

# Synergistic Antineoplastic and Immunomodulatory Effects of Hesperidin in Ehrlich Ascites Carcinoma Tumor Model Treated with Cisplatin

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## Abstract

Despite substantial advancements in cancer research in recent years, the treatment of cancer remains fraught with difficulties. Cisplatin is a strong chemotherapeutic drug used to treat a variety of cancers, but its efficacy is limited by drug resistance and toxic effects on non-tumor tissues. The flavonoid hesperidin was reviewed to have anticancer properties. In the present study, a tumor model was established, cisplatin plus hesperidin synergism was conducted, antineoplastic and immunomodulatory effects were evaluated; oxidative stress markers and liver and kidney function tests were measured after mono and combined treatments. The mechanisms of hesperidin synergistic role with cisplatin chemotherapy were explored using ninety mice divided equally into nine groups. The findings indicated that hesperidin treatment alone, or in combination with cisplatin, inhibited tumor growth by causing cell cycle arrest, stimulating apoptosis, and reducing tumor cell proliferation. Furthermore, it stimulated anti-tumor immunity by increasing the proportion of T cytotoxic ( $CD3^+CD8^+$ ) and T helper ( $CD3^+CD4^+$ ) cells in the spleen with modulating effect on  $CD4^+CD25^+$  regulatory cells accompanied by improving spleen cells proliferation. Moreover, a significant increase in IFN- $\gamma$  and granzyme B levels of tumorized mice co-treated with 100 or 200 mg/kg hesperidin plus cisplatin was demonstrated. Interestingly, the use of hesperidin in combined treatment succeeded to counteract toxic effects induced by cisplatin on normal healthy tissues. In conclusion, the combined use of hesperidin and cisplatin has a synergistic effect that enhances the efficacy of cancer treatment by inducing apoptosis and regulating the immune response against cancer cells. Additionally, this combination therapy reduces the harmful effects of cisplatin on healthy tissues.

**Keywords:** Hesperidin; Antineoplastic; Immunomodulatory; antitoxic; Cisplatin; Tumor model.

## 1. Introduction

The malignant nature of cancer is a great challenge in modern medicine and scientific research. Despite the tremendous efforts dedicated to discovering methods for treating cancer and overcoming its poor prognosis and recurrence, it remains the most common cause of global mortality accounting for approximately ten million deaths in 2020 (Garcia et al., 2020; Sung et al., 2021). Chemotherapeutics are the most common and powerful drugs used in elimination of rapidly proliferating tumor cells even in sites far from their primary origin (Schirmacher, 2019; Sarbaz et al., 2022). The use of chemotherapeutics is clinically limited due to their severe side and toxic effects in recipient body during treatment of malignant tumors (Liu et al., 2016; Brown et al., 2019; Aliwaini et al., 2020; Zavattaro et al., 2021; Fouad et al., 2022).

Cisplatin (Cis) is an inorganic compound comprising two chlorine atoms and two ammonia groups surrounding a central platinum atom (Pourmadadi et al., 2022). It is considered as a potent chemotherapeutic drug used for treating various human cancers, such as ovarian, breast, bladder, esophageal, cervical, head and neck, brain, and

lung cancer, alone or combined with other medications (Sleijfer et al., 1985; Pourmadadi et al., 2022). The cytotoxic effect of Cis stands on its ability to inhibit the replication of rapidly proliferating cells through the formation of Cis-DNA adducts and induction of apoptosis (Siddik, 2003). Unfortunately, there are many challenges facing Cis in treating cancer including drug resistance and the potential for toxic effects on non-tumor tissues (Al-Kharusi et al., 2013; Dasari and Tchounwou, 2014; Dasari et al., 2022). Therefore, many studies aimed to develop strategies to overcome the chemotherapy drawbacks using natural products or drugs to modulate one or more mechanisms of chemoresistance, enhance their antineoplastic efficiency, and prevent toxic effects on non-tumor tissue (Osman et al., 2015; Liu et al., 2016; Donia et al., 2018; Schirmacher, 2019; Xu et al., 2023).

Flavonoids are one of the fundamental subtypes of dietary polyphenols present in plants, vegetables, and fruits (Liu et al., 2014; Rodríguez-García et al., 2019). These are regarded as the most crucial phytochemical compounds that exhibit beneficial effects on various cancerous tissues (Spatafora and Tringali, 2012; Darband et al., 2018; Sudhakaran et al., 2019). Hesperidin (HDN) is one of the most remarkable flavonoids abundant in

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numerous citrus fruits such as oranges and lemons (Devi et al., 2015). There have been reports indicating that HDN exhibits a broad spectrum of biological characteristics, functioning as a strong anti-inflammatory, antioxidant, cardioprotective, neuroprotective, antimicrobial, immunomodulatory, antiviral, antiallergic, antiatherosclerotic, and anticancer compound (Aishatwi et al., 2013; Hassouna et al., 2015; Roohbakhsh et al., 2015; Ahmadi and Shadboorestan, 2016; Barreca et al., 2017). More importantly, Aggarwal et al. (2020) stated that HDN can reverse cancer cells resistance against antineoplastic drugs when used in combination with them, which makes it a promising anticancer candidate. Thus, the aim of our study was to investigate the potential anticancer properties and immunomodulatory effects of two doses of HDN supplementation in mice inoculated with Ehrlich ascites carcinoma (EAC) tumor cells and subsequently treated with Cis.

## 2. Material and methods

### 2.1. Chemicals:

Hesperidin was purchased from Sigma-Aldrich (St. Louis, MO, USA) and was dissolved in a solution of 0.9% sodium chloride. Cisplatin was purchased from Mylan SAS pharmaceutical company (Saint-Priest, France).

### 2.2. Tumor cell line preparation:

The EAC cell line was generously supplied by the National Cancer Institute (NCI), Cairo University, Egypt. To maintain the cell line, we performed successive intraperitoneal (i.p.) injections of  $2.5 \times 10^5$  viable tumor cells in 200  $\mu$ L saline, carried out every two weeks, as outlined in Ibrahim et al. (2018). These injections were administered into healthy naïve mice within our laboratory.

### 2.3. Animals:

Adult female Swiss albino mice (*Mus musculus*), with an age range of 6 to 8 weeks and a weight of 25-30 g, were purchased from NCI, Cairo University, Egypt. The animals were adapted for two weeks before the experiment in standard cages under optimum housing conditions, with free and unlimited access to both food and water. The study was performed after the consent of the institutional Animal Ethical Committee, Menoufia University, with approval ID: (MUFS/ F / PH /1/23).

### 2.4. Experimental design:

For the intended protocol, nine experimental groups were established each comprising ten randomly chosen female mice. Mice in all groups were i.p. inoculated with  $2.5 \times 10^5$  EAC cells at day (d0) except in group I, II & III.

Group I: control group, injected with 0.2mL saline i.p. at d0.

