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Genoprotective effect of D-Pinitol isolated from aerial parts of Soybean plants against Doxorubicin-induced genotoxicity evaluated by *in vitro* comet assay in Vero cell lines

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ABSTRACT

Doxorubicin is a chemotherapeutic agent with a genotoxic effect on normal cells at its therapeutic dose itself. D-Pinitol is an abundantly available carbohydrate in Soybean plants and has been proven for antioxidant and anti-inflammatory activities. Our investigation was examined by *in vitro* comet assay to explore the genoprotective effect of D-Pinitol in normal cells against Doxorubicin-induced genotoxicity in Vero cell lines. *In vitro* comet assay treatment groups were: Vero Cell lines with culture medium (control group), Doxorubicin (0.15μg/ml), D-Pinitol (0.05×10^3 mM, 0.125×10^3 mM, and 0.25×10^3 mM) alone, and pretreatment with D-Pinitol (0.05×10^3 mM, 0.125×10^3 mM, and 0.25×10^3 mM) before Doxorubicin (0.15μg/ml) treatment. When compared to the control group, D-Pinitol alone treated groups showed no significant changes in the percentage of DNA damage. For the evaluation of the genoprotective effect of D-Pinitol, the % DNA damage in the D-Pinitol, and Doxorubicin simultaneously treated groups were compared to the Doxorubicin alone treated group. The results showed that Doxorubicin-induced genotoxic effect in Vero cell lines was significantly reduced by D-Pinitol in a dose-dependent manner by reducing DNA damage. Our findings confirmed that D-Pinitol had no genotoxic effect and it showed a genoprotective effect against Doxorubicin-induced genotoxicity.

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INTRODUCTION

Doxorubicin (DOX) is a potent antibiotic of anthracycline class in anticancer drugs. Although DOX is very useful for treating various types of human cancers, having severe side effects at its therapeutic dose is one of the most undesired outcomes of using it. Hence, management of its side effects is essential to achieve patient's treatment, tolerability, and overall quality of life (Remesh, 2012). Previous reports suggested that oxidative stress and free radicals production are the main reasons for DOX-induced cardiotoxicity, cytotoxicity, and genotoxicity (Hajra *et al.*, 2018). Apart from these side effects, DOX also induces severe inflammatory responses in various organs, including the kidney, liver, blood

vessels, and intestine. The previous reports also showed an increase in pro-inflammatory cytokine levels after DOX administration (Wang *et al.*, 2016; Xu *et al.*, 2008). Since DOX has an important role in cancer treatment, it is very crucial to minimize its toxic effects on normal cells. This reduction in DOX-induced toxicity to normal cells can be attained by simultaneous administration of free radical scavenging agents, antioxidants and anti-inflammatory agents. Decreasing DOX's toxicity by attenuating oxidative stress and pro-inflammatory mediators is a forthcoming therapeutic approach against DOX-induced toxicity (Hajra *et al.*, 2018).

Hence, the compounds that can scavenge free radicals and reduce the levels of pro-inflammatory mediators will safeguard the normal cells from DNA damage when they subjected to the genotoxic agents (Čabarkapa *et al.*, 2014). D-Pinitol has been identified as a major carbohydrate present in *Glycine max* L. Merr. (Streeter, 2001; Sripathi and Poongothai, 2013). It has been reported for its free radical scavenging capacity (Orthen *et al.*, 1994), antioxidant activity (Rengarajan *et al.*, 2014; Sivakumar *et al.*, 2010), anti-inflammatory activity (Kim *et al.*, 2005a; Singh *et al.*, 2001), hepatoprotective effect (Choi *et al.*, 2009; Zhou *et al.*, 2008), and Cardioprotective effect (Kim *et al.*, 2005b). These therapeutic effects of D-Pinitol may be useful in the prevention of the genotoxic effect of DOX on normal cells. This research's main goal was to evaluate the genoprotective effect of D-Pinitol in the Vero cell line from the DNA damage induced by DOX through *in vitro* comet assay. The *in vitro* Comet assay presents a visual technique to analyze DNA damage in the cells when treated with chemicals inducing toxic effects (Visvardis *et al.*, 2000). Hence this assay is an advantageous, fast, and effective *in vitro* method for studying the genotoxicity of chemical agents (Tice *et al.*, 2000; Anderson *et al.*, 1994).

