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ORIGINAL ARTICLE **OPEN ACCESS**

Genoprotective efficacy of D-Pinitol isolated from aerial parts of Soybean plant against Doxorubicin-induced genotoxicity assessed by *in vivo* comet assay.

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ABSTRACT
The current study's objective was to evaluate D-Pinitol's genoprotective potential against Doxorubicin-mediated genotoxicity assessed by in vivo Comet test. Swiss albino mice (n=6) were grouped into ten groups. 0.9% NaCl was treated to Group I (Control) for fifteen days. DOX (5 mg/kg i.p.) was treated to Group I (Positive control) for three days on 1st, 8th and 15th days. D-P of various concentrations (100 mg/kg, 200 mg/kg, 300 mg/kg & 400 mg/kg p.o.) were given to mice for 15 days and the groups were noted as group III, IV, V & VI with respect to the increase in doses of D-P. In Group VII, D-P 100 mg/kg was given beforehand DOX (5 mg/kg) treatment. D-P 200 mg/kg was administered prior to DOX (5 mg/kg) treatment in Group VIII. D-P 300 mg/kg was administered prior to DOX (5 mg/kg) treatment in Group IX. D-P 400 mg/kg was administered prior to DOX (5 mg/kg) treatment in Group X. EDTA blood (50 µl) samples from the vehicle control and all experimental groups were taken and DNA damage was evaluated using the in vivo Comet test. In the DOX-only treated group, there was an increase in DNA damage that was highly significant (P<0.001). D-P didn't have negative or positive effect on DNA damage compared to Control group. The animal group that received D-P before DOX treatment showed a significant (P<0.001) dose-dependent decrease in DNA damage compared to positive control (DOX 5 mg/kg) group. Thus, D-P's anti-inflammatory, antioxidant, and free radical scavenger abilities revealed how well it shielded normal cells from DNA damage brought on by DOX. D-P can be used as a genoprotective agent since it has shown resistance to the genotoxicity caused by DOX in DNA of normal cells.
Keywords: *D-Pinitol, Doxorubicin, Genoprotective effect, Genotoxicity, In vivo Comet assay.*

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INTRODUCTION

An antibiotic belonging to the anthracycline family called doxorubicin (DOX) is utilised to treat a wide range of human carcinomas [1]. Patients using DOX treatments, however, have genotoxicity and bonemarrow suppression, and it could result in secondary tumours and cardiac toxicity. The underlying mechanisms for the toxicities caused by DOX include inflammation and oxidative damage tonormal cells[2]. Since DOX is vital for the therapy of cancer, it is imperative to lessen its harmful impact to normal cells. Reducing oxidative stress and inflammation is thus a potential treatment strategy for DOX-induced genotoxicity. A novel strategy for treating DOX-induced genotoxicity involves mitigating oxidative stress, pro-inflammatory mediators, and subsequently the inflammatory process [3].Several plants, including soybean plants, contain the naturally occurring substance named D-Pinitol. It belongs to the inositol family. It is fortunate that plenty of identified multi-purpose attributes of D-Pinitol have widened the therapeutic attention in this substance. Its protective action was proven through its antioxidant property and anti-inflammatory activity [4] viz., hepatoprotective effect [5], renal protective effect [6] [7] and spinal card injury protective effect [8].

The alkaline comet test, one of the most effective techniques for finding genetic damage, has become of greater importance in recent years [9]. The information that above explained provoked to examine D-Pinitol's potential for protective action on genetic material of normal cells against Doxorubicin-mediated genotoxicity by preventing the generation of free radicals, oxidative stress, and inflammation.

MATERIAL AND METHODS

In vivo Comet Assay

Materials Required

Doxorubicin HCl (CIPLA, India), Isolated D-Pinitol for Soybean plant, Olympus BX 50 microscope (Olympus Optical Co., Germany), Ethidium Bromide (Merck, India), Normal agarose (Merck, India), and Low melting agarose (Merck, India).

Animal Care and Handling

Swiss albino mice of weight 25 to 30 g were acclimatized with a twelve - hour light/dark cycle in animal house according to CPCSEA criteria before the study began [10].Research study of Hajra *et al*. and Navaaro *et al*., were utilized to choose the DOX and D-P doses, respectively [2][11].