Group II and III: HDN 100mg and 200mg groups, received 14 consecutive oral doses of HDN (100mg/kg b.w./day) and (200mg/kg b.w./day), respectively, from d1 to d14 according to (Berköz et al., 2021).

Group IV: EAC group, inoculated i.p. with  $2.5 \times 10^5$  EAC cells at d0 (Ibrahim et al., 2018).

Group V: EAC+Cis group, EAC bearing mice injected i.p. with Cis (2mg/kg b.w.) at d3, d5 & d7 according to El-Bolkiny et al. (2021).

Group VI and VII: EAC+HDN 100mg and EAC+HDN 200mg groups, EAC bearing mice orally administrated with 14 consecutive doses of HDN as in group II and III, respectively.

Group VIII and IX: EAC+Cis+HDN 100mg and EAC+Cis+HDN 200mg groups, treated with Cis as in group V in addition to the 14 oral doses of HDN as in group II and III, respectively.

### 2.5. Sampling:

On the 15<sup>th</sup> day following the i.p. injection of EAC cells, samples of blood were assembled from retro-orbital sinuses of each mouse. The collected blood was divided into two tubes one mixed with EDTA for CBC analysis, and the other was allowed to coagulate for serum separation. The obtained serum was stored at -80°C for future use. Subsequently, mice were sacrificed with cervical dislocation and dissected to harvest ascitic fluid from the peritoneal cavity. In addition, the spleen was extracted from each mouse for further analysis.

### 2.6. EAC growth response:

Tumor growth was evaluated by changes in the total count of EAC cells, the count of viable and nonviable EAC cells, using trypan blue dye exclusion assay and the rate of tumor growth inhibition as detailed in Ibrahim et al. (2018).

### 2.7. Blood count analysis:

Hematological parameters, platelet count, total and differential white blood cell counts, red blood cell count, hemoglobin concentration, and hematocrit value, were manually assessed in EDTA-treated blood samples from each mouse. The methodology followed was in accordance with Dacie and Lewis (1984).

### 2.8. Apoptosis detection in EAC cells:

Using flow cytometer (BD Accuri C6, San Jose, CA, USA) along with appropriate software (San Jose, CA, USA), tumor early and late apoptotic cells were discriminated as mentioned previously by Ibrahim et al. (2018). Shortly, tumor cells were harvested, washed, and incubated in PBS at 4°C for thirty minutes before staining them with annexin-V (FITC)/propidium iodide (PI) at 25°C for fifteen minutes according to the manufacturing instructions of the commercial kit (Abcam, Canada).

### 2.9. EAC cell cycle detection:

The percentage of EAC cells in different phases of cell cycle was measured using MODFIT DNA analysis program (Verity Software House, Topsham, ME, USA, version: 2.0). A BD Accuri C6 flow cytometer and the suitable software (San Jose, CA, USA) were used for phase distribution analysis. After fixation and washing of tumor cells, they were treated with RNAase A and stained finally with PI according to Morsi et al. (2022).

### 2.10. Preparation of spleen cell suspension and its phenotypic analysis:

A single-cell suspension of splenocytes was prepared according to Ibrahim et al. (2010). After cell counting, using hemocytometer and viability exclusion method by trypan blue dye, T helper ( $CD3^+CD4^+$ ), T cytotoxic ( $CD3^+CD8^+$ ), and T regulatory ( $CD4^+CD25^+$ ) cells were detected using anti-mouse monoclonal antibodies including Fluorescein isothiocyanate (FITC)-labelled CD3

(clone: 17A2), Phycoerythrin (PE)-labelled CD25 (clone: PC61), Allophycocyanin (APC)-labelled CD4 (clone: RM4-5), and Phycoerythrin-cyanine 5 (PE.Cy5)-labelled CD8 (clone: 53-6.7) according to their manufacturing instructions. The monoclonal antibodies were purchased through BD Bioscience Company (BD Bioscience CO, USA), and the assessments were conducted using flow cytometer (BD Accuri C6, San Jose, CA, USA) with the suitable software (San Jose, CA, USA).

### 2.11. Proliferation marker Ki67:

Flowcytometric analysis to detect the Ki67 nuclear protein in both tumor and spleen cells was conducted using BD Accuri C6 flow cytometer and the suitable software (San Jose, CA, USA). Cells were suspended in PBS at a concentration of one million cells per milliliter, and flowcytometric analysis was carried out using mouse Ki67 antibody (Santa Cruz Biotechnology, Inc., Texas, USA) according to manufacturer's instructions.

### 2.12. Granzyme B and interferon $\gamma$ (IFN- $\gamma$ ) detection:

Sandwich enzyme linked immunosorbent assay (ELISA) was used to detect serum granzyme B (CUSABIO, CSB-E08720m, USA) and IFN- $\gamma$  (CUSABIO, CSB-E04578m, USA). The ELISA procedure was conducted in accordance with the instructions outlined in the manufacturer's kit.

### 2.13. Biochemical analysis:

Serum samples were utilized for the assessment of oxidative stress biomarkers, including the levels of malondialdehyde (MDA; Elabscience, E-BC-K025-M, USA) and reduced glutathione (GSH; Elabscience, E-BC-K030-M, USA). The colorimetric assays were conducted in accordance with the instructions furnished by the manufacturer's kit. Moreover, the collected serum was employed to determine further biochemical parameters, including the activity of alanine aminotransferase (ALT; Linear Chemicals, REF 1105000, Spain), aspartate aminotransferase (AST; Linear Chemicals, REF 1109000, Spain), as well as the levels of total protein (SPINREACT, Ref: 1001291, Spain), albumin (SPINREACT, Ref: MX1001020, Spain), urea (SPINREACT, Ref: 1001333, Spain), and creatinine (SPINREACT, Ref: MD1001111, Spain).

### 2.14. Statistical analysis:

Data are expressed as mean  $\pm$  standard error of mean (SEM). Statistical disparities among the groups were assessed through a one-way analysis of variance (ANOVA) test using the IBM SPSS Statistics software for Windows, Version 22 (IBM Corp., Armonk, NY USA) followed by least significant differences (LSD) post hoc test for multiple comparisons.  $P < 0.05$  was considered to be statistically significant.

## 3. Results

### 3.1. Hesperidin and cisplatin synergistic effect on tumor burden:

Mono-treatment of EAC-bearing mice with Cis or with the two different doses of HDN and dual treatment of

EAC-bearing mice with Cis and each dose of HDN led to a highly significant ( $P < 0.001$ ) reductions in ascitic fluid volume, total tumor cell count, and tumor cell viability in comparison to the untreated EAC group (Table 1) with the greatest tumor scavenging effect induced by combined treatment with 200 mg/kg HDN and Cis, while comparison with Cis treated group showed that tumorized mice co-treated with Cis and 100 mg/kg of HDN had no significant ( $P > 0.05$ ) effect on ascitic fluid volume, total tumor cell count or tumor cell viability. On the contrary, co-treatment with 200 mg/kg HDN (in EAC+Cis+HDN 200mg/kg group) exerted a significant ( $P < 0.05$ ) reduction in the volume of ascitic fluid ( $P < 0.05$ ), total tumor cell count ( $P < 0.001$ ), and their viability ( $P < 0.05$ ). Moreover, our study clarified that the highest rate of tumor inhibition was achieved by dual-treatment of animals bearing EAC with Cis and 200 mg/kg of HDN to be 77.88%, while the rate of tumor inhibition was nearly similar in EAC+Cis group (54.07%) and EAC+Cis+HDN100 mg/kg group (57.76%) as shown in Table 1.