MATERIALS AND METHODS

Materials Required

Doxorubicin HCL (TCI chemicals, India), D-Pinitol isolated from aerial parts of Soybean (*Glycine max* L. Merr.,) plants, DMEM medium (Gibco, USA), Fetal Bovine Serum (Gibco, USA), Antibiotic solution (Gibco, USA), Ethidium Bromide (Merck, India), Low melting agarose (Merck, India), Normal agarose (Merck, India), Phosphate Buffer Saline Solution (Himedia, India) and Olympus BX 50 microscope (Olympus Optical Co., Germany).

Methodology - *In vitro* Comet Assay

Cell line and cell culture

Vero cell lines (African green monkey kidney cells) were cultured in liquid Dulbecco's Modified Eagle's medium (DMEM) enriched with 10 percentage Fetal Bovine Serum (FBS), 100 u/ml penicillin and 100 μg/ml streptomycin, and maintained under an atmosphere of 5 percentage CO_2 stored at 37^{o}C.

Briefly, Vero cells were incubated at 37°C for 24 h in a humidified 5% CO_2 incubator after they were seeded (density of 10,000 cells/well) in a six-well plate. After the wells were washed with sterile Phosphate Buffer Saline Solution (PBS), the cell lines were treated with D-Pinitol and DOX as per the treatment protocol given in Table 1. Trypsinization was done before harvesting of cells in a 1.5 ml tube. The microscopic slides were coated first with 200 μl of 0.75 % normal melting agarose (the first layer) and 100 μl of 0.5% low melting agarose (the second layer). Next, the slides were distributed with 20 μl cell suspensions in 60 μl of 0.5% low melting agarose (the third layer). Then incubation of slides was done in cell lysis buffer (2.5 M NaCl, 0.2 M NaOH, 100 mM Na_2EDTA, 10 mM Tris–HCl, 1% Triton X-100 and 10% dimethyl sulfoxide, pH =10.0) for overnight at 4°C. After that, the slides were immersed in double- distilled water three times, followed by 20 min incubation with unwinding solution (3M NaOH). The slides were subsequently placed in a horizontal gel electrophoresis tank containing electrophoresis solution (1 mM Na_2EDTA and 300 mM NaOH, pH =13). The electrophoresis was conducted at 25 V (1 V/cm, 300 mA) for 25 min. The incubation of slides was done in neutralization buffer (0.4 M Tris–HCl, pH = 7.5) for 10 min followed by immersion in ultrapure water for three times and then air-dried. The staining of cells was done with 50 μl of ethidium bromide (5 mg/L) and then the cells were observed under a fluorescent microscope. 15 minutes after staining, the comets of each group were examined on the microscope at 100 X magnification. In order to minimize extra DNA damage, all steps in the procedure were carried out in dim light. The percentage of DNA damage events was calculated by manual counting. Comets were visually scored and classified into five classes, 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)based on the level of DNA migration (Anderson *et al.*, 1994). The experiment was performed in triplicate for each group and, the analysis was performed on100 cells for each experiment (Singh *et al.*, 1988; Nandhakumar *et al.*, 2011).

Statistical analysis

Table 1: Treatment protocol

Groups	Labeled	Treatment
I	Control	Vero Cell lines with culture medium
II	Positive Control	Doxorubicin 0.15μg/ml
III		D-Pinitol 0.05$\times 10^3$ mM
IV		D-Pinitol 0.125$\times 10^3$ mM
V		D-Pinitol 0.25$\times 10^3$ mM
VI	Tests	Doxorubicin 0.15μg/ml + D-Pinitol 0.05$\times 10^3$ mM
VII		Doxorubicin 0.15μg/ml+D-Pinitol 0.125$\times 10^3$ mM
VIII		Doxorubicin 0.15μg/ml +D-Pinitol 0.25$\times 10^3$ mM

Selection of doses of Doxorubicin and D-Pinitol were based on the articles Abd-ElAziz et al., and Al-Ashaal et al., respectively (Al-Shdefat *et al.*, 2014; Al-Ashaal *et al.*, 2012).

