Protective efficacy of isolated D-Pinitol from Soybean aerial parts on Doxorubicin-induced genotoxicity and cytotoxicity in normal bone marrow cells of mice assessed via *in vivo* Bone marrow chromosomal aberration assay

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ABSTRACT

The current study aimed to ascertain whether D-Pinitol has a protective effect against Doxorubicin-induced genotoxicity and cytotoxicity on normal bone marrow cells of mice by *in vivo* bone marrow chromosomal aberration assay and histopathological studies of bone tissue and bone marrow. The following ten groups of sixty Swiss Albino mice formed: Vehicle Control, Doxorubicin (5 mg/kg), D-Pinitol (400, 300, 200 & 100 mg/kg), and D-Pinitol (400, 300, 200 & 100 mg/kg) + Doxorubicin (5 mg/kg). Twenty-four hours following the last treatment, four hundred microlitre of colchicine (4 mg/kg) was intraperitoneally administered to the animals in all groups to inhibit cell division. Two hours after being given the injection, the animals were killed by inhaling carbon dioxide. Bone marrow was extracted from the femur for the analysis of chromosomal aberrations. In the current research, it was observed that Doxorubicin significantly (P>0.001) enhanced the frequency of chromosomal abnormalities and significantly reduced the mitotic index, which is associated with apoptosis. The mitotic index and chromosomal abnormalities did not change substantially in the D-Pinitol alone treated groups. Pre-administration of D-Pinitol with Doxorubicin reduced DNA damage by minimizing the occurrence of chromosomal abnormalities in mouse bone marrow cells, and improving the mitotic index. Further, histological analysis of bone and bone marrow demonstrates that doxorubicin's toxicity to bone marrow is reduced in mice treated with D-Pinitol and doxorubicin. Genetic stability will invariably be disturbed by DNA damage brought on by Doxorubicin mediated inflammation and Reactive Oxygen Species production. It has been suggested that D-Pinitol's anti-inflammatory and antioxidant characteristics explain its protective mechanism against DOX-induced genotoxicity and cytotoxicity. As a result, D-Pinitol was proven to have the best genoprotective and cytoprotective effect against Doxorubicin-induced genotoxicity and cytotoxicity.

KEYWORDS: Genotoxicity; Cytotoxicity; Doxorubicin; D-Pinitol; *in vivo* bone marrow chromosomal aberration assay.

INTRODUCTION:

Cancer is a more life-threatening and serious illness that threatens people's health everywhere [1]. Increasing age and population growth, as well as the growing prevalence of cancer-causing habits in the nations that are developing in economic terms, are the main causes of the ongoing rise in cancer rates [2].

With an estimated nearly nine million deaths globally, cancer ranks as the second most common reason for mortality [3]. Scientists are still searching for an efficient anticancer medication since the International Agency for Research on Cancer (IARC) estimates that by 2030, there would be about 13 million deaths from cancer [1]. The most efficient method available to medical practitioners for treating neoplasms is chemotherapy [1] [4]. Several neoplastic diseases are treated with a variety of chemotherapeutic drugs. One characteristic that distinguishes anticancer medications from alternative therapies is the intensity and recurrent illness of adverse consequences at therapeutic dosages [5]. In addition to killing cancer cells, chemotherapy could also harm healthy cells [1]. Therefore, oncological scientific investigation is concentrating on creating new, efficient medications that might lessen the harmful side effects caused by chemotherapeutic drugs [6].

Doxorubicin (DOX), a daunorubicin derivative that has been hydroxylated, provides a broad therapeutic spectrum in the treatments of Haematological tumours and various solid tumours [7]. In several tissues, DOX induces the generation of free radicals, oxidative stress, and substantial inflammatory responses [8]. Since DOX is a key component of cancer therapy, it's important to limit its adverse effects on normal cells and tissues [9]. Henceforth, we require a DOX chemotherapy adjuvant treatment that possesses all the anti-inflammatory, reactive oxygen species scavenging, antioxidant effects, and required to safeguard normal cells and tissues against DOX-induced genotoxicity and cytotoxicity [10],[11].

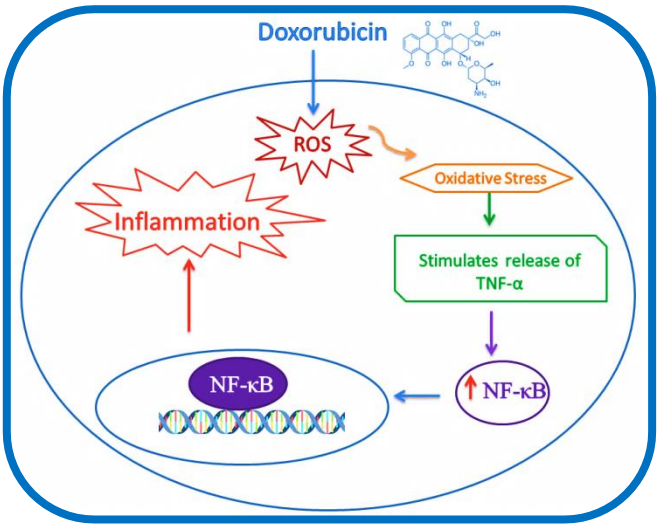

Figure.1. Schematic diagram representing DOX-induced oxidative stress and inflammatory pathway [8]