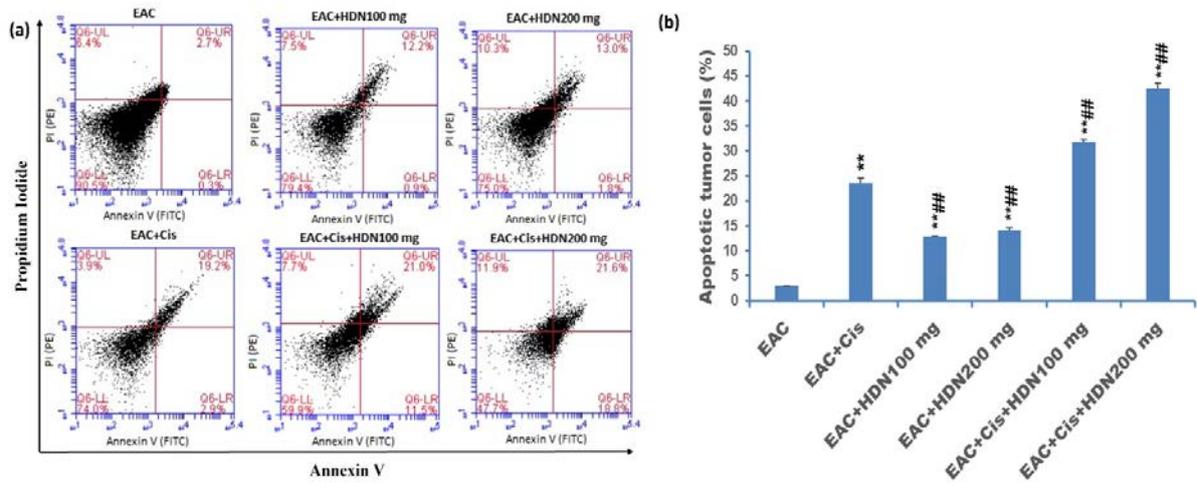
**Table 1.** Anti-tumor effects of mono- or combined-treatments in EAC-bearing mice

	Ascitic fluid volume (mL)	Tumor cells count ( $10^6$ /mL)	Tumor cells viability (%)	Tumor inhibition rate (%)
EAC	18.5 $\pm$ 0.44	75.30 $\pm$ 1.25	99.33 $\pm$ 0.04	0
EAC+Cis	2.13 $\pm$ 0.21 **	34.58 $\pm$ 0.98 **	68.90 $\pm$ 2.05 **	54.07
EAC+HDN 100 mg	13.16 $\pm$ 0.40 *** <sup>#</sup>	66.13 $\pm$ 2.15 *** <sup>#</sup>	92.78 $\pm$ 1.23 *** <sup>#</sup>	12.17
EAC+HDN 200 mg	10.00 $\pm$ 0.36 *** <sup>#</sup>	63.78 $\pm$ 1.75 *** <sup>#</sup>	76.79 $\pm$ 0.60 *** <sup>#</sup>	15.29
EAC+Cis+H DN100 mg	1.71 $\pm$ 0.10 **	31.80 $\pm$ 0.82 **	69.93 $\pm$ 3.01 **	57.76
EAC+Cis+H DN200 mg	1.21 $\pm$ 0.08 *** <sup>#</sup>	16.65 $\pm$ 0.57 *** <sup>#</sup>	59.72 $\pm$ 2.80 *** <sup>#</sup>	77.88

Values are displayed as mean  $\pm$  standard error of mean (SEM), n = 6. \*  $P < 0.05$  significantly different from EAC group. \*\*  $P < 0.001$  significantly different from EAC group. #  $P < 0.05$  significantly different from Cis-treated mice. ##  $P < 0.001$  significantly different from Cis-treated mice. EAC: Ehrlich ascites carcinoma, HDN: hesperidin, Cis: cisplatin.

### 3.2. Hesperidin and cisplatin synergistic effect on tumor cell apoptosis:

To determine the mechanism of action of HDN and/or Cis on EAC tumor cells, flowcytometric analysis of annexin-V/PI (apoptotic marker) expression was evaluated. The influence of Cis and/or HDN on tumor cell apoptosis was demonstrated in Fig. 1. The percentage of total apoptotic cells (annexin-V<sup>+</sup> plus annexin-V<sup>+</sup>/PI<sup>+</sup>) increased significantly ( $P < 0.001$ ) after mono and dual treatments of EAC mice when compared with EAC untreated group. Furthermore, dual treatment with Cis and 100 or 200 mg/kg HDN significantly ( $P < 0.05$ ) raised the rate of tumor cell apoptosis being nearly doubled in EAC+Cis+HDN 200mg/kg (42.45 $\pm$ 1.18%) when compared with EAC+Cis group which recorded an apoptosis rate of 23.65 $\pm$ 0.89%.



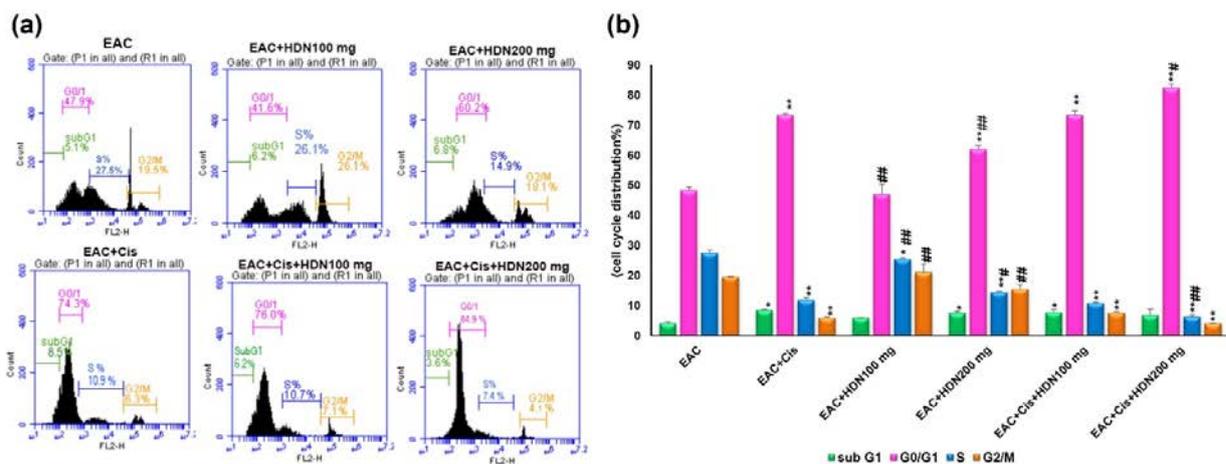
**Figure 1.** Hesperidin and cisplatin synergistically induced tumor cells apoptosis. Apoptotic EAC cells were distinguished by flow cytometry based on their PI/Annexin-V staining patterns. (a) The presented dot plot is representative of one trial from a total of six conducted independently. (b) Pooled data from the six experiments are presented as mean % of apoptotic tumor cells (annexin-V<sup>+</sup> plus annexin-V<sup>+</sup>PI<sup>+</sup>) ± SEM. \*\* *P*<0.001 significantly different from EAC group. ### *P*<0.001 significantly different from Cis-treated mice. EAC: Ehrlich ascites carcinoma, HDN: hesperidin, Cis: cisplatin, PI: propidium iodide.