Statistical analysis was performed by One way ANOVA method. Values were expressed as mean and for n = 6. All data were analyzed with The GraphPad Prism 8.0.1 software. A difference at $P < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

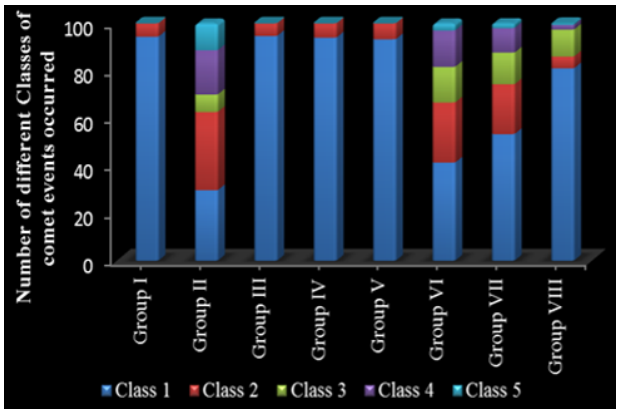

Figure 1: Histogram of number of different Classes of Comet events occurred in Vero cell lines

We assessed the effect of D-Pinitol for its ability to prevent DOX-induced genotoxicity. The values were represented as the mean number of different classes of comets that occurred in all groups (Table 2 and Figure 1) and as the percentage of DNA damage (Table 3 and Figure 2). Images of comets that occurred in all groups were shown in Figure 3. The obtained data elucidates that all three doses of D-Pinitol did not express any significant differences in the percentage of DNA damage values in D-Pinitol alone treated groups when compared to the control group. While increased levels of DNA damage was detected in cells treated only with DOX (0.15μg/ml), a significant reduction in the levels of DNA damage was seen in the Vero cell lines treated simultaneously with DOX (0.15μg/ml) and three different concentrations of D-Pinitol (0.05$\times 10^3$ mM, 0.125$\times 10^3$ mM and 0.25$\times 10^3$ mM) when compared with DOX alone treated group (Tables 2 and 3 & Figures 1 and 2). All three doses of D-Pinitol hopefully decreased the induction of DNA damage by DOX. Concentration-response of D-Pinitol indicated that protection against DOX-induced DNA damage was more intense, with an increase in D-Pinitol concentration. The increased DNA damage reduction was observed in the Vero cell lines pretreated with 0.25$\times 10^3$ mM concentration of D-Pinitol. Hence, the higher concentration of D-Pinitol showed a more significant protective effect.

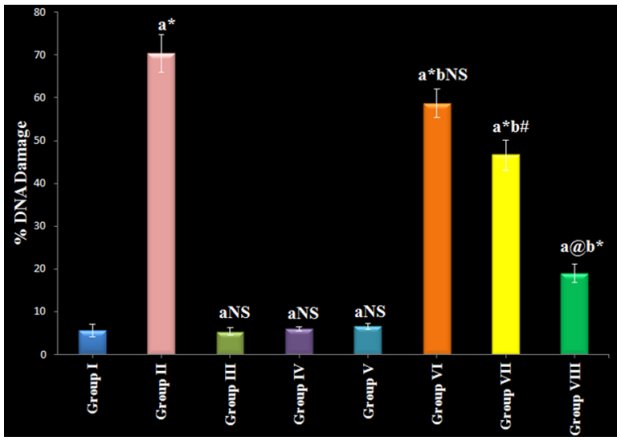

Figure 2: Histogram of genoprotective effect of D-Pinitol on Doxorubicin-induced genotoxicity by *in vitro* comet assay in Vero cell lines

The examination of dietary components with antioxidant and anti-inflammatory activities has gotten much consideration because the essential factors in the generation and progression of numerous chronic diseases were oxidative stress and inflammation (Arts and Hollman, 2005). D-Pinitol has been shown its beneficial effects in several experimental models of diseases emerging and worsening because of oxidative stress and inflammation.

Table 2: Number of different classes of comet events occurred in Vero cell lines

Nature of Comet events	Group I(Vero Cell lines and culture medium)	Group II(DOX 0.15 μg/ml)	Group III(D-P 0.05$\times$ 10^3 mM)	Group IV(D-P 0.125$\times$ 10^3 mM)	Group V(D-P 0.25$\times$ 10^3 mM)	Group VI(DOX 0.15μg/ml +D-P 0.05$\times$ 10^3mM)	Group VII(DOX 0.15μg/ml +D-P 0.125$\times$ 10^3mM)	Group VIII(DOX 0.15μg/ml +D-P 0.25$\times$ 10^3mM)
Class 1	94.33 $\pm$ 1.453	29.67 $\pm$ 4.372	94.67 $\pm$ 0.882	94 $\pm$ 0.577	93.33 $\pm$ 0.667	41.33 $\pm$ 3.283	53.33 $\pm$ 3.528	81 $\pm$ 2.082
Class 2	5.667 $\pm$ 1.453	33 $\pm$ 0.577	5.333 $\pm$ 0.882	6 $\pm$ 0.577	6.667 $\pm$ 0.667	25.33 $\pm$ 1.764	21 $\pm$ 1.155	5 $\pm$ 0.577
Class 3	0	7.333 $\pm$ 0.667	0	0	0	15 $\pm$ 1.528	13.33 $\pm$ 0.882	11.33 $\pm$ 0.882
Class 4	0	18.67 $\pm$ 2.333	0	0	0	15.33 $\pm$ 0.882	10.33 $\pm$ 1.202	2 $\pm$ 0.577
Class 5	0	11.33 $\pm$ 1.764	0	0	0	3 $\pm$ 0.577	2 $\pm$ 0.577	0.667 $\pm$ 0.333