Methodology

The comet assay was done using a procedure given by Singh *et al.* with minor changes. The microscopic slides were coated by two layers, the first with 0.75 percent normal melting agarose (200 µl) and the second with 0.5 percent low melting agarose (100 µl). The next stage was to combine EDTA blood (50 µl) from the vehicle control and experimental groups with 60 microliters of 0.5 percent low melting agarose, which was then dispersed as the third layer on the slides. The slides were then incubated overnight at 4°C in cell lysis buffer (0.2 M NaOH, 2.5 M NaCl, 10 mM Tris–HCl, 100 mM Na2EDTA, 10% dimethyl sulfoxide, and 1%Triton X-100; pH =10.0). The slides were then inserted in a horizontal gel electrophoresis tank with electrophoresis solution (300 mM NaOH and 1 mM Na2EDTA; pH = 13). The electrophoresis was carried out for 25 minutes at 25 V (1 V/cm, 300 mA). The slides were then submerged in ultrapure water three times and air-dried after incubated 10 minutes in neutralization buffer (0.4 M Tris–HCl; pH =7.5). Under a fluorescence microscope, the cells were examined after being stained with 50 µl of ethidium bromide (5 mg/L). To avoid further DNA damage, all procedures were performed in low light. The percentage of DNA damage events was calculated by manual counting [12].

Statistical Analysis

Statistics were evaluated to be significant at P values under 0.05 ($P<0.05$). One-way ANOVA for this research was performed statistically using GraphPad Prism software version 8.01.

RESULTS AND DISCUSSION

In vivo comet assay results are depicted in Table.2. & Figure.1. for the number of different classes of comet events that occurred and in Table.3. & Figure.2. for the percentage of DNA damage. Classes 1, 2, 3, 4, and 5 were indicating the extent of DNA damage that occurred during the experimentation (Figure.3.). Figure.4. showed images of comets that appeared in all groups. According to the research study's findings, none of the D-P doses substantially altered the DNA damage values compared to the vehicle control group at all concentrations. Contrarily, in the positive control group, the DNA damage increased substantially ($P<0.001$). Comparing DOX alone treated group to the D-P prior-administration with DOX treated group exposed a significant ($P<0.001$) dose-dependent reduction in DNA damage. As the concentration of D-P increased, the protection against DOX-induced DNA damage was also increased. As a result, it was revealed that higher D-P concentrations were shown to have greater protection.Due to its potential to cause secondary tumours, the antineoplastic medication DOX is believed to trigger a particular sort of toxicity in normal tissues (12). The most serious acute toxicities caused by DOX are genotoxicity [2], cardiotoxicity and suppression of bone marrow (13). The comet test is a quick and reliable tool to find out whether some cells have DNA damage that are brought on by genotoxic substances [14]. To determine the extent of DNA damage, the generated images from the comet test consisting of comets are examined. In the *in vivo* comet experiments, DOX treatment alone increased DNA damage enormously. The majority of comets detected after DOX treatment belonged to classes 2, 3, 4, and 5. D-P only treated groups demonstrated a high number of class 1 comets. D-P at all concentrations when pre-administered with DOX, effectively reduced the percentage of DNA damage that occurred due to DOX-induced genotoxicity. Hence D-P prior treatment to DOX is having genoprotection against DOX-induced genotoxicity. This genoprotective action of D-P would be due to its antioxidant [15] andanti-inflammatory properties [16].