**3.3. Hesperidin and cisplatin synergistic effect on cell cycle and proliferation of EAC cells:**

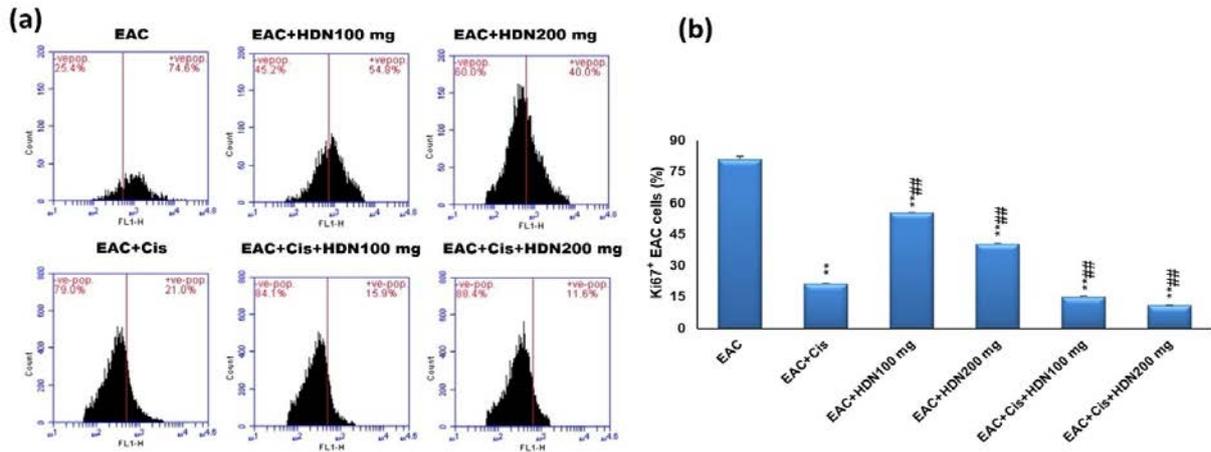
Using flowcytometry, it was possible to investigate the percentage of tumor cells across different phases of cell cycle depending on their DNA content. Results demonstrated in Fig. 2 showed appreciable changes in cell cycle after treating EAC bearing mice with Cis with or without HDN. In comparison with EAC non-treated group, there was highly significant (*P*<0.001) increase in G0/G1 cell population after mono-treatment with Cis (73.43±0.49%) or HDN 200mg/kg (62.10±1.09%) and co-treatment with Cis and HDN 100mg/kg (73.50±1.44%) or Cis and HDN 200mg/kg (82.45±1.41%). Simultaneously, these treatments led to a highly significant (*P*<0.001) decrease in cell fractions at S and G2/M phases except the effect of EAC+HDN 200 mg/kg on G2/M that was not significant (*P*>0.05). Co-treatment with Cis and 200 mg/kg HDN achieved a significant (*P*<0.05) arrest or increase of

tumor cell fraction at G0/G1 phase and significant (*P*<0.01) decline of cell fraction in S phase and non-significant (*P*>0.05) decrease at G2/M phase, when compared with EAC+Cis. Thus, the influence of combined treatment with Cis and 200 mg/kg of HDN produced noticeable cell cycle specificity that highlights the synergetic role of high dose of HDN with Cis.

Furthermore, flowcytometric analysis of Ki67, a marker for cell proliferation, expression in tumor cells was evaluated. Compared with non-treated EAC group, where percentage of Ki67<sup>+</sup> tumor cells was 80.38±1.60 %, all treated groups achieved highly significant (*P*<0.001) reduction in the percentage of Ki67<sup>+</sup> tumor cells and the greatest rate of reduction was recorded in EAC+Cis+HDN 200mg/kg group to be 11.06±0.21% (Fig. 3). Moreover, combined treatment with Cis and 100 or 200 mg/kg of HDN showed a significant (*P*<0.001) decrease in Ki67<sup>+</sup> tumor cells % when compared with EAC+Cis group.



**Figure 2.** Hesperidin and cisplatin synergistically arrest tumor cells' cell cycle. (a) The presented histogram is representative of one trial from a total of six conducted independently showing distribution of tumor cells in different phases of cell cycle. (b) Pooled data showing percentage of EAC cells in different phases of cell cycle. Data are displayed as mean ± SEM of six mice. \* *P*<0.05 significantly different from EAC group. \*\* *P*<0.001 significantly different from EAC group. # *P*<0.05 significantly different from Cis-treated mice. ## *P*<0.001 significantly different from Cis-treated mice. EAC: Ehrlich ascites carcinoma, HDN: hesperidin, Cis: cisplatin.



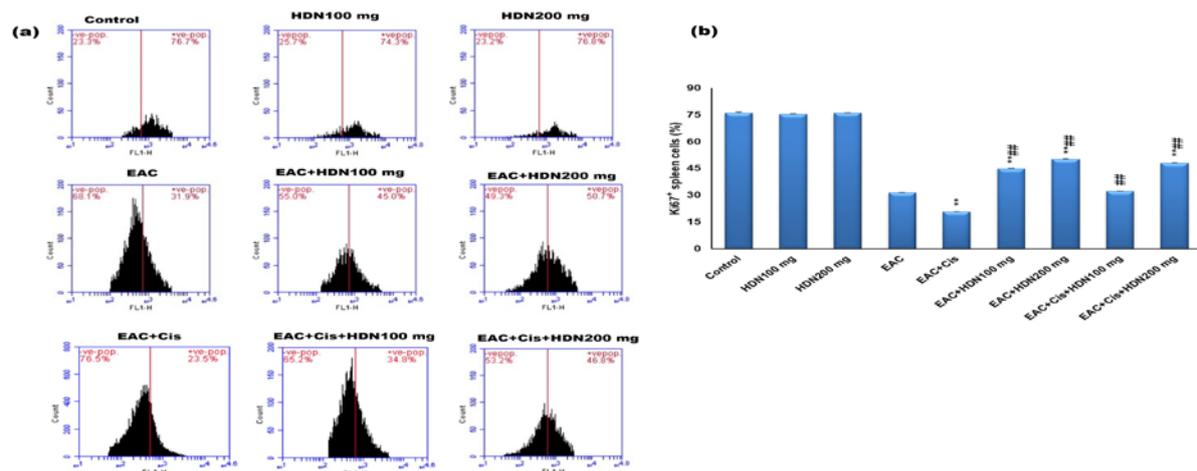
**Figure 3.** Hesperidin and cisplatin synergistically inhibit tumor cells' proliferation. Tumor cells proliferation was evaluated using flow cytometry depending on their Ki67 protein staining patterns. (a) The presented histogram is representative of one trial from a total of six conducted independently. (b) Pooled data from the six experiments are presented as mean % of Ki67<sup>+</sup> cells ± SEM. \*\*  $P < 0.001$  significantly different from EAC group. ###  $P < 0.001$  significantly different from Cis-treated mice. EAC: Ehrlich ascites carcinoma, HDN: hesperidin, Cis: cisplatin.

