and of dominant lethality transmitted through the spermatozoa. The effects of ADR on mutations, cytotoxicity and sperm head abnormalities were compared with those of radiation. The cytotoxic effect of 6 Gy of gamma radiation on stem spermatogonia was equivalent to about 4.5 mg ADR/kg. Chromosomal translocations were observed in 0.6% of the spermatocytes of mice treated with ADR (2-6 mg/kg). (Meistrich 1985). In the results of Larramendy et al (1980) the frequency of chromatid-type aberrations exhibited a direct-correlation with the dose in mice treated for 6h but not for 12 h. On the other hand, chromosome-type aberrations detected 12 hrs after injection were directly correlated with the dose of adriamycin, the genotoxic effects of the metacentric-like chromosomes induced by adriamycin arise either from translocations involving entire chromosomes arms or from aberrations of the exchange type between 2 short arms of acrocentric chromosomes of doxorubicin induced a significant increase ($p < 0.01$) the frequency of chromosome abnormalities, these results being consistent with those reported by other authors (Anderson et al 1998) In fact, according to Ling et al. (1996) [doxorubicin could induce apoptosis by promoting cyclin B accumulation.

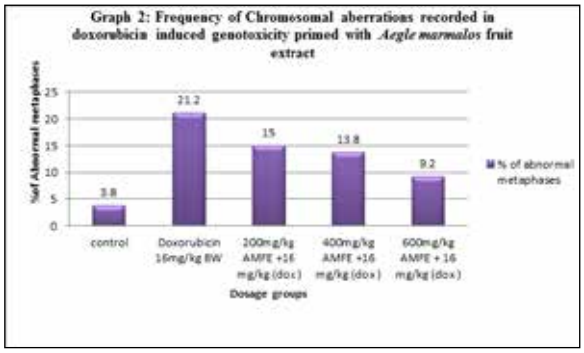
Doxyrubicin is a potent antitumor agents used for the treatment of many cancer. It is demonstrated that this drug has the potential for initiating genetic events in nontumor cells in human and animal systems. The results showed that doxyrubicin (Dox)induced micronuclei in polychromatic erythrocytes male and female mice. The results are in agreement with other reports of Doxyrubicin cytotoxicity (Anderson, *et al*, 1998. Prahalathan, *et al* 2005. The biochemical nmechanism of adriamycin causes cytotoxicity is unclear. However when it intercates with DNA generates free radicals. Two pathway of mechanisms have been proposed. Two different pathways of free radical formation of Dox have been described. First is the formation of semiquinone free radical the semi quinine can be transferred to a C7 radical that can also mediate cellular damage. The reduction of doxorubicin by 2 electrons generates a secondary alcohol metabolite doxorubicinol. The second pathway doxorubicin free radicals come from an enzymatic mechanism that involves reactions with iron. For example Fe3+reacts with doxorubicin in a redox reaction after which the iron atom accepts an electron and a Fe2+ deoxyribicin free radical complex is produced. This iron doxorubicin complex can reduce oxygen to hydrogen peroxide and other active species Granados *et al*, 2010 , Xu et al 2005)

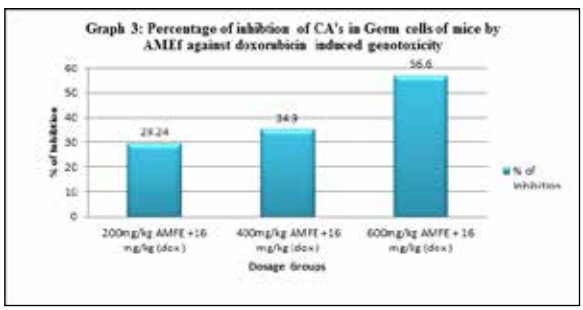
Jagatia et al (2004) investigated the radioprotective activity of a leaf extract of bael leaf (*Aegle marmelos*) (AME) in mice exposed to different doses of gamma-radiation. For radioprotection studies, mice were administered different doses, 0, 5, 10, 15, 20 or 40 mg kg(-1), of AME or sterile physiological saline intraperitoneally once daily consecutively for 5 days before exposure to 10 Gy 60Co gamma-radiation or five doses of 15 mg kg(-1) AME before exposure to 6, 7, 8, 9, 10 or 11 Gy. The animals were monitored for symptoms of radiation sickness and mortality up to 30 days post-irradiation. The irradiation caused a dose-dependent decline in survival, while treatment of mice with AME enhanced survival. The dose reduction factor was 1.15. Irradiation caused a dose-dependent decline in the level of glutathione accompanied by an elevation in lipid peroxidation. AME pretreatment arrested glutathione decline and lipid peroxidation significantly. The compounds, 6-methyl-4-chromanone, isolated from *Aegle marmelos* by Nicolis et al (2009) showed inhibition of IL-8 in the IB3-1 CF cells in vitro. Cardenolide, periplogenin, isolated from the leaves of *Aegle marmelos* protected the doxorubicin induced cardiotoxicity and lipid peroxidation in rats by reversing the increase in serum creatine kinase-MB, glutamate-pyruvate transaminase, and tissue LPO (Panda, 2009.)