DOX – Doxorubicin;D-P – D-Pinitol

Table 3: Effect of D-Pinitol on Doxorubicin-induced genotoxicity by *in vitro* comet assay in Vero cell lines

Criterion	Group I(Vero Cell lines and culture medium)	Group II(DOX 0.15 μg/ml)	Group III(D-P 0.05$\times$ 10^3 mM)	Group IV(D-P 0.125$\times$ 10^3 mM)	Group V(D-P 0.25$\times$ 10^3 mM)	Group VI(DOX 0.15μg/ml + D-P 0.05$\times$ 10^3 mM)	Group VII(DOX 0.15μg/ml + D-P 0.125$\times$ 10^3 mM)	Group VIII(DOX 0.15μg/ml + D-P 0.25$\times$ 10^3 mM)
% DNA Damage	5.667$\pm$ 1.453	70.33$\pm$ 4.372 $a*$	5.333 $\pm$ 0.882 aNS	6 $\pm$ 0.577 aNS	6.667 $\pm$ 0.667 aNS	58.67 $\pm$ 3.283 $a*bNS$	46.67 $\pm$ 3.528 $a*b\#$	19 $\pm$ 2.082 $a@b*$

DOX – Doxorubicin;D-P – D-Pinitol. Mean $\pm$ SEM, n=6, where, a- Group II, III, IV, V, VI, VII &VIII compared with Group I. b- Group VI, VII & VIII compared with Group II.* P < 0.001, $^\#$ P < 0.01 & $^@$ P <0.05

It's antioxidant (Rengarajan *et al.*, 2014; Sivakumar *et al.*, 2010) and anti-inflammatory properties (Singh *et al.*, 2001; Kim *et al.*, 2005a) were well reported.

DOX, an anthracycline anticancer agent, can induce reactive oxygen species (ROS) generation with severe inflammatory responses. DNA damage caused by inflammatory Reactive Oxygen Species can inevitably lead to disturbance of genetic stability. Hence the production of free radicals and pro-inflammatory mediators is the primary mechanism responsible for DOX genetic toxicity and DNA damage to normal cells (Quiles *et al.*, 2002).

The genotoxic evaluation of D-Pinitol by *in vitro* comet assay in Vero cell lines indicated that this compound did not induce any genotoxic effects. This investigation also showed the capacity of D-Pinitol to protect oxidative DNA damage caused by DOX in Vero cell lines. In our results, all concentrations of D-Pinitol showed a genoprotective effect against DOX-induced genotoxicity. The protective effect of D-Pinitol is possibly due to the result of the expression of several molecular pathways such as ROS scavenging effect through its antioxidant capacity and atten-