**3.4. Hesperidin and cisplatin synergistically affect splenic proliferation and immunophenotyping in EAC bearing mice:**

In comparison with EAC group, the percentage of Ki67<sup>+</sup> spleen cells exhibited a significant ( $P < 0.001$ ) decrease after mono treatment with Cis. On the other hand, mono treatments with both doses of HDN significantly ( $P < 0.001$ ) succeeded in increasing its percentage. Remarkably, mono treatments (100 or 200 mg/kg HDN) and combined treatments (Cis with 100 or 200 mg/kg HDN) exerted a significant ( $P < 0.001$ ) improvement in splenic proliferation when compared with Cis mono treated group (Fig. 4).

To investigate the effect of Cis and/or HDN on the alterations of splenocytes immunophenotyping, the expressions of CD3<sup>+</sup>CD4<sup>+</sup>, CD3<sup>+</sup>CD8<sup>+</sup>, and CD4<sup>+</sup>CD25<sup>+</sup>

immune cells were presented in Table 2. Tabular data shows that, CD3<sup>+</sup>CD4<sup>+</sup>, and CD3<sup>+</sup>CD8<sup>+</sup> cell percentages declined significantly ( $P < 0.001$ ) in untreated EAC group, parallel with the significant rise in CD4<sup>+</sup>CD25<sup>+</sup> cells as compared with normal control group. Chemotherapeutic treatment with Cis alone resulted in highly significant ( $P < 0.001$ ) decrease in CD3<sup>+</sup>CD4<sup>+</sup>, CD3<sup>+</sup>CD8<sup>+</sup>, and CD4<sup>+</sup>CD25<sup>+</sup> cells percentage compared to untreated EAC group. In comparison with Cis mono-treated group, mono and combined treatments achieved a significant ( $P < 0.001$ ) improvement in the % of CD3<sup>+</sup>CD4<sup>+</sup>, CD3<sup>+</sup>CD8<sup>+</sup>, and CD4<sup>+</sup>CD25<sup>+</sup> spleen cells. Importantly, the demonstrated results proved the strong modulatory role of HDN doses in conjunction with Cis to alter the immune response against tumor.



**Figure 4.** Hesperidin and cisplatin synergistically improve spleen cells proliferation. Spleen cells proliferation was evaluated by flow cytometry depending on their Ki67 protein staining patterns. (a) The presented histogram is representative of one trial from a total of six conducted independently. (b) Pooled data from six experiments are presented as mean % of Ki67<sup>+</sup> cells ± SEM. \*\*  $P < 0.001$  significantly different from EAC group. ###  $P < 0.001$  significantly different from Cis-treated mice. EAC: Ehrlich ascites carcinoma, HDN: hesperidin, Cis: cisplatin.

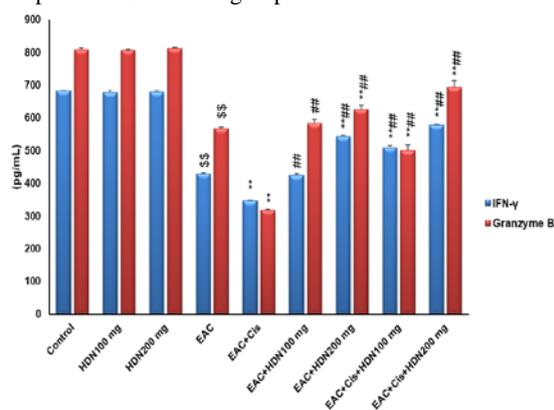
**Table 2.** Hesperidin and cisplatin synergistically improve splenocytes phenotypic analysis in tumor-bearing mice.

	CD3 <sup>+</sup> CD4 <sup>+</sup> (%)	CD3 <sup>+</sup> CD8 <sup>+</sup> (%)	CD4 <sup>+</sup> CD25 <sup>+</sup> (%)
Control	51.15±0.81	36.96±0.36	17.68±0.25
HDN100 mg	50.96±0.93	35.95±0.55	17.15±0.32
HDN200 mg	51.23±0.86	37.03±0.92	17.18±0.39
EAC	15.33±0.13 <sup>SS</sup>	26.15±0.21 <sup>SS</sup>	39.31±0.27 <sup>SS</sup>
EAC+Cis	10.11±0.19 <sup>**</sup>	19.95±0.29 <sup>**</sup>	4.80±0.11 <sup>**</sup>
EAC+HDN 100 mg	18.95±0.30 <sup>**##</sup>	30.20±0.19 <sup>***##</sup>	32.51±0.16 <sup>**##</sup>
EAC+HDN 200 mg	37.10±0.34 <sup>**##</sup>	33.13±0.20 <sup>***##</sup>	22.11±0.09 <sup>**##</sup>
EAC+Cis+HDN 100 mg	22.31±0.18 <sup>**##</sup>	25.61±0.19 <sup>##</sup>	11.63±0.21 <sup>**##</sup>
EAC+Cis+HDN 200 mg	45.65±0.21 <sup>**##</sup>	41.78±0.16 <sup>***##</sup>	23.88±0.18 <sup>**##</sup>

Data are displayed as mean ± SEM, n = 6. <sup>SS</sup> P<0.001 significantly different from control group. <sup>\*\*</sup> P<0.001 significantly different from EAC group. <sup>##</sup> P<0.001 significantly different from Cis-treated mice. EAC: Ehrlich ascites carcinoma, HDN: hesperidin, Cis: cisplatin.