Subramaniam.et al. (2008) reported that marmelin, an ethyl acetate fraction of *Aegle marmelos* extracts suppressed TNF-alpha-mediated activation and translocation of NF-kappaB, inhibited AKT and ERK phosphorylation both in-vitro and in tumor xenografts . A. marmelos leaf, seed and fruit is known to affect male fertility in reversible manner. A. marmelos bark extract is a rich source of marmin and fagarine known for reducing male fertility. Agarwal et al 2012 found that methanolic extract of A. marmelos causes a dose and duration dependent infertility via reducing reproductive organ weight and serum testosterone levels. They also report reduction in sperm density, motility, viability and sperm acrosomal integrity. Exfoliation of elongated spermatids, nuclear chromatin condensation and degeneration were found in testes histopathological studies and presence of spaces within the germinal epithelium signifying testicular cytotoxicity and necrosis. Finally time dependent complete infertility was observed in that study. The authors also reported that after the withdrawal of treatment, complete restoration of the morphological as well as physiological parameters in extract treated rats[Agrawal et al., 2012]. These findings suggest that A. Marmelos extract is a strong candidate for male contraceptive via its ability to produce complete inhibition of pregnancy, rapid restoration of fertility after withdrawal from treatment[Chauhan and agarwal., 2008].

GRAPHS:







It is important to reduce its toxicity in normal cells, a goal that can be achieved by concurrent administration of free radical scavenging agents, such as antioxidants [Amara-Mokrane et al 1996, Miyata et al 2004) Natural antioxidants in the human diet can attenuate the effects of mutagens and genotoxic carcinogens. Some antioxidants, such as vitamins A, C and E, minimize the side effects of antineoplastic drugs and can improve cancer chemotherapy. An increase in the dietary content of antioxidants through the increased ingestion of fruits and vegetables rich in these compounds can decrease the oxidation of DNA by free radicals, thereby preventing cancer and other degenerative diseases. Oxidative damage to biomolecules caused by stress is one of the major risk factors for atherosclerosis, mainly through the oxidation of low density lipoprotein (LDL) in the blood

The present results are comparable with venkatesh 2007 who reported the protective effects of *Aegle Marmelos* in mouse bone marrow cells at 350 mg/kg dose level. Earlier we have reported on the protective effects of *phyllanthus emblica* fruit extract on adriamycin induced genotoxicity in somatic cells of mice [(Rudrama Devi K. & Kusum Latha Chamyal 2012.)The protective effects against DOX induced genotoxicity by AME may be due to inhibition. of free radicals formed by DOX in cytoplasm of cells and increased antioxidiant status by addition of fruit extract. The fruit of *Aegel marmelos* contains marmelosin, luvangetin, aurapten, psoralen, marmelide, tannins and phenols. The AMF extract has been used in for treating diarrhea, diabetic constipation heart disease, ulcers wood healing because of its medicinal properties. Lupeol, a compound present in *A. marmelos* possess antineoplastic effects on various human neoplastic cell lines (Baliga et al 2012) Marmelin (1-hydroxy-5, 7-dimethoxy- naplhale necarboxy aldehyde) present in *A. marmelos* inhibiting growth of epithelial cancer cells, but not normal cells (mouse embryo fibroblasts) further it decreases cell survival, proliferation and invasiveness (Baliga et al 2012). It is well known that consumption of fruits and vegetables is associated and are known to prevent chromosomal and DNA damage in animals (Nerseyan et al., 2004, Miyata et al., 2004). Usually antimutagens acting in rodents are active in human too(Weishurger. et al 2001) Our results have a practical decline of genotoxic effects of doxyrubicin in cancer patients some health care workers as nurse and pharmaceutical plant workers handle this drug which may alternate the higher risks for development of secondary malignancy and for abnormal reproductive outcomes.

CONCLUSION

From the above studies it is concluded that AMF Extract as protective agent against doxyrubicin induced genotoxic effect in germ cells of mice. It is concluded that *Aegle mamelos* can be used as a major chemopreventive agent against doxyrubicin induced mutagenicity.

ACKNOWLEDGEMENT

Mr. Prabakar Reddy is thankful to Prof. L. Rajeswari Anand, Head, Department of Zoology, Osmania University, Hyderabad, Andhra Pradesh for providing Laboratory facilities and Prof. T. Nagaraju, Chairman, Board of Studies, Department of Zoology, Osmania University, Hyderabad, Andhra Pradesh for his encouragement to carry out this study.

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