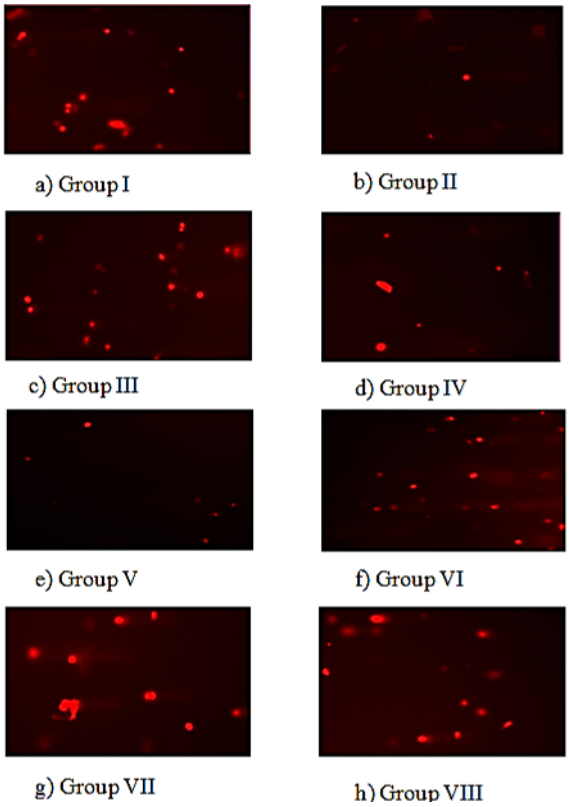

Figure 3: Images of Comets visualized by comet assay in Vero cell lines

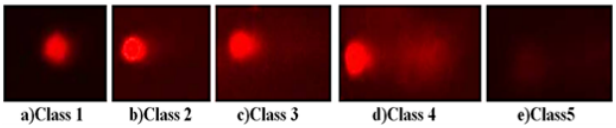

Figure 4: Images of Classes of Comet events

uation of pro-inflammatory mediators by its anti-inflammatory property. Genoprotective effect of D-Pinitol was more pronounced at the larger concentration than smaller concentrations.

CONCLUSIONS

Our findings showed that D-Pinitol was not genotoxic to Vero cell lines. Our investigation confirmed the genoprotective effect of D-Pinitol against DOX-induced genotoxicity by protecting normal cell lines from DNA damage.

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Conflict of Interest

The authors declare that they have no conflict of interest for this study.

REFERENCES

Al-Ashaal, H. A., Fahmy, M. A., Melek, F. R., Aly, N. H., Hasan, Z. M. 2012. Effect of supplemented soybean (Glycine max L) diet and extracts on aluminum sulfate-induced genotoxicity. *Toxicological & Environmental Chemistry*, 94(5):965–986. ISSN 0277-2248, 1029-0486.

Al-Shdefat, R. I., Abd-ElAziz, M. A., Al-Saikhan, F. I. 2014. Genoprotective and Genotoxic Effects of Thymoquinone on Doxorubicin-Induced Damage in Isolated Human Leukocytes. *Tropical Journal of Pharmaceutical Research*, 13(12):2015–2020. ISSN 1596-5996, 1596-9827.

Anderson, D., Yu, T., Phillips, B. J., Schmezer, P. 1994. The effect of various antioxidants and other modifying agents on oxygen-radical-generated DNA damage in human lymphocytes in the Comet assay. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, 307:261–271. ISSN 0027-5107.

Arts, I. C., Hollman, P. C. 2005. Polyphenols and disease risk in epidemiologic studies. *The American Journal of Clinical Nutrition*, 81(1):317S–325S. ISSN 0002-9165, 1938-3207.

Choi, M. S., Lee, M. K., Jung, U. J., Kim, H. J., Do, G. M., Park, Y. B., Jeon, S. M. 2009. Metabolic response of soy pinitol on lipid-lowering, antioxidant and hepatoprotective action in hamsters fed-high fat and high cholesterol diet. *Molecular Nutrition & Food Research*, 53(6):751–759.

Hajra, S., Patra, A. R., Basu, A., Bhattacharya, S. 2018. Prevention of doxorubicin (DOX)-induced genotoxicity and cardiotoxicity: Effect of plant derived small molecule indole-3-carbinol (I3C) on oxidative stress and inflammation. *Biomedicine & Pharmacotherapy*, 101:228–243. ISSN 0753-3322.

Kim, J. C., Shin, J. Y., Shin, D. H., Kim, Y. B., Park, S. C., Park, R. D., Shin, Y. C. 2005a. Synergistic antiinflammatory effects of pinitol and glucosamine in rats. *Phytotherapy Research*, 19(12):1048–1051.

Kim, J. I., Kim, J. C., Kang, M. J., Lee, M. S., Kim, J. J., Cha, I. J. 2005b. Effects of pinitol isolated from soybeans on glycaemic control and cardiovascular risk factors in Korean patients with type II diabetes mellitus: a randomized controlled study. *European Journal of Clinical Nutrition*, 59(3):456–458. ISSN 0954-3007, 1476-5640.

Nandhakumar, S., Parasuraman, S., Shanmugam, M. M., Rao, K. R., Chand, P., Bhat, B. V. 2011. Evaluation of DNA damage using single-cell gel electrophoresis (Comet Assay). *Journal of Pharmacology and Pharmacotherapeutics*, 2(2):107–111. ISSN 0976-500X.