### 3.5. Hesperidin and cisplatin synergistic effect on IFN- $\gamma$ and granzyme B levels in EAC bearing mice:

The impacts of HDN and Cis mono or dual treatments on serum IFN- $\gamma$  and granzyme B levels are depicted in Fig. 5. The data showed that there was highly significant (P<0.001) decrease in their levels in untreated EAC group compared with control group. Additionally, a significant (P<0.001) decline in the level of IFN- $\gamma$  and granzyme B was recorded after chemotherapeutic treatment with Cis alone when compared with EAC group. Conversely, mono-treatment of mice bearing EAC with 200mg of HDN, as well as co-treatment with Cis plus 200mg of HDN, exerted highly significant (P<0.001) increase as compared to EAC group. Moreover, there was significant (P<0.001) increase in IFN- $\gamma$  and granzyme B levels after different mono and combined HDN treatments when compared to Cis treated group.



**Figure 5.** Hesperidin and cisplatin synergistically improve serum IFN- $\gamma$  and granzyme B levels. Data are displayed as the mean ± SEM (n=6). <sup>SS</sup> P<0.001 significantly different from control group. <sup>\*\*</sup> P<0.001 significantly different from EAC group. <sup>##</sup> P<0.001 significantly different from Cis-treated mice. EAC: Ehrlich ascites carcinoma, HDN: hesperidin, Cis: cisplatin.

### 3.6. Hesperidin combination with cisplatin ameliorated hematological changes in EAC bearing mice:

Oral administration of HDN alone, at doses of 100 or 200 mg/kg, did not induce significant (P>0.05) changes in the whole set of hematological parameters (Table 3 and 4) when compared with normal control group. However, inoculation of  $2.5 \times 10^5$  cell of EAC cell line led to a significant (P<0.05) decrease in Hb content (11.76±0.30), RBCs count (6.05±0.24), and Ht value (29.05±0.51) accompanied by a significant increase in platelet count (835.00±10.24) as compared with normal control group (14.24±0.10), (8.08±0.34), (42.80±1.06), and (673.33±9.58), respectively. Chemotherapeutic treatment of EAC bearing mice with Cis caused a significant (P<0.001) reduction in Hb content, RBCs count (P<0.05), Ht value (P<0.001), and platelets count (P<0.001) as compared to EAC untreated group. In comparison with Cis treated group, mono treatment of EAC bearing mice with 100 or 200 mg/kg HDN or combined treatment with Cis and 100 or 200 mg/kg HDN recovered these changes significantly (P<0.001) toward normal values, except the impact of combined treatment with 100 mg/kg HDN and Cis on thrombocytes that was not significant (P>0.05) (Table 3).

Total and relative differential counts of leukocytes were around normal range of control group following treatments of non-tumorized mice with 100 or 200 mg/kg HDN alone as demonstrated in Table 4, although a significant (P<0.05) variation in total and differential leukocyte counts was observed in the untreated EAC group with respect to control group. Treatment with Cis alone resulted in a significant (P<0.001) decrease in total leukocyte count and relative lymphocytes, which was associated with a significant (P<0.001) rise in relative granulocyte count when compared to EAC group. Mono treatment with 100 or 200 mg/kg HDN and combined treatment with Cis and 100 or 200 mg/kg HDN revealed a significant (P<0.05) dose dependent recovery in total and relative differential WBC counts almost toward normal count as compared to Cis treated group. However, the effect on relative monocytes was not significant (P>0.05) when compared to the Cis mono-treated mice.

**Table 3** Alterations in erythrogram of EAC bearing mice upon treatment with cisplatin and/or hesperidin.

	Hb (g/dL)	RBCs (10 <sup>6</sup> /mm <sup>3</sup> )	Ht (%)	Platelets (10 <sup>3</sup> /mm <sup>3</sup> )
Control	14.24±0.10	8.08±0.34	42.80±1.06	673.33±9.58
HDN100 mg	14.23±0.10	7.99±0.15	42.41±0.44	668.16±6.52
HDN200 mg	14.40±0.10	8.23±0.14	42.60±0.28	662.00±7.69
EAC	11.76±0.30 <sup>SS</sup>	6.05±0.24 <sup>SS</sup>	29.05±0.51 <sup>SS</sup>	835.00±10.24 <sup>SS</sup>
EAC+Cis	9.03±0.14 <sup>**</sup>	5.20±0.07 <sup>*</sup>	22.88±0.15 <sup>**</sup>	564.33±7.98 <sup>**</sup>
EAC+HDN 100 mg	12.28±0.22 <sup>###</sup>	6.53±0.15 <sup>##</sup>	32.83±0.65 <sup>*##</sup>	796.00±10.44 <sup>*##</sup>
EAC+HDN 200 mg	13.66±0.26 <sup>*##</sup>	7.21±0.09 <sup>*##</sup>	39.98±1.67 <sup>*##</sup>	668.50±6.27 <sup>*##</sup>
EAC+Cis+H DN100 mg	11.02±0.25 <sup>*##</sup>	6.45±0.15 <sup>##</sup>	30.41±0.81 <sup>**</sup>	576.00±10.44 <sup>**</sup>
EAC+Cis+H DN200 mg	13.78±0.30 <sup>*##</sup>	7.65±0.06 <sup>*##</sup>	43.45±0.46 <sup>*##</sup>	620.33±9.60 <sup>*##</sup>

Data are displayed as mean ± SEM, n = 6. <sup>SS</sup> P<0.001 significantly different from control group. \* P<0.05 significantly different from EAC group. \*\* P<0.001 significantly different from EAC group. ## P<0.001 significantly different from Cis-treated mice. EAC: Ehrlich ascites carcinoma, HDN: hesperidin, Cis: cisplatin, Hb: hemoglobin, RBCs: red blood cells, Ht: hematocrit.

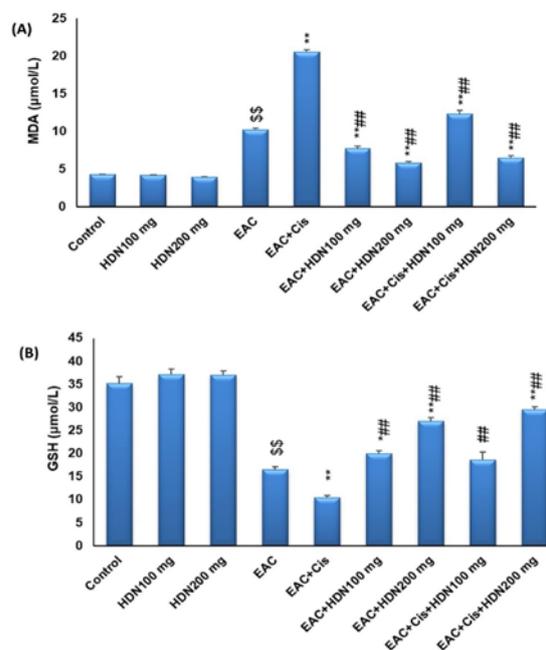
**Table 4.** Alterations in total and differential leukocytic counts of EAC bearing mice upon treatment with cisplatin and/or hesperidin.