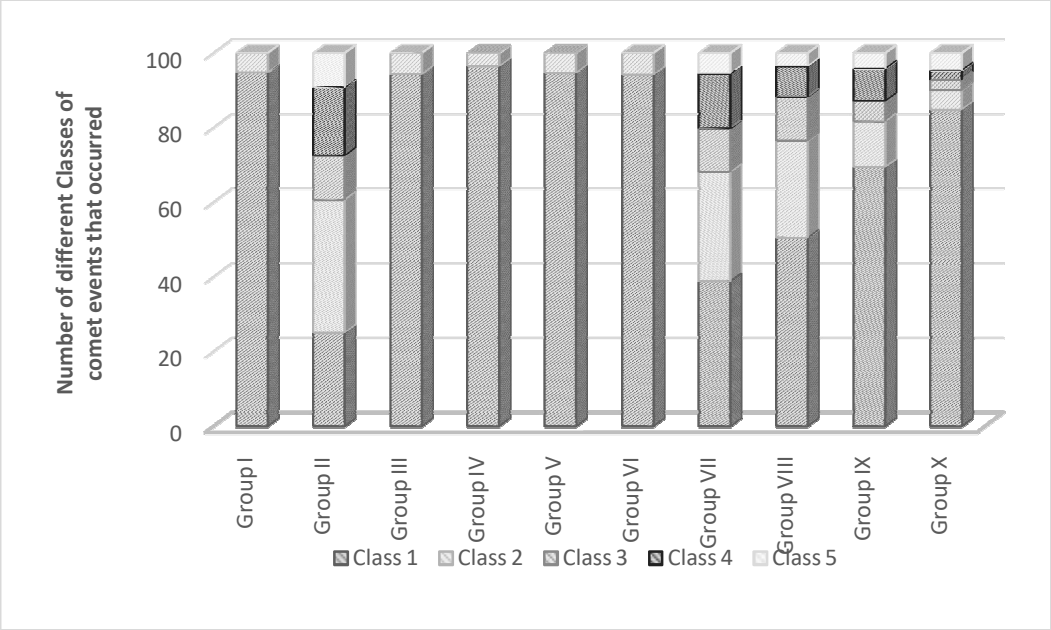

Figure.1. Histogram - Nature of comet events occurred in treated groups of mice

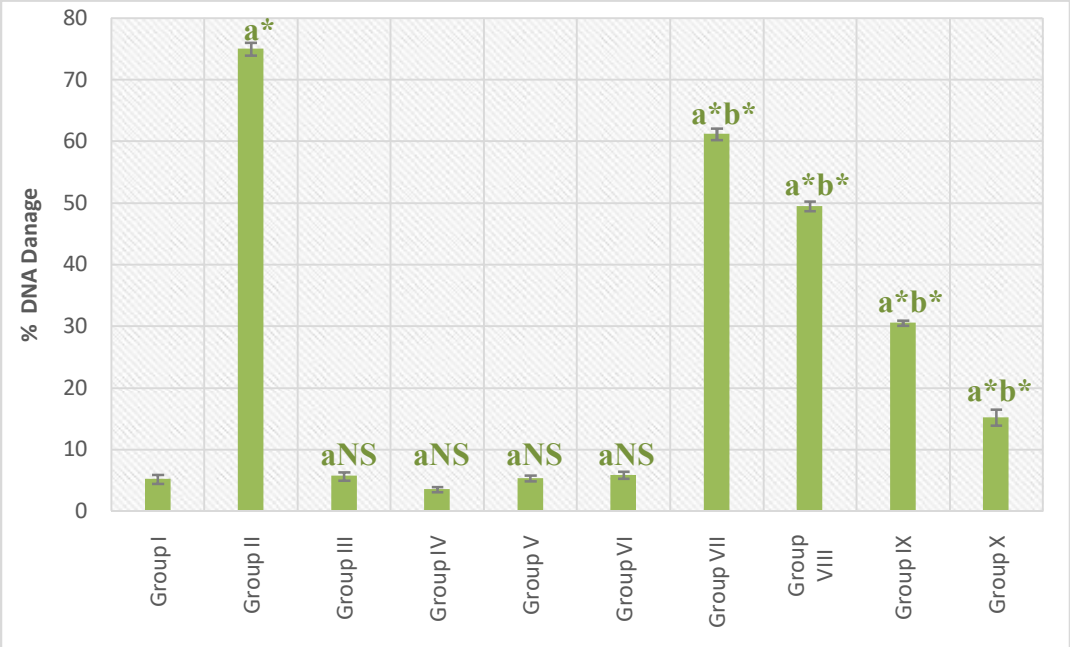

Figure.2. Histogram - Percentage of DNA Damage

Figure.3. Images of Classes of Comet events: a) Class 1 - no damage, <5%; b) Class 2 - low level damage, 5–20%; c) Class 3 - medium level damage, 20–40%; d) Class 4 - high level damage, 40–95%; e) Class 5 - total damage, >95%.