Orthen, B., Popp, M., Smirnoff, N. 1994. Hydroxyl radical scavenging properties of cyclitols. *Royal Society of Edinburgh*, 102:269–272. ISSN 0269-7270, 2053-5910.

Quiles, J. L., Huertas, J. R., Battino, M., Mataix, J., Ramírez-Tortosa, M. C. 2002. Antioxidant nutrients and adriamycin toxicity. *Toxicology*, 180(1):79–95. ISSN 0300-483X.

Remesh, A. 2012. Toxicities of anticancer drugs and its management. *International Journal of Basic & Clinical Pharmacology*, 1(1):2–12. ISSN 2319-2003.

Rengarajan, T., Rajendran, P., Nandakumar, N., Balasubramanian, M. P., Nishigaki, I., Haribabu, L. 2014. Free radical scavenging and antioxidant activity of D-Pinitol against 7, 12 dimethylbenz (a) anthracene induced breast cancer in sprague dawley rats. *Asian Pacific Journal of Tropical Disease*, 4(5):384–390. ISSN 2222-1808.

Singh, N. P., McCoy, M. T., Tice, R. R., Schneider, E. L. 1988. A simple technique for quantitation of low levels of DNA damage in individual cells. *Experimental Cell Research*, 175(1):184–191. ISSN 0014-4827.

Singh, R. K., Pandey, B. L., Tripathi, M., Pandey, V. B. 2001. Anti-inflammatory effect of (+)-Pinitol. *Fitoterapia*, 72(2):168–170. ISSN 0367-326X.

Sivakumar, S., Palsamy, P., Subramanian, S. P. 2010. Attenuation of oxidative stress and alteration of hepatic tissue ultrastructure by D-Pinitol in streptozotocin-induced diabetic rats. *Free Radical Research*, 44(6):668–678. ISSN 1071-5762, 1029-2470.

Sripathi, S. K., Poongothai, G. 2013. A review on Insulinomimetic Pinitol from plants. *International Journal of Pharma and Bio Sciences*, 4(2):992–1009.

Streeter, J. G. 2001. Simple partial purification of D-Pinitol from soybean leaves. *Crop Science*, 41(6):1985–1987. ISSN 0011-183X.

Tice, R. R., Agurell, E., Anderson, D., Burlinson, B., Hartmann, A., Kobayashi, H., Miyamae, Y., Rojas, E., Ryu, J. C., Sasaki, Y. F. 2000. Single cell gel/comet assay: Guidelines for in vitro and in vivo genetic toxicology testing. *Environmental and Molecular Mutagenesis*, 35(3):206–221. ISSN 0893-6692.

Čabarkapa, A., Živković, L., Žukovec, D., Djelić, N., Bajić, V., Dekanski, D., Spremo-Potparević, B. 2014. Protective effect of dry olive leaf extract in adrenaline induced DNA damage evaluated using in vitro comet assay with human peripheral leukocytes. *Toxicology in Vitro*, 28(3):451–456. ISSN 0887-2333.

Visvardis, E. E., Haveles, K. S., Pataryas, T. A., Margaritis, L. H., Sophianopoulou, V., Sideris, E. G. 2000. Diversity of peripheral blood mononuclear cells as revealed by a novel multiple microgel "Comet assay". *Environmental and Molecular Mutagenesis*, 36:32–39. ISSN 0893-6692, 1098-2280.

Wang, L., Chen, Q., Qi, H., Wang, C., Wang, C., Zhang, J., Dong, L. 2016. Doxorubicin-Induced Systemic Inflammation Is Driven by Upregulation of Toll-Like Receptor TLR4 and Endotoxin Leakage. *Cancer Research*, 76(22):6631–6642.

Xu, Z., Lin, S., Wu, W., Tan, H., Wang, Z., Cheng, C., Lu, L., Zhang, X. 2008. Ghrelin prevents Doxorubicin-induced cardiotoxicity through TNF-alpha/NF-κB pathways and mitochondrial protective mechanisms. *Toxicology*, 247(2-3):133–138.

Zhou, Y., Park, C. M., Cho, C. W., Song, Y. S. 2008. Protective Effect of Pinitol against D-Galactosamine-Induced Hepatotoxicity in Rats Fed on a High-Fat Diet. *Bioscience, Biotechnology, and Biochemistry*, 72(7):1657–1666.