	Leukocytes (10 <sup>3</sup> /mm <sup>3</sup> )	Granulocytes (%)	Lymphocytes (%)	Monocytes (%)
Control	8.35±0.13	32.50±0.61	63.16±0.47	4.33±0.21
HDN100 mg	8.23±0.11	32.83±0.47	62.66±0.55	4.50±0.22
HDN200 mg	8.28±0.11	32.66±0.55	63.16±0.70	4.16±0.30
EAC	10.90±0.15 <sup>SS</sup>	51.83±1.07 <sup>SS</sup>	42.83±1.19 <sup>SS</sup>	5.33±0.21
EAC+Cis	5.35±0.06 <sup>**</sup>	65.66±0.42 <sup>**</sup>	29.66±0.33 <sup>**</sup>	4.66±0.21
EAC+HDN100 mg	9.85±0.25 <sup>*##</sup>	51.50±0.42 <sup>##</sup>	44.00±0.93 <sup>##</sup>	4.50±0.76
EAC+HDN200 mg	8.91±0.13 <sup>*##</sup>	40.16±0.30 <sup>*##</sup>	56.00±0.57 <sup>*##</sup>	3.83±0.30 <sup>*</sup>
EAC+Cis+HDN100 mg	6.13±0.13 <sup>*##</sup>	47.00±0.68 <sup>*##</sup>	48.50±0.61 <sup>*##</sup>	4.50±0.42
EAC+Cis+HDN200 mg	7.91±0.25 <sup>*##</sup>	33.83±0.47 <sup>*##</sup>	61.00±0.36 <sup>*##</sup>	5.16±0.30

Data are presented as mean ± SEM, n = 6. <sup>SS</sup> P<0.001 significantly different from control group. \* P<0.05 significantly different from EAC group. \*\* P<0.001 significantly different from EAC group. # P<0.05 significantly different from Cis-treated mice. ## P<0.001 significantly different from Cis-treated mice. EAC: Ehrlich ascites carcinoma, HDN: hesperidin, Cis: cisplatin.

### 3.7. Effect of hesperidin combination with cisplatin on oxidative stress markers in EAC-bearing mice

Mice that received HDN alone showed no significant (P>0.05) change in serum MDA and GSH levels in respect to normal group. On the other hand, EAC bearing mice revealed a significant (P<0.001) rise in MDA levels accompanied with a significant (P<0.001) decline in GSH levels as compared to normal mice; this negative effect was nearly duplicated significantly (P<0.001) after treating EAC mice with Cis alone as compared to EAC untreated mice. On contrary, mono- or combined treatment with HDN or HDN with Cis was able to ameliorate induced oxidative stress through a significant (P<0.001) decline in MDA levels and a rise in GSH levels in a dose dependent manner when compared with Cis treated group (Fig. 6A & Fig. 6B).



**Figure 6.** Hesperidin and cisplatin enhancing effect on serum concentrations of oxidative stress marker MDA (A) and the antioxidant GSH (B). Data are displayed as mean ± SEM, n = 6. \$\$ P<0.001 significantly different from control group. \* P<0.05 significantly different from EAC group. \*\* P<0.001 significantly different from EAC group. ## P<0.001 significantly different from Cis-treated mice. EAC: Ehrlich ascites carcinoma, HDN: hesperidin, Cis: cisplatin, MDA: malonaldehyde, GSH: reduced glutathione.

### 3.8. Hesperidin and cisplatin enhancing effect on other biochemical parameters in EAC bearing mice:

The data presented in Table 5 demonstrates that EAC bearing mice revealed a significant ( $P<0.001$ ) increase in ALT, AST, urea, and creatinine levels associated with a significant ( $P<0.001$ ) decrease in total protein and albumin levels as compared to normal control group. Moreover, mono-treatment with Cis resulted in a significant ( $P<0.05$ ) increase in these hepato-renal toxicity parameters in

**Table 5.** Hesperidin and cisplatin enhancing effect on biochemical changes in EAC bearing mice.

	ALT (U/L)	AST (U/L)	Total protein (g/dL)	Albumin (g/dL)
Control	26.00±4.61	60.66±0.66	6.68±0.06	4.14±0.03
HDN100 mg	27.50±0.99	58.50±0.99	6.55±0.09	4.05±0.04
HDN200 mg	24.16±0.74	58.33±0.84	6.56±0.07	4.03±0.07
EAC	89.83±0.54 <sup>SS</sup>	103.33±1.30 <sup>SS</sup>	5.13±0.08 <sup>SS</sup>	2.61±0.12 <sup>SS</sup>
EAC+Cis	118.33±2.78 <sup>**</sup>	130.33±2.26 <sup>**</sup>	4.17±0.04 <sup>**</sup>	2.25±0.09 <sup>*</sup>
EAC+HDN100 mg	49.33±1.60 <sup>###</sup>	89.16±1.60 <sup>###</sup>	5.58±0.09 <sup>###</sup>	2.98±0.06 <sup>###</sup>
EAC+HDN200 mg	36.16±1.19 <sup>###</sup>	76.33±2.02 <sup>###</sup>	6.03±0.07 <sup>###</sup>	3.35±0.09 <sup>###</sup>
EAC+Cis+HDN100 mg	56.66±1.45 <sup>###</sup>	111.33±1.85 <sup>###</sup>	4.83±0.05 <sup>###</sup>	2.78±0.07 <sup>###</sup>
EAC+Cis+HDN200 mg	42.16±0.83 <sup>###</sup>	76.50±1.23 <sup>###</sup>	6.03±0.06 <sup>###</sup>	3.53±0.12 <sup>###</sup>

Data are displayed as mean ± SEM, n = 6. <sup>SS</sup>  $P<0.001$  significantly different from control group. <sup>\*</sup>  $P<0.05$  significantly different from EAC group. <sup>\*\*</sup>  $P<0.001$  significantly different from EAC group. <sup>###</sup>  $P<0.001$  significantly different from Cis-treated mice. EAC: Ehrlich ascites carcinoma, HDN: hesperidin, Cis: cisplatin, ALT: alanine aminotransferase, AST: aspartate aminotransferase.

## 4. Discussion

Despite substantial advancements in cancer research in recent years, the treatment of cancer remains fraught with difficulties (Du and Shim, 2016; Xu et al., 2023). One of the main reasons for the failure of chemotherapy and radiotherapy to effectively treat cancer in clinics is due to tumor resistance (Bhosle et al., 2005). Therefore, innovative approaches to cancer treatment are crucial. Recently, combining multiple anti-cancer agents has emerged as a promising method in cancer therapies, known as the synergistic effect (Zhong et al., 2019; Kubota et al., 2023). In this investigation, the role of HDN in enhancing the sensitivity of Ehrlich cancer cells to Cis chemotherapy while reducing its side effects on the normal healthy tissues was demonstrated. In the present study, EAC tumor model of albino mice was established, Cis chemotherapy plus HDN synergism were conducted, antineoplastic and immunomodulatory effects were evaluated, oxidative stress markers and liver and kidney function tests were measured after mono and combined treatments and mechanism of HDN synergistic role with Cis chemotherapy were explored.