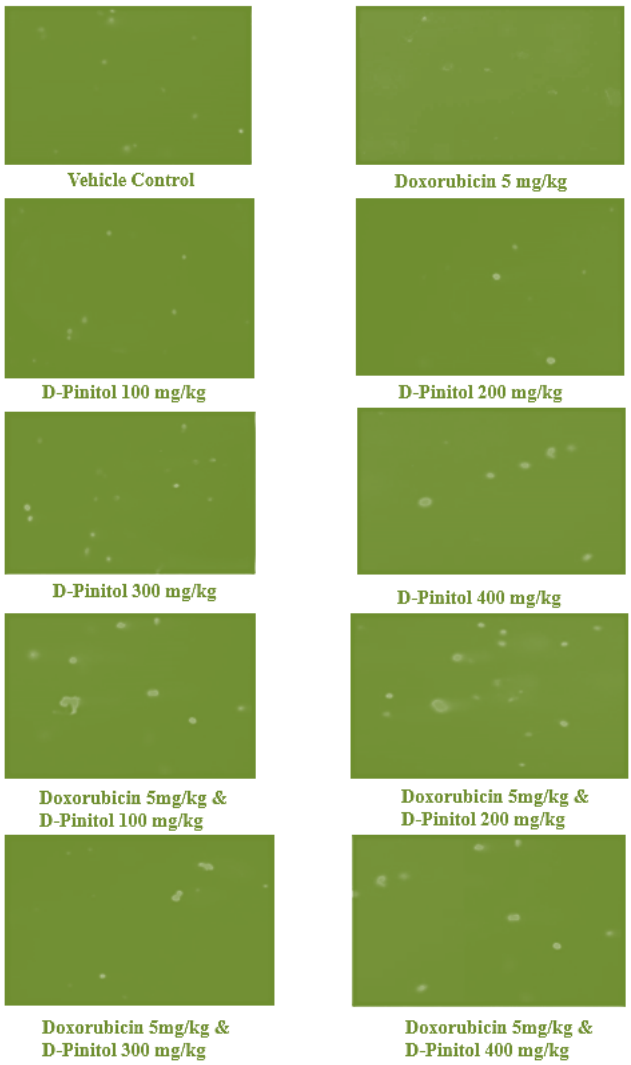

Figure.4. Images of Comets visualized by *in vivo* comet assay

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

Table.2. Number of different classes of comet events that occurred in treated groups of mice

Nature of Comet events	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)
Class1	94.83± 0.703	25± 1.065	94.33± 0.667	96.5± 0.428	94.67± 0.494	94.17± 0.6	38.83± 0.946	50.5± 0.764	69.5± 0.428	84.83± 1.302
Class2	5.167± 0.703	35.67± 0.803	5.667± 0.667	3.5± 0.428	5.333± 0.494	5.833± 0.6	29.33± 0.919	26± 1.693	12.17± 0.654	5.167± 0.6
Class3	0	11.83± 0.601	0	0	0	0	11.67± 0.558	11.83± 0.703	5.5± 0.764	2.833± 0.477
Class4	0	18.33± 0.882	0	0	0	0	14.67± 0.667	8.167± 0.601	8.667± 0.803	2.5± 0.224
Class5	0	9.167± 0.98	0	0	0	0	5.5± 1.258	3.5±0.0 .847	4.167± 1.014	4.667± 1.382

Mean ± SEM, n=6.

Table.3. Effect of DOX and D-P on Percentage DNA damage in 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)
% DNA Damage	5.167± 0.703	75± 1.065 a*	5.667± 0.667 aNS	3.5± 0.428 aNS	5.333± 0.494 aNS	5.833± 0.601 aNS	61.17± 0.946 a*b*	49.5± 0.764 a*b*	30.5± 0.428 a*b*	15.17± 1.302 a*b*

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. * $P < 0.001$. # $P < 0.01$. @ $P < 0.05$.

CONCLUSION

In germ cells, D-P exhibits a genoprotective role on DOX-induced genotoxicity. The genotoxic evaluation of D-P revealed that it did not induce any genotoxic effects. The antioxidant and anti-inflammatory properties of D-P would be the foremost reason for its genoprotective effect.

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LIST OF ABBREVIATIONS

DOX – Doxorubicin
D-P – D–Pinitol

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

CONFLICT OF INTEREST

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

INFORMED CONSENT

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

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