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ROSMARINIC ACID ENHANCES CISPLATIN CYTOTOXICITY IN HEPG2 CELL LINE AND ATTENUATES ITS NEPHROTOXICITY IN MICE

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ABSTRACT: The present study aimed to investigate the anticancer effect of rosmarinic acid (RA), both alone and in combination with cisplatin (cis). Also, the potential nephroprotective effects of RA against cis-induced nephrotoxicity in mice and the possible mechanisms underlying this protection were explored. Treatment of HepG2 cells with RA produced a significant decline in cellular proliferation. RA induced cell cycle arrest at G_0 / G_1 phase and S phase, significantly inhibited vascular endothelial growth factor (VEGF) concentration and induced caspase-3 level in cell lysate. While pre-treatment with IC_{50} RA before cis addition significantly enhanced cis cytotoxic activity in HepG2 cells. Moreover, Pre-treatment with RA significantly mitigated cis-induced elevation of blood urea nitrogen (BUN) and serum creatinine levels. The elevated levels of malondialdehyde (MDA), tumor necrosis factor- alpha (TNF-α), Bax and caspase-9 in kidney tissues were significantly reversed. Additionally, RA significantly abrogated cis-induced reduction in reduced glutathione level (GSH). The histopathological examination emphasized the obtained results. **Conclusion:** RA possesses cytotoxic activity against HepG2 cell line through cell cycle arrest, induction of caspase-3 and inhibition of VEGF. In addition, RA enhances cis-induced cytotoxicity in HepG2 cells. Also, it ameliorates cis-induced nephro-toxicity in mice; an effect which could be attributed to inhibition of oxidative stress, inflammation and apoptosis.

INTRODUCTION: Chemotherapy using anti-cancer drugs is usually accompanied by many problems such as severe side effects and expression of drug resistance which may require the discontinuation of therapy although it has been shown to represent a significant role in clinical cancer treatment.

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Consequently, increasing the therapeutic index of anticancer drugs without toxicity to normal cells is considered an important concern in chemotherapy [1]. This approach is achieved by using naturally occurring dietary compounds [2]. Despite cis is one of the most effective chemotherapeutic agents for the treatment of several human malignancies [3], yet the inevitable risk of nephrotoxicity limit its use in clinical practice as a powerful anticancer agent [4].

Many pathways are involved in the pathophysiology of cis-induced nephrotoxicity such as oxidative stress, apoptosis, inflammation and necrosis [5]. Oxidative stress occurs as a result of excessive free radicals generation and impaired

antioxidant status and is mostly implicated in the pathogenesis of cis-induced acute renal injury [6]. Several studies have elucidated that TNF-α; a pro-inflammatory cytokine, plays a vital role in the pathogenesis of cis-induced renal injury through binding to its receptors and activating inflammatory and apoptotic signaling pathways [7].

Consequently, it is of great importance to explore ways for the alleviation of the dose-limiting adverse effects of cis at its tumoricidal doses for safer clinical application. Evidence from earlier studies has demonstrated that different antioxidants and anti-inflammatory agents achieve a great effectiveness against deleterious effects of cis on kidney [8]. Rosmarinic acid (RA); a widely occurring natural antioxidant which is found in numerous plant including Lamiaceae species has been proven to possess a broad area of applications [9]. Numerous biological activities of RA are demonstrated in many studies such as antioxidative, anti-inflammatory, neuroprotective, antitumor, anti-allergic and antibacterial [10 - 15]. It exerts antiproliferative activity through induction of cell cycle arrest in G_0/G_1 and G_1/S phase [16] and induces apoptosis in cancer cells *via* mitochondrial pathway [17]. In addition, it has antiangiogenic activity [18].

The aim of the present study was to investigate the cytotoxic effects of RA alone and in combination with cis on HepG2 cell line and to investigate the possible chemotherapeutic effects of RA. Also, the possible protective effect of RA against cis-induced nephrotoxicity in mice through the assessment of oxidative stress, inflammatory, apoptotic markers in the kidney.