The results showed that the combination of HDN with Cis effectively suppressed tumor burden through decreasing ascitic fluid volume, tumor cell count and viability, resulting in the highest tumor inhibition rate

comparison with the EAC untreated group. Interestingly, treatment of EAC bearing mice with HDN alone or combined with Cis significantly ( $P<0.001$ ) relieve the negative biochemical effects upon HDN mono-treatment when compared to EAC group and decreased toxic effects induced by Cis on these biochemical parameters when compared to Cis treated group. This positive effect exhibited a dose-dependent trend with HDN treatment, moving the values toward the normal range.

observed. This means that when cancer cells are treated with both chemotherapy drug and HDN, the response to chemotherapy is improved. This enhanced chemosensitivity has been attributed to various mechanisms, including the induction of apoptosis, cell cycle arrest of cancer cells, and the suppression of tumor cell proliferation as demonstrated in our results. The anticancer properties of HDN have been previously documented (Dona et al., 2018; Du et al., 2018; Aggarwal et al., 2020; Jeong et al., 2022).

Interestingly, Wang et al. (2015) documented that HDN was discovered to halt the cell cycle at the G0/G1 phase by decreasing cyclin D1 and increasing p21 and p53 expression. It was also found to activate the endoplasmic reticulum stress pathway, leading to apoptosis in HeLa cells (a type of immortal cervical cancer cells). This process is achieved by decreasing the protein levels of cyclin D1, cyclin E1, and cyclin-dependent kinase 2, which are involved in the cell cycle regulation. In the same line, Pandey et al. (2019) reported that HDN induced ROS-mediated apoptosis in human gall bladder carcinoma, but they found cell cycle arrest at G2/M phase.

Undoubtedly, the immune system plays a crucial role in defending against cancer (Vesely et al., 2011). It has the ability to recognize and destroy abnormal cells, such as cancer cells that are potentially harmful to the body. The process is known as immunosurveillance and involves the activation of immune cells, such as T cells and natural killer cells, to identify and destroy cancer cells. In some cases, the immune system may also stimulate an immune response that directly targets cancer cells, such as the production of cytokines and the activation of an immune response (Pandya et al., 2016).

Within the scope of this study, flow cytometric analysis showed that HDN combined treatment with Cis induced an antitumor immune response by increasing the percentage of splenic T helper ( $CD3^+CD4^+$ ) and T cytotoxic ( $CD3^+CD8^+$ ) cells, while concurrently orchestrating a modulatory effect on  $CD4^+CD25^+$  regulatory cells accompanied by improving spleen proliferation, which regulate the immune response against tumor. Similarly, earlier investigations revealed the synergistic role of combined treatments with Cis on antitumor immunity (Ye et al., 2018; El-Bolkiny et al., 2021; Wang et al., 2022). Hassouna et al. (2015) and Berköz et al. (2021) documented that HDN can improve the immunosuppressive impact of diazinon and cyclophosphamide, respectively. This was achieved by enhancing both adaptive and innate immune responses, and so it is considered as a useful immunomodulatory agent during cancer chemotherapy; these findings strongly support results of the current study.

Cytotoxic  $CD8^+$  T cells are the final executors of antitumor immune action due to their direct cytotoxicity on

cancer cells via secreting IFN- $\gamma$ , triggering Fas-mediated cytotoxicity or perforin induced cell lysis (Martinez-Lostao et al., 2015). IFN- $\gamma$  consistently orchestrates both pro-tumorigenic and antitumor immunity within the tumor microenvironment. It plays a role as a cytotoxic cytokine combined with granzyme B and perforin to initiate tumor cells apoptosis (Tau et al., 2001; Maimela et al., 2018). Interestingly, Jorgovanovic et al. (2020) reviewed the fundamental role of IFN- $\gamma$  in activating cellular immunity and subsequently induction of anti-tumor immune response. The present study revealed a significant increase in IFN- $\gamma$  and granzyme B serum levels of tumorized mice mono-treated with 200 mg/kg HDN and co-treated with 100 or 200 mg/kg HDN plus Cis chemotherapy. These observations collectively suggest that HDN treatment promotes the efficacy of CD8<sup>+</sup> T cells in combating tumors and strengthens their role in anti-tumor immunity. Additionally, these findings shed light on one of the mechanisms through which HDN triggers apoptosis in tumor cells.

The previous results demonstrated the potential of HDN as a standalone agent in cancer treatment, although its effect was enhanced when used in combination with Cis chemotherapy. Thus, while HDN alone may have some potential in treating cancer, its true strength lies in its ability to enhance the efficacy of chemotherapy drugs, making it a promising complementary therapy for cancer treatment.

Chemotherapy exerts severe and long-lasting side effects on normal, healthy cells and tissues. These side effects can significantly impact the quality of life for patients undergoing chemotherapy, and in some cases, they may even be more harmful than the cancer itself. For example, studies have shown that cardiotoxicity induced by chemotherapy can reach to heart failure and death, even in patients with no prior history of heart disease (CadedduDessalvi et al., 2018). Therefore, it is important for healthcare providers to closely monitor patients receiving chemotherapy and take steps to minimize and manage these harmful effects.

In the current study, tumorized animals treated only with Cis exhibited severe anaemia characterized by a notable reduction in RBCs count, Hb content, and Ht value. This anemic condition was accompanied by a sharp decrease in total leukocytes, relative lymphocyte count, and total platelet count. Moreover, in this study chemotherapeutic treatment resulted in oxidative stress that was detected by elevated serum MDA levels and reduced serum GSH levels and, induced liver and kidney toxicity. On the contrary, combined treatment with (HDN and Cis) improved these hematological alterations, hepatorenal toxicity, and oxidative stress toward normal values. Previous studies have reported toxic effects of Cis on bone marrow cell colonies that substantially affect blood cells and induce hematotoxicity (Das et al., 2003; Aldossary 2019; Ibrahim et al., 2020; El-Bolkiny et al., 2021).

The review article by Aldossary (2019) on the toxic effects of Cis highlights several major toxicities associated with the use of this chemotherapy drug which include nephrotoxicity, hepatotoxicity, cardiotoxicity, and neurotoxicity. Recent investigations have demonstrated that hesperidin and other flavonoids can be utilized safely to counter the harmful cytotoxic effects brought on by chemotherapy drugs. This is due to their antioxidant, anti-

tumor, and immunostimulant properties (Korga et al., 2019; Rodríguez-García 2019; Berköz et al., 2021).

## 5. Conclusion

The synergistic effect between hesperidin and cisplatin chemotherapy augments the effectiveness of cancer treatment through inducing apoptosis, suppressing the proliferation of tumor cells, and regulating the immune response. Hesperidin not only successfully enhanced chemosensitivity of cancer cells but also reduced the severity of cisplatin toxic effects on normal tissues. Despite these promising current results, further investigations are required to fully comprehend the molecular mechanisms underneath the current role of hesperidin in enhancing chemosensitivity and to determine the optimal dosing and administration strategies.

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