D-Pinitol (D-P), a well-known natural cyclitol molecule, has long been used in the conventional practice of Ayurveda medicine [12]. It is the most common soluble carbohydrate found in each segment of soybean plants, which belong to the Leguminosae family (*Glycine max* L. Merr.) [13], [14]. As an herbal remedy, D-P has attracted a lot of attention due to its diverse pharmacological characteristics. It has a lot of therapeutically advantageous benefits, like cardioprotective [15], spinal cord injury protection [16], preventive and ameliorative of Alzheimer's disease [17], hepatoprotective [18], renal protective [19], preventative of osteoporosis [20], and anti-neoplastic activity [21] due to its anti-inflammatory, [22] and antioxidant [23] properties. Hence, the impact of D-P in the reduction of adverse effects after DOX treatment was investigated in this study.

MATERIALS AND METHODS:

Animal Care and Handling:

In a 12-hour dark /light cycle, Swiss Albino mice of weight around 25–30 g (both sexes), were housed. As per CPCSEA requirements, the animals were acclimated by being kept in a hygienic environment [24].

In vivo Bone marrow chromosomal aberration assay:

Materials Required:

Doxorubicin HCl (CIPLA, India), Colchicine (Zydus Healthcare Ltd., India), Giemsa stain (Hi-media, India), Isolated D-Pinitol (from soybean plant aerial parts) and Microscope (Olympus Optical Co., Germany).

Methodology:

Each animal group was separated into 10 groups, each consisting of six mice.

Table.1. Treatment Protocol

Groups	Labeled	Treatment
I	Vehicle Control	0.5 ml of 0.9% normal saline
II	Positive Control	Doxorubicin (5 mg/kg), i.p. on 1st, 8th and 15 th days (Positive Control)
III	Test	D-Pinitol (100 mg/kg), p.o. daily
IV		D-Pinitol (200 mg/kg), p.o. daily
V		D-Pinitol (300 mg/kg), p.o. daily
VI		D-Pinitol (400 mg/kg), p.o. daily
VII		Doxorubicin (5 mg/kg), i.p. on 1st, 8th and 15 th days+ D-Pinitol (100 mg/kg), p.o. daily
VIII		Doxorubicin (5 mg/kg), i.p. 1st, 8th and 15 th days + D-Pinitol (200 mg/kg), p.o. daily
IX		Doxorubicin (5 mg/kg), i.p. 1st, 8th and 15 th days + D-Pinitol (300 mg/kg), p.o. daily
X		Doxorubicin (5 mg/kg), i.p. 1st, 8th and 15 th days + D-Pinitol (400 mg/kg), p.o. daily

DOX and D-P doses selection were according to Hajra *et al.* and Navaaro *et al.*, respectively [25],[26].

According to the treatment regimen, mice received D-P (at all dosages, i.e., 100, 200, 300, and 400 mg/kg) for a period of fifteen days, and DOX (on the first, eighth, and fifteenth day) [27] for three days. Thirty minutes before the injection of DOX, D-P was treated. To halt division of cells, mice in every group administered intraperitoneal injection of 400 microliters of colchicine (4 mg/kg), 24 hours following the last treatment. Two hours following the injection, the animals were exposed to carbon dioxide inhalation and then euthanized. Bone marrow was collected from the femur and placed in a syringe with 1 ml of 0.075 M KCl. The marrow suspension was incubated for 20 minutes at 37°C before centrifuged for 10 minutes at 62 x g. The pellet was mixed with a fixative (3:1 Methanol/acetic acid solution which is prepared at the time of usage) after the supernatant had been removed. The suspension described above was allowed to stand for 15 minutes before centrifugation. Two fixative washes (500 µl each) were performed on the preparation. A few drops of the suspension were dropped from a height onto a clean glass slide after the resulting pellet was mixed with 500 µl of fixative. After being air dried, the slides were stained for 5 minutes at pH 6.8 with 10% Giemsa. Fragments, Gaps, exchange, Break, and Ring were analyzed under the microscope for chromosomal aberration at 100X. The number of mitotic cells in 100 cells examined per mouse was utilized to calculate the mitotic index [28].

Histopathological Studies:

Materials Required:

Eosin stain and Hematoxylin (Himedia, India), Paraffin Wax (Labogens, India), Giemsa (Himedia, India), Diluent for smear preparation: 5% bovine serum albumin (BSA) in Phosphate buffered saline (Himedia, India) and Formaldehyde (Labogens, India).

Methodology:

Bone tissue:

The bones were cleaned of most attached skeletal muscle, ligaments, and tendons. The tissue was fixed by immersing in neutral buffered 10% formalin, and then it was decalcified; it was embedded in paraffin; it was sectioned at 4–8 μm, and finally, it was stained with hematoxylin and eosin (H&E) [29].

Bone marrow smear:

The freshly harvested femur bone was broken to expose marrow using forceps and bone roungers after muscle was removed from the femur. To collect bone marrow from the open cavity, a paintbrush was soaked in the diluent; the excess liquid was wiped, and a pointed tip was formed. To spread cells, the brush was gently pushed from the top of the glass slide down the length of the glass microscope slide in an "S"-wave motion. The slides were allowed to air-dry for 5 minutes before fixing them in 100% Methanol. Then, the slides were stained with Giemsa [30].

Statistical Method:

Graph pad prism software version 8.01 was used to do statistical analysis using one-way ANOVA for this study. The variation of data was described using SEM (Standard Error of the Mean). $P<0.05$ (P values of less than 0.05) was statistically significant.

RESULTS AND DISCUSSION:

***In vivo* Bone marrow chromosomal aberration assay:**

The results of antigenotoxicity study, *in vivo* Bone marrow chromosomal assay performed with various concentrations of D-P in combination with DOX are represented in Table.2. & Figure.2. Various chromosomal abnormalities that were present in every group with a total number of aberrations presented in Table.3. Figure.3 and Figure.4 represents % chromosomal aberrations and mitotic index respectively. Only a few abnormal metaphases among hundred metaphases were seen in both the Group I (vehicle control) and Group III,IV,V & VI (D-P

alone treated groups). The DOX alone administered group presented a statistically significant (P<0.001) percentage of chromosomal abnormalities and a significantly higher number of aberrant metaphases when compared to the vehicle control group. The most common chromosomal abnormalities in all treatment groups were fragments, exchanges, gap, break, dicentric, and ring formation, as illustrated in Figure.4. In comparison to the DOX alone treated group, D-P co-treated with DOX demonstrated a considerable significant reduction (P<0.001) in aberrations in a dose-dependent manner. When D-P was given along with DOX, the overall number of chromosomal abnormalities and aberrant metaphases caused by DOX were significantly reduced.

Mice treated with D-P at all dosages showed a mitotic index that was similar to the vehicle control group, with a large number of dividing cells (Metaphase – M) and resting cells (nucleus-N). In mice administered with DOX, the mitotic index displayed a large number of nuclei (N) and a small number of dividing cells (M). Mice given DOX showed a high number of nuclei (N) and a low number of dividing cells (M) in their mitotic index. In the mitotic index of mice treated with both DOX and D-P, many cells resumed normal division, while other cells (nucleus) remained at rest. This occurred in a dose-dependent manner, ranging from low to high dosage. The normal division of many cells was restored at a high dosage of 400 mg/kg of D-P. Compared to the Group I (vehicle control) mice, Group II (DOX-injected) mice showed considerably reduction in mitotic index (P<0.001). When animals treated with DOX and D-P, there was significant raise (P<0.001) in the mitotic index which was represented in the Figure.3 in a dose-dependent manner.

Table.2. The number of various chromosomal aberrations observed

Nature of Chromosomal Aberrations	Group I (Control-0.9% Normal saline)	Dose in mg/kg								
		Group II (DOX 5)	Group III (D-P 100)	Group IV (D-P 200)	Group V (D-P 300)	Group VI (D-P 400)	Group VII (DOX 5+ D-P 100)	Group VIII (DOX 5+ D-P 200)	Group IX (DOX 5+ D-P 300)	Group X (DOX 5+ D-P 400)
Normal	86.33± 2.2	45.83± 5.076	86.5± 2.141	88.67± 1.116	87.83± 1.352	87.5± 2.062	48.5± 2.335	52.5± 1.803	65.33± 2.404	76.67± 2.275
Fragment	4.167± 0.477	19.17± 2.414	3.667± 0.882	3.333± 1.054	2± 0.365	3± 0.577	23.33± 0.919	23.67± 2.974	11.17± 0.401	9.833± 0.872
Gap	2.667± 0.558	20.17± 2.561	4.333± 0.615	3.5± 0.563	3.333± 0.715	3.5± 0.428	16.17± 1.778	10.67± 1.085	13.83± 1.621	6.5± 0.764
Chromatid Exchange	2.167± 0.872	5.333± 1.054	1.833± 0.477	2.167± 0.477	2.667± 0.494	1± 0	4.667± 0.76	6.333± 0.667	2.833± 0.307	3.333± 0.615
Ring	2.833± 0.946	2.833± 0.6	2.167± 0.792	2.333± 0.333	2.333± 0.422	2.333± 0.494	2.333± 0.803	0.5± 0.342	3.667± 0.333	2.5± 0.428
Break	1.833± 0.792	2.167± 0.703	0.5± 0.224	0	1.667± 0.333	0	0.5± 0.224	3± 0.516	1.5± 0.428	1.167± 0.307
Others	0	4.5± 1.176	1±0	0	0.167± 0.167	1.667± 0.333	4.5± 0.671	3.333± 0.667	1.667± 0.333	0

Mean ± SEM, n=6.

Figure.2. Photomicrograph shows Chromosomal aberrations in the treated groups of mice at 100X

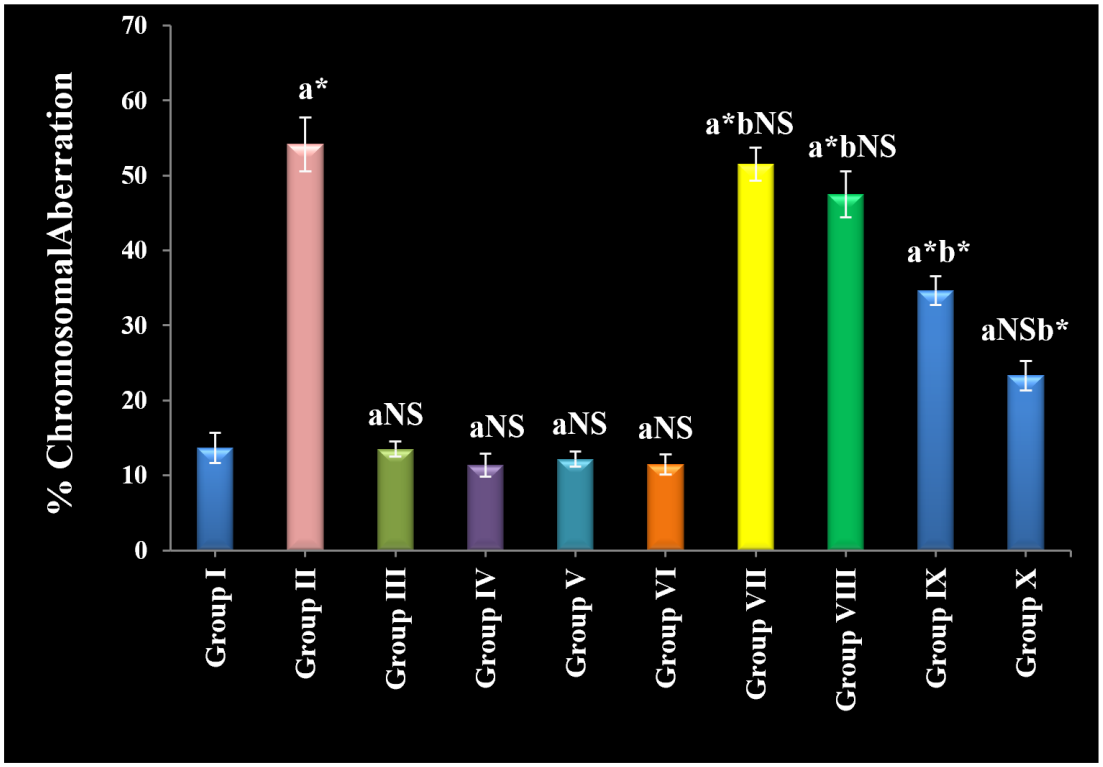

Table.3. Percentage of Chromosomal Aberrations and Mitotic Index of treated mice.

Criterion	Group I (Control-0.9% Normal saline)	Dose in mg/kg								
		Group II (DOX 5)	Group III (D-P 100)	Group IV (D-P 200)	Group V (D-P 300)	Group VI (D-P 400)	Group VII (DOX 5+ D-P 100)	Group VIII (DOX 5+ D-P 200)	Group IX (DOX 5+ D-P 300)	Group X (DOX 5+ D-P 400)
%Chromosomal aberration	13.67± 2.028	54.17± 3.61 a*	13.5± 1.025 aNS	11.33± 1.52 aNS	12.17± 1.014 aNS	11.5± 1.335 aNS	51.5± 2.187 a*bNS	47.5± 3.106 a*bNS	34.67± 1.891 a*b*	23.33± 1.961 aNSb*
Mitotic Index	3.43± 0.156	1.28± 0.114 a*	3.38± 0.07 aNS	3.41± 0.06 aNS	3.4± 0.097 aNS	3.28± 0.101 aNS	1.68± 0.18 a*bNS	2.13± 0.143 a*b#	2.38± 0.101 a*b*	3.08± 0.095 aNSb*

Mean ± SEM, n=6, where a- Group II, III, IV, V, VI, VII, VIII, IX & X compared with Group I. b- Group VII, VIII, IX & X compared with Group II. * $P < 0.001$, $^{\#}$ $P < 0.01$ & $^{@}$ $P < 0.05$.

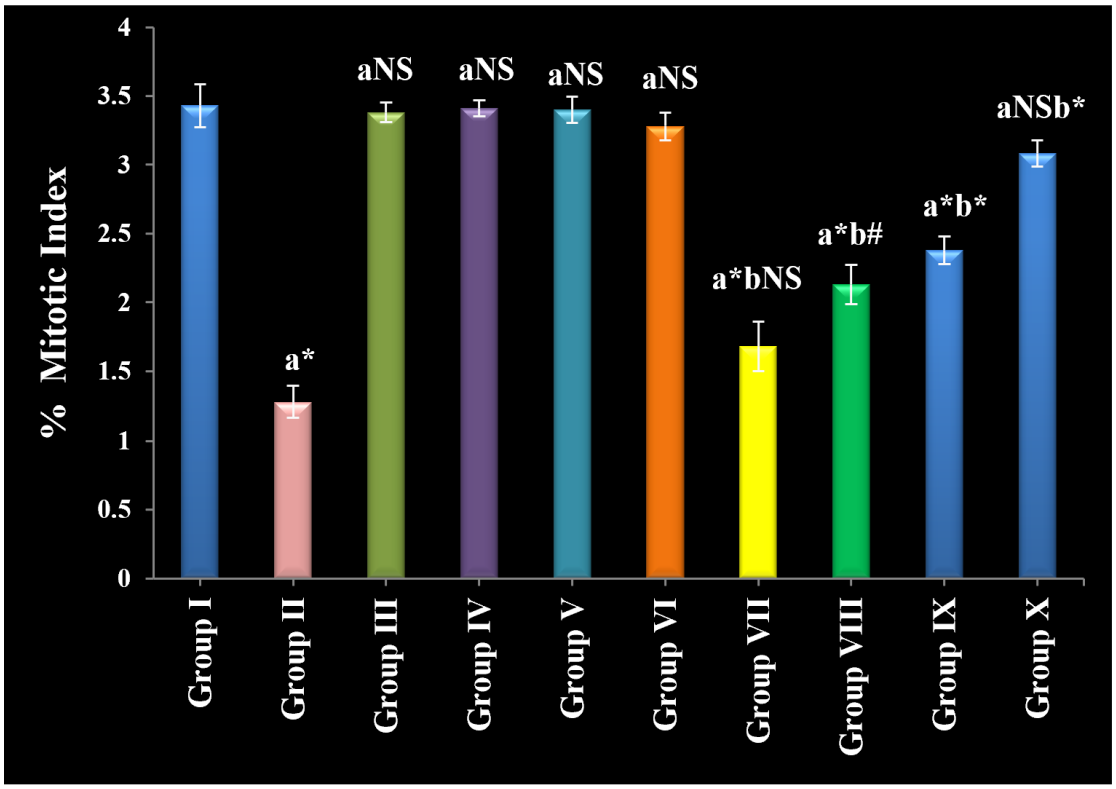

Figure.3. Histogram - Percentage of Chromosomal Aberrations

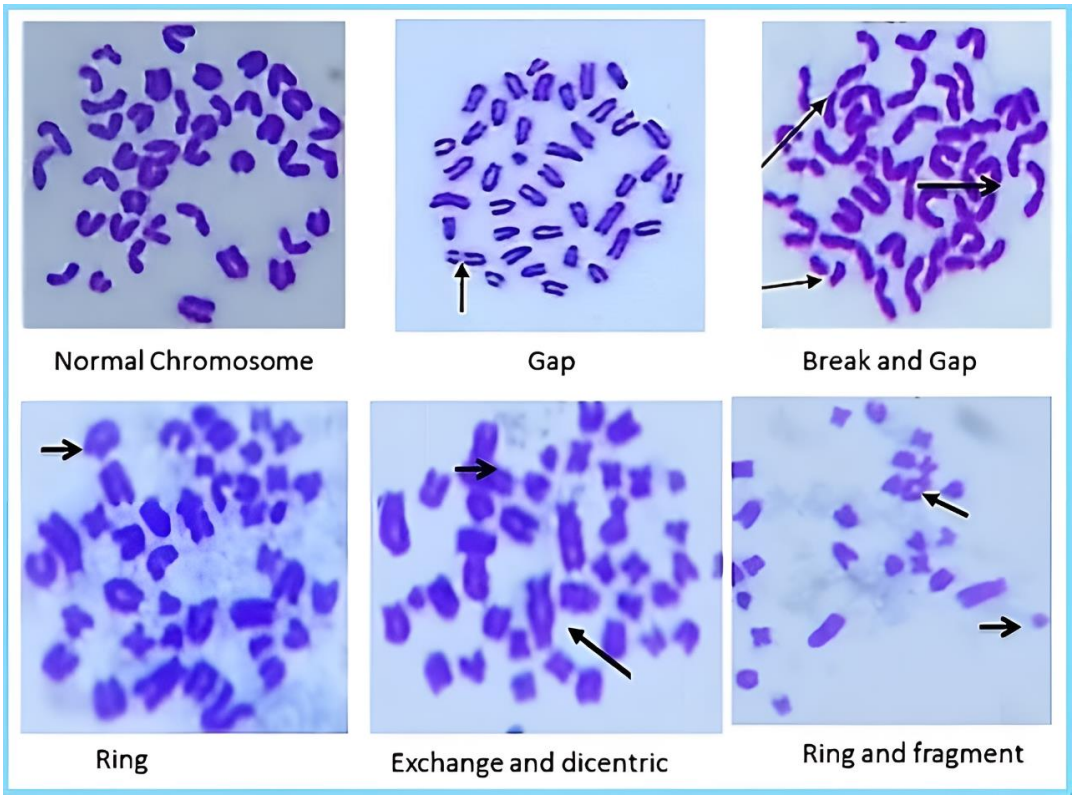

Figure.4. Photomicrograph shows Chromosomal aberrations in the treated groups of mice at 100X

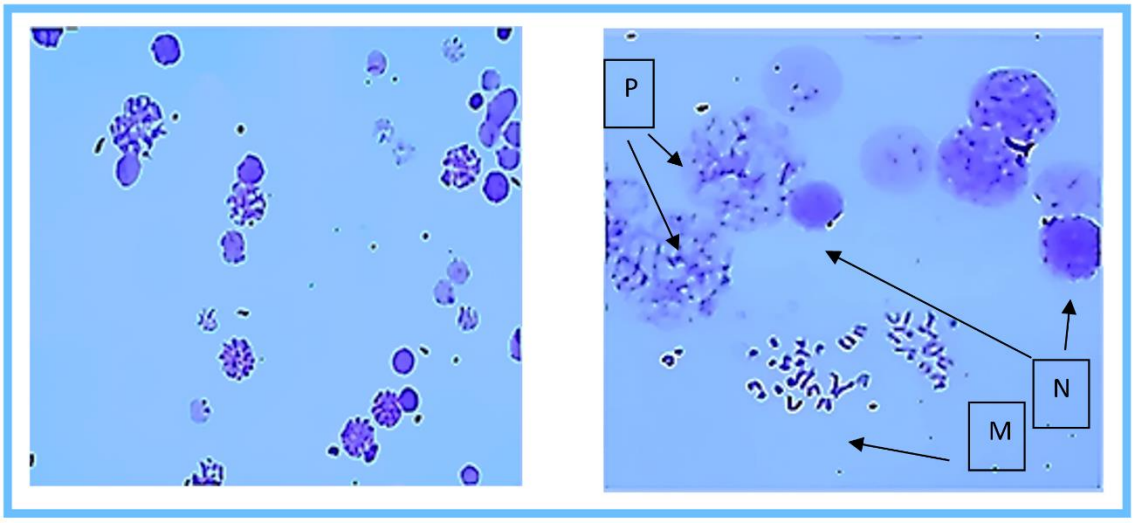

Figure.5. Photomicrograph shows Mitotic index showing stages of cell cycle in bone marrow of mice at 10X & 40X - M: metaphase, P: prophase, N: nucleus

Histopathological Studies in Swiss albino mice:

Bone tissue and Bone marrow smear:

Histopathological examination of bone tissue (Figure.6) and bone marrow smears (Figure.7) revealed good cell density of bone marrow with cellularity of hypercellular type. Considerable aggregation and overlaying of cells with negligible cellular necrosis were visible in the Group I (vehicle control). Animalss administered with D-P alone displayed cell densities that were comparable to those with hypercellular cellularity. There was no cellular necrosis visible, although there were overlapping clusters of cells. Thus, D-P was shown to have no negative impact on bone marrow smears. In DOX alone treated group, there was poor cell density with the hypocellular type of cellularity. There were clumps with few cells, or else no clump in bone marrow smears. Wide cells with cellular necrosis were seen in large quantity among the bone marrow cells. However, in the DOX and D-P co-treated groups, cell density was fair, and cellularity was normocellular in nature. There was a great reduction in cellular necrosis in a dose-dependent manner. Well distributed cells with few small clumps to none were seen. These drastic changes in histological studies revealed the protective effect of D-P against DOX-induced cytotoxicity to bone marrow cells. Based on the above findings of this investigation, it was revealed that DOX administration caused abnormal histological changes in bone marrow cell structures. When D-P was given priorly along with DOX therapy at doses 100, 200, 300, 400 mg/kg, these aberrant alterations in the bone tissues in the positive control were reversed. Bone tissue in the DOX and D-P (400 mg/kg) treated group had a restored structure of bone marrow cells and tissue due to the protection utmost given by D-P in this group.

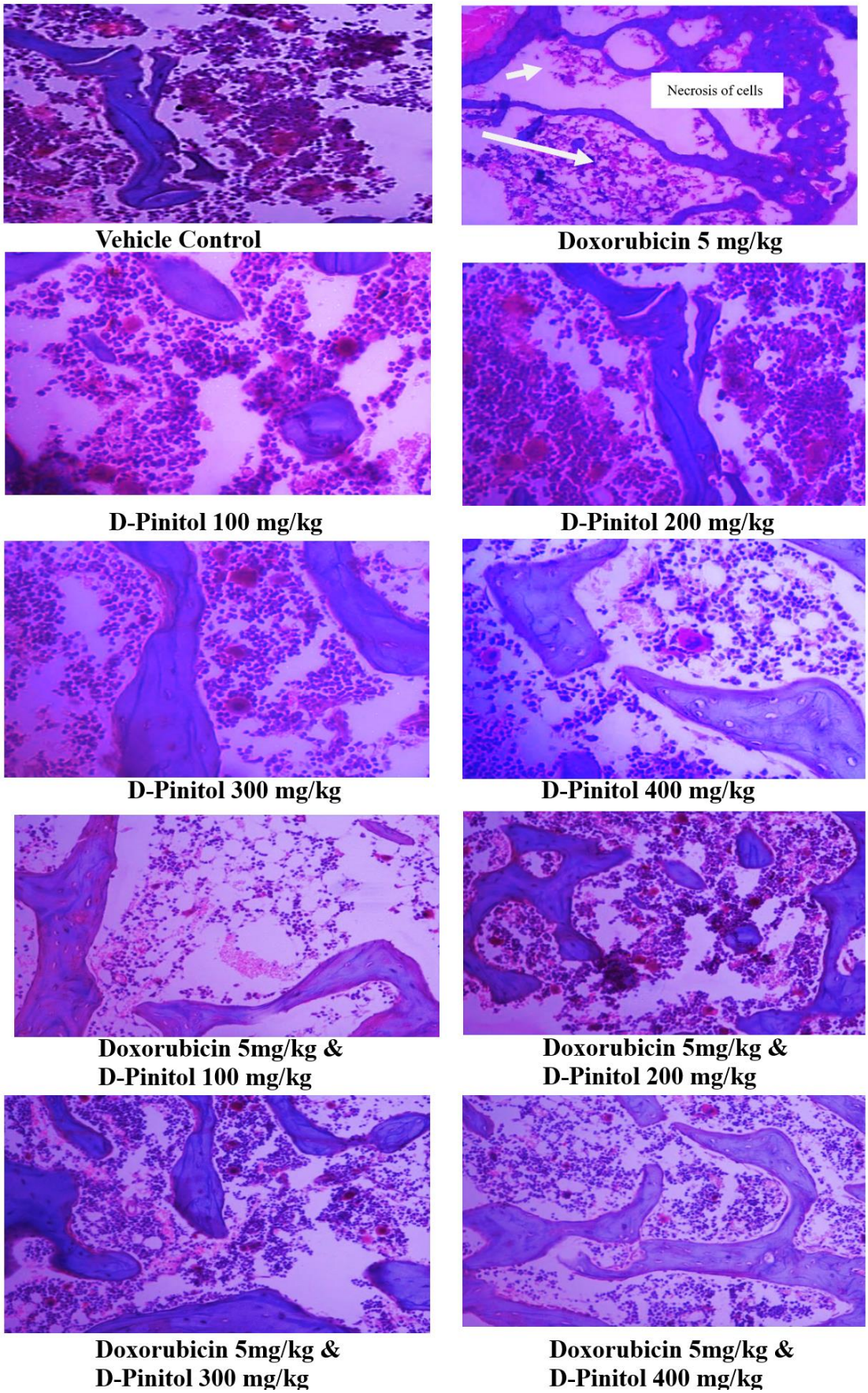

Figure.6. Photomicrograph of a section of Bone tissue at 40X

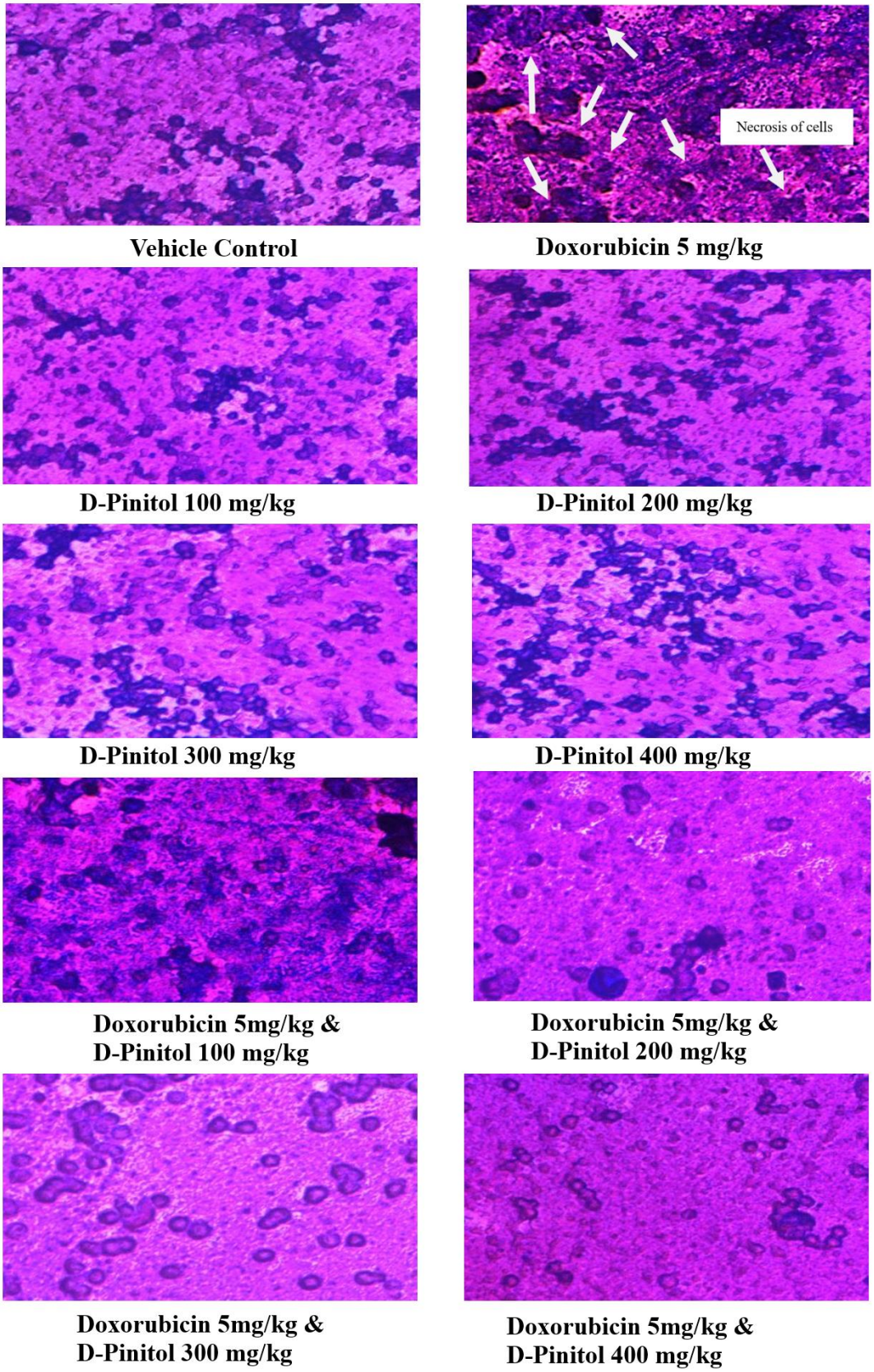

Figure.7. Photomicrograph of Bone marrow smears at 10X

DISCUSSION:

Genoprotective effect of D-P against the genotoxic effects of DOX in the bone tissue of mice:

The current research focused on D-P's genoprotective properties when administered alone and in conjunction with DOX. Chromosomal aberrations assay in bone marrow was chosen to assess genotoxicity [28] [31], since this battery of test will confirm a 100% correct genotoxicity analysis [28].

DOX is genotoxic and causes chromosomal aberrations in various cell types, which include human cells [32]. We noticed that DOX increased the prevalence of chromosome abnormalities significantly. Furthermore, we observed that DOX caused a substantial decrease in mitotic index, which is correlated with apoptosis [33]. D-P alone treated groups did not result in a significant rise in the mitotic index and chromosome aberrations. D-P pre-administration with DOX inhibited the chromosomal aberrations and DNA damage and also increased the mitotic index in bone marrow cells of mice caused by DOX administration. This therapeutic effect of D-P complies with its genoprotective activity against genotoxicity caused by DOX.

Cytoprotective effect of D-P on DOX-induced cytotoxicity in bone and bone marrow of mice:

Previous studies found that DOX caused significant deterioration of bone [34] and bone marrow [11]. Additionally, the current study discovered that DOX produced histological deterioration in bone and bone marrow cells after 15 days of administration. By interacting with cell macromolecules, DOX-induced ROS, oxidative stress [35], [36], and mediators of inflammation [9] might result in cytological destruction. When used alone, D-P has no harmful effects on bone marrow cells, or tissues. The histological changes in the bone and bone marrow cells under investigation might be effectively reversed by D-P administration due to its antioxidant activity, ability to quench free radicals and anti-inflammatory properties,.

CONCLUSION:

In conclusion, the current study proved D-P’s protective role against DOX-induced genotoxicity and cytotoxicity as a genoprotective and cytoprotective agent through its potent antioxidant and anti-inflammatory property; Hence, D-P could be used as adjuvant therapy for DOX chemotherapy to mitigate DOX-mediated genotoxicity and cytotoxicity.

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ETHICS STATEMENT:

The Institutional Animal Ethics Committee (IAEC) of Adhiparasakthi College of Pharmacy (Reg. No. 409/PO/Re/S/01/CPCSEA) approved the experimental protocol. The approval number was APCP/IAEC/2019-2020/1.

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The author declare that she has no funding support for this study.

CONFLICT OF INTEREST:

The author declare that she has no conflict of interest for this study.

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