MATERIALS AND METHODS:

Drugs and Chemicals: Cisplatin was obtained from Merk Ltd., Cairo, Egypt. Rosmarinic acid, Dimethyl sulfoxide (DMSO) and sulphorhodamine-B (SRB) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Fetal bovine serum, RPMI-1640 medium, trypsin 0.25% and penicillin/ streptomycin were purchased from GIBCO (USA). Anti-human VEGF monoclonal antibody and biotin-labeled anti-human VEDF polyclonal antibody were purchased from R&D System Inc. (Minneapolis, MN). Peroxidase conjugated streptavidin and polyclonal Goat anti-rabbit peroxidase conjugate were purchased from Jackson Immunsearch Lab. (USA). Antibodies to rabbit polyclonal to caspase-3 was purchased from Abcam Inc. (Cambridge, MA, USA). All other chemicals were of highest grade commercially available.

Biodiagnostic Kits: Kits used for determination of blood urea nitrogen level and creatinine level in serum were purchased from Diamond Diagnostic, Cairo, Egypt. Malondialdehyde (MDA) level was assessed using a kit obtained from North-West Life Science Specialties, Vancouver. Kit used for determination of reduced glutathione (GSH) content was purchased from Amsbio AMS, Biotechnology, UK. Protein content in the kidney homogenate was assessed using a kit purchased from Biodiagnostic, Cairo, Egypt. Tumor necrosis factor-α (TNF-α) level was detected using ELISA kit for TNF- α (Uscn Life Science Inc., Wuhan, Hubei 430056, PRC). Bax and Caspase-9 levels were measured using specific ELISA kits for Bax and caspase-9 respectively (Cloud-Clone Corp, USA).

***In-vitro* Studies:**

Cell Lines and Culture Condition: Human liver tumor cell line (HepG2) and human breast cancer cell line (MCF-7) were obtained frozen in liquid nitrogen from the American Type Culture Collection (ATCC, Manassas, VA, USA). They were cultured in RPMI-1640 medium supplemented with 10% FBS, 2 mM L-glutamine, 100 µg/ml penicillin/streptomycin and 1.5 g/L sodium bicarbonate and incubated at 37 ºC in 5 % carbon dioxide. The cell lines were maintained by serial sub-culturing in the Egyptian Organization for Biological Products and Vaccines (VACSERA, Giza, Egypt).

Cytotoxicity Assay: HepG2 and MCF-7 cancer cells were seeded at density of 5×10^3 cells/well in 96-well microtiter plates. After 24 h, Cells were incubated with different concentrations were incubated with different concentrations of RA (100 - 800 µM) completed to total of 200 µl volume/well using fresh medium and incubation was continued for 48 h. Also, HepG2 cells were incubated with different concentrations of CIS (0.8 - 15 ug/ml) for 24 h. For combination analysis, HepG2 cells were pretreated with IC_{50} of RA for 24 h before addition of different concentrations of Cis (0.8-15µg/ml).

Cytotoxicity was determined using Sulphorhod-amine-B (SRB) method according to a previously explained method [19]. After treatment, cells were fixed with 10% trichloro-acetic acid for 1 h at 4 °C. Wells were washed with water and then stained with 50 µl 0.4% SRB in 1% acetic acid for 30 min at 25 °C. The dye was solubilized with 50 µl 10 mM tris base (pH 7.4). Optical density (OD) of each well was measured spectrophotometrically at 570 nm with an ELISA microplate reader (Dynatec medical products, England). MCF-7 cells have shown to be insensitive to the cytotoxic effect of RA. RA 800 µM resulted in only 16.5% decrease in survival fraction compared to control (Data is not shown).

Determination of IC_{50} Values: The percentage of cell survival was calculated as follows:

Survival fraction = O.D. (treated cells) / O.D. (control cells).

Where: O.D. is optical density.

The IC_{50} values (the concentration of RA or cis required to produce 50% inhibition of cell growth) were calculated using sigmoidal dose response curve-fitting models (GraphPadPrizm software, version 5). The experiment was repeated 3 times.

Cell Cycle Analysis: The method was carried out as previously described [20]. HepG2 cells were incubated with various concentrations of 0.5 IC_{50} and IC_{50} of RA for 48 h. Cells were fixed by 70% ethanol and then stored at 4 ºC, washed twice and re-suspended in PBS containing 40 µg/mL PI, 0.1 mg/ml RNase A in darkroom. After 30 min at 37ºC, cells were immediately analyzed with a flow-cytometry (Becton-Dickinson, San Jose, CA, USA). The S-phase fractionation (SPF) was estimated as the percentage of cells occupying the region between the mean channel number for G_0/G_1 and that of G_2/M, measured by the software program for DNA analysis.

Determination of Caspase-3 by Indirect ELISA: The level of caspase-3 in total cell lysate was measured according to a previously described method [21]. Cell lysates were prepared after their respective treatment with 0.5 IC_{50} and IC_{50} of RA for 24 h. The assay was performed by coating 96-well plate with cell lysate and incubation for 1 h at 37 ºC then overnight at 4 ºC. Cell lysates were aspirated and wells were washed 3 times with washing buffer. 200 µl of blocking buffer/well was added and incubated for 1.5 h at 37 ºC. The plate was washed one time, antibodies to rabbit polyclonal to caspase-3 were incubated for 1 h at 37 ºC then washed. Polyclonal Goat anti-rabbit peroxidase conjugate was incubated for 1 h at 37 ºC then washed. The enzymatic reaction was carried out by adding 100 µl/well of substrate buffer. Color development was stopped by adding 100 µl of stopping buffer and the plate was read spectrophotometrically at 450 nm. The level of caspase-3 was expressed as optical densities values.

Determination of VEGF by Sandwich ELISA: The concentration of VEGF in total cell lysate was measured according to a previously described method [22]. Cell lysates were prepared after their respective treatment with 0.5 IC_{50} and IC_{50} of RA for 48 h. The assay was performed by coating a 96-wellplates with anti-human VEGF monoclonal antibody. 50 µl of sample and standard were added in duplicates in the plate and incubated for 2 h at 37ºC then the plates were washed 3 times with washing buffer.

50 µl of prepared biotin-labelled anti-human VEGF polyclonal antibody were added to the wells and incubated for 1 h at 37 ºC then washed. 50 µl of prepared peroxidase conjugated streptavidin were added to the wells and incubated for 1 h at 37 ºC. Then 50 µl of the substrate buffer were added. Color development was stopped by adding 50 µl of stopping buffer and the plate was read spectrophotometrically at 450 nm.

***In vivo* Studies:**

Animals: Male Swiss albino mice weighting 20 - 25 gm obtained from the Egyptian Organization for Biological Products and Vaccines (VACSERA, Giza, Egypt). Animals were randomized and housed (10 - 12 mice/cage) in stainless steel wire bottom cages in conditioned atmosphere at 22 ± 2°C, given tap water *ad libitum*, fed with standard commercial mice chow and left to accommodate for one week before the experiment with 12 h dark / light cycle. Animal care and experimental protocols were approved by the animal care and use ethical committee at the Faculty of Pharmacy, Helwan University.

Experimental Design:

Group 1: Control group; mice received 1% ethanol in distilled water orally for 7 days.

Group 2: RA control group; mice received RA (200 mg/kg, p.o) [23] for 7 days.

Group 3: Cis group; mice received cis (14 mg/kg, i.p.) as a single dose [24] 1 h prior administration of 1% ethanol in distilled water administered orally for 7 days (3 days before cis administration).

Group 4: RA + cis group; mice received v (14 mg/kg, i.p.) as a single dose 1 h prior administration of RA (200 mg/kg, p.o) administered for 7 days (3 days before cis administration).

At the end of the experiment (24 h after the last dose of RA), blood samples were collected from animals by retro-orbital puncture. Animals were then sacrificed, kidney tissues were dissected and washed with cold normal saline. Portions of the kidney tissues were homogenized in ice cold PBS (0.1 M, pH 7.4) using teflon homogenizer (Glas-Col, USA). The homogenates were centrifuged at 10,000 rpm for 30 min at 4 ºC, and the supernatants were stored at - 80 ºC until biochemical assays could be performed. Another portion of kidneys were stored in 10% neutral buffered formalin for histopathological studies.

Determination of Nephrotoxicity Markers: The level of BUN and creatinine were determined according to previously described methods [25, 26].

Determination of Tissue Lipid Peroxidation (Oxidative Stress): MDA is formed from the breakdown of polyunsaturated fatty acids which is considered as a convenient index for the determination of the extent of the peroxidation reaction. The assay is based on the reaction between MDA and thiobarbituric acid (TBA) that results in the formation of an MDA-TBA2 adduct which absorbs strongly at 532 nm [27].

Determination of Tissue Glutathione (GSH) Contents: GSH assessment was carried out as previously described [28], where o-phthalaldehyde (OPA probe) reacts with GSH (not GSSG) generating fluorescence measured at Ex/Em = 340/420 nm.

Determination of Protein Content: The principle of total protein assay is based on the formation of a Cu^{+2}- protein complex under alkaline conditions, forming a purple-blue complex which can be detected photometrically at 550 nm [29].

Determination of Inflammatory Marker: The involvement of inflammation in cis-induced nephrotoxicity was assessed by measuring the level of TNF-α in kidney homogenate of different treated groups. Assessment was performed according to the manufacturer's instructions where samples and standards were added to pre-coated wells with the convenient antibodies and then kept for 2 h. Detection reagents A and B and the substrate were added and incubated according to the manufacturer's instructions. After the addition of the stop solution, the plates were measured at 450 nm spectrophotometrically.

Determination of Apoptotic Markers: The impact of apoptosis in cis-induced nephrotoxicity was determined by measuring Bax and caspase-9 levels in kidney homogenate of different treated groups. Detection was performed according to the manufacturer's instructions where samples and standards were added to precoated wells with the convenient antibodies and then kept for 2 h. Detection reagents A and B and the substrate were added and incubated according to the manufacturer's instructions. After the addition of the stop solution, the plates were measured at 450 nm spectrophotometrically.

Histopathological Examination: Autopsy samples were taken from the kidney of mice indifferent groups, fixed in 10% neutral buffered formalin and embedded in paraffin. Histopathological studies were performed as previously described [30].

Statistical Analysis: Data are presented as mean ± SD. Multiple comparisons were performed using one-way ANOVA followed by Tukey–Kramer test for post hoc analysis, as appropriate. Unpaired t-test and Mann-Whitney test were used to compare two different treatment groups. The 0.05 level of probability was used as the criterion for significance.

All statistical analyses were performed using Graph Pad Prism (ISI®, USA) version 5 software.

RESULTS: *In vitro* Results:

RA Synergistically Enhances the Antitumor Effects of Cisplatin: Cell viability was expressed as the survival fraction compared with untreated control cells as shown in **Fig. 1A**.

Fig. 1A showed the effect of different concentrations of RA (100 - 800 µM) on the percentage of survival of HepG2 cells after 48 h exposure to the RA. There was a significant dose-dependent decrease in cellular proliferation compared to its control.

The treatment of HepG2 cell line with different concentration of Cis (0.8-15 µg/ml) for 24 h significantly reduced the survival fraction of cells in a concentration-dependent manner **Fig. 1B**. IC_{50} of cis was obtained from the fitted survival curve and was found to be 7.75 µg/ml for HepG2 human cancer cells. The sensitivity of the cancer cells to cis cytotoxicity was significantly enhanced when combined with IC_{50} of RA (500 µM). It was shown that pre-treatment of HepG2 cells with RA for 24 h before CIS addition significantly reduced CIS IC_{50} to 1.51 µg/ml **Fig. 1B**.

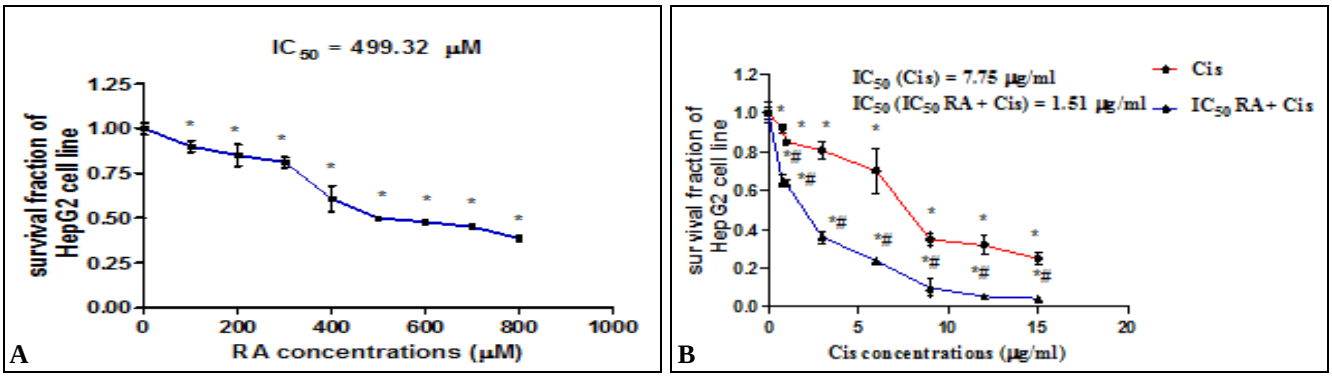

FIG. 1: EFFECT OF PRE-TREATMENT WITH (A) VARIOUS CONCENTRATIONS OF RA (100 - 800 µm) FOR 48 H, (B) CIS (0.8 - 15 µg/ml) ALONE OR IN COMBINATION WITH IC_{50} RA (500 µm) ON CELLULAR PROLIFERATION OF HepG2 CELLS USING SULPHORHODAMINE-B (SRB) METHOD

Each point is the mean ± SD of three independent experiments performed in triplicate. $^{*}P< 0.05$: Statistically significant when compared to the control using one way analysis of variance (ANOVA) followed by Tukey Kramer multiple comparison test. $^{\#}$ $P< 0.05$: Statistically significant when compared to the corresponding group treated with cis alone using Mann-Whitney test.

RA-Induced Cell Cycle Arrest in HepG2 Cells: Treatment with IC_{25} of RA (250 µM) for 48 h caused the accumulation of Go/G1 phase in HepG2 cell which was associated with a considerable drop in S phase population while treatment with IC_{50} RA for 48 h caused the accumulation of S phase in HepG2 cell with a decrease in the proportion of cells in G2/M phase **Table 1**, **Fig. 2**.

TABLE 1: EFFECT OF VARIOUS CONCENTRATIONS OF RA ON THE PERCENTAGE OF G_0/G_1, S, AND G_2/M CELLS AT 48 h FOLLOWING TREATMENT IN HepG2 CELLS

RA concentration (µM)	Percentage of total cells		
	G_0/G_1 phase	S phase	G_2/M phase
Control	74.740 ± 1.489	20.780 ± 0.942	4.480 ± 0.779
1/2 IC_{50} of RA (250 µM)	80.780 ± 0.1607*	15.020 ± 2.431*	4.200 ± 2.590
IC_{50} of RA (500 µM)	74.920 ± 1.199	25.080 ± 1.199*	0*

Data are presented as means ± SD of 3 independent experiments in which the effect of 1/2 IC_{50} and IC_{50} of RA on cell cycle distribution of HepG2 cells was determined by flow cytometric analysis of DNA content using propidium iodide (PI) staining. *: Significantly different from control, $p < 0.05$ using one way analysis of variance (ANOVA) followed by Tukey Kramer multiple comparison test.

RA Increased Caspase-3 Level of the Apoptotic Pathway: The level of caspase-3 in HepG2 cells was significantly increases when the cells were incubated with RA (250 and 500 µM) for 24 h in a dose-dependent manner. HepG2 cells showed 2.72 and 3.94-fold increase in caspase-3 levels after treatment with RA (250 and 500 µM) respectively **Fig. 3**.

RA Inhibited VEGF Concentration in HepG2 Cells: 1/2 IC_{50}, IC_{50} of RA significantly decreased the secretion of VEGF from HepG2 cells. The inhibition of the VEGF concentration by 36.3% and 41.66% at 1/2 IC_{50} and IC_{50} of RA, respectively **Fig. 4**.

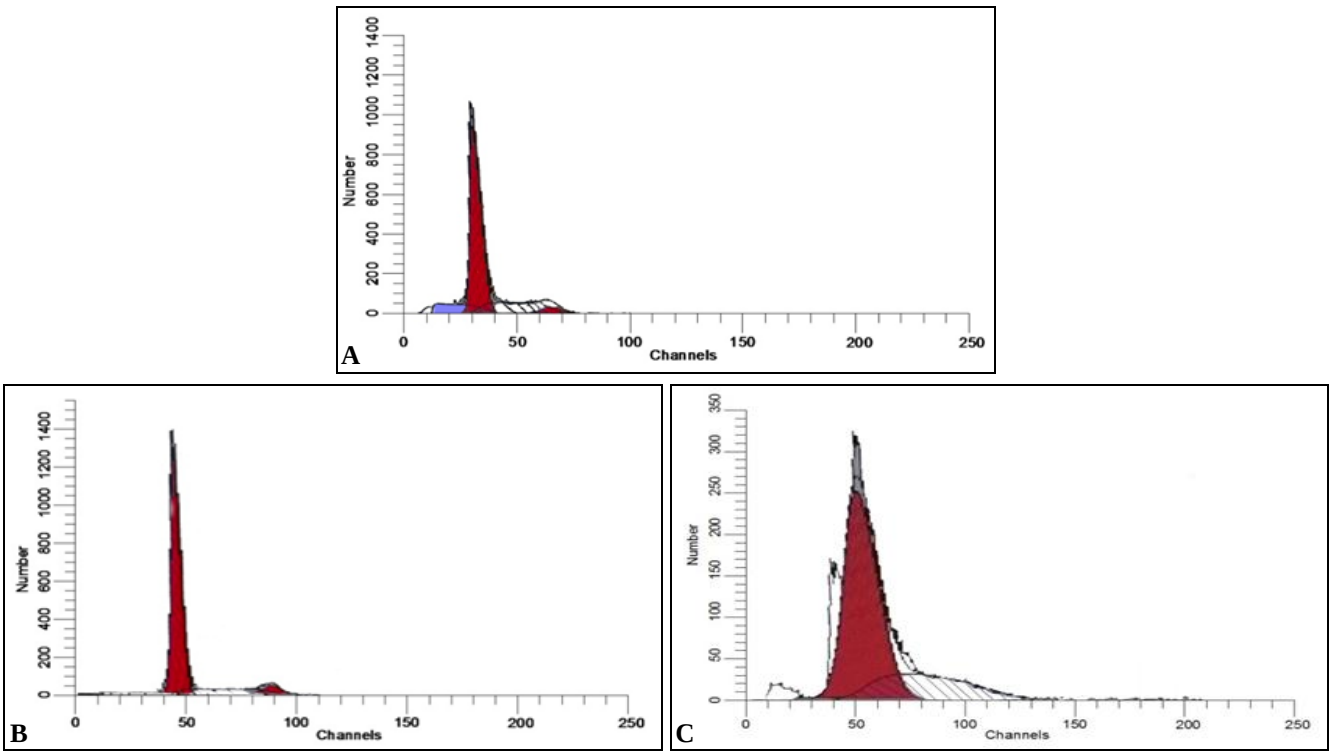

FIG. 2: EFFECT OF RA TREATMENT ON CELL CYCLE PHASE DISTRIBUTION OF HepG2 CELLS. CELL CYCLE DISTRIBUTION WAS ANALYZED AFTER 48 h OF EXPOSURE TO RA BY STAINING WITH PROPIDIUM IODIDE. (A) CONTROL, (B) 0.5 IC_{50} RA (250 µM), (C) IC_{50} RA (500 µM). THE DISTRIBUTION OF CELLS IN THE VARIOUS PHASES OF THE CELL CYCLE WAS DETERMINED USING THE CELLQUEST HISTOGRAM ANALYSIS PROGRAM. Data are mean ± SD from triplicate experiments.

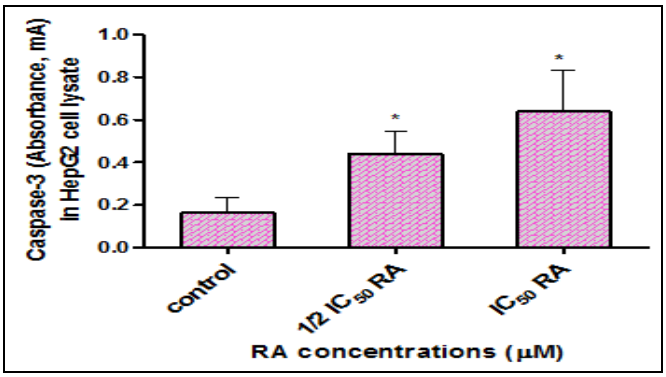

FIG. 3: EFFECT OF VARIOUS CONCENTRATIONS OF RA (250 AND 500 µM) ON THE LEVEL OF CASPASE-3 IN HepG2 CELLS AT 24 h FOLLOWING TREATMENT USING INDIRECT IMMUNOASSAY ELISA TECHNIQUE. Data are presented as mean ± SD of 4 independent experiments, * *P*< 0.05 indicates significant difference from control using one way analysis of variance (ANOVA) followed by Tukey Kramer multiple comparison test.

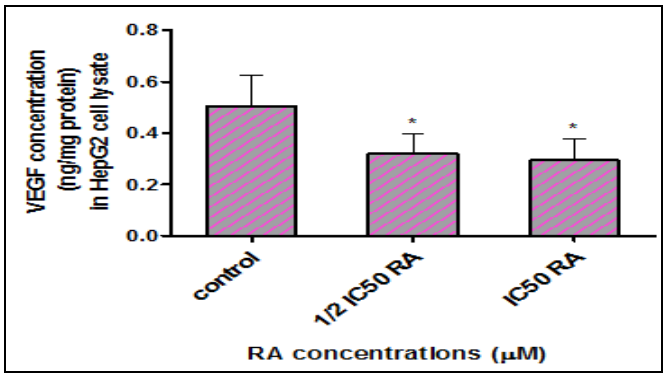

FIG. 4: EFFECT OF VARIOUS CONCENTRATIONS OF RA (250 AND 500 µM) on VEGF CONCENTRATION IN HepG2 CELLS AT 48 h FOLLOWING TREATMENT USING SANDWICH ENZYME LINKED IMMUNO-SORBENT ASSAY (SANDWICH ELISA). Data are presented as mean ± SD of 4 independent experiments.* *P*< 0.05 indicates significant difference from control using one way analysis of variance (ANOVA) followed by Tukey Kramer multiple comparison test.

***In vivo* Results:**

Effect of RA on Nephrotoxicity Parameters: As shown in **Table 2** Cis-treated group showed 5.04- and 5.37- fold increase in serum creatinine and BUN levels, respectively compared to control group. On the other hand, pre-treatment of cis-injected mice with RA (200 mg/Kg, p.o) significantly improved nephrotoxicity markers as manifested by a significant decrease in serum creatinine and BUN levels by 50.80% and 36.42% respectively as compared to the cis group.

TABLE 2: EFFECT OF PRE-TREATMENT WITH RA (200 mg/kg, P.O.) FOR 7 DAYS ON SERUM CREATININE AND BUN IN CIS-INJECTED MICE

Treated groups	Serum creatinine (mg/dl)	BUN (mg/dl)
Control	0.272 ± 0.046	29.120 ± 4.765
RA 200 mg/Kg	0.345 ± 0.039	27.830 ± 3.426
Cis 14 mg/Kg	1.372 ± 0.535*	156.500 ± 57.850 *
RA+ Cis 200 mg/Kg	0.675 ± 0.109#	99.490 ± 33.300 *,#

Values are expressed as Mean ± SD of (n=7). *: Significantly different from control group,#: Significantly different from Cis group, respectively, $p < 0.05$ using ANOVA followed by Tukey-Kramer as post-hoc test, BUN: Blood urea nitrogen

Effect of RA on Oxidative Stress Markers: Cis administration significantly depleted GSH levels in kidney by 67.8% as well as a significantly increased MDA levels by 3.8 - folds compared to control values. In contrast, Cis group pre-treated with RA (200 mg/Kg, p.o) significantly elevated GSH levels by about 148.45% as compared to cis group. Also, MDA levels were significantly reduced by 40.93% compared to cis group. Additionally, compared to control group, animals receiving RA (200 mg/kg, p.o) showed a significant increase in GSH content mounted to 152.7% **Table 3**.

Effect of RA on Inflammatory Markers: Table 3 illustrates the effects of RA treatment on TNF-α level in kidneys of cis-intoxicated mice. Cis treatment resulted in a significant 2.2-fold increase in TNF-*α* levels compared to control group. However, pre-treatment of cis -injected mice with RA (200 mg/Kg, p.o) significantly reduced TNF-α level by 19.5% compared to cis group.

TABLE 3: EFFECT OF PRE-TREATMENT WITH RA (200 mg/kg, P.O) FOR 7 DAYS ON OXIDATIVE STRESS MARKERS AND INFLAMMATORY MARKER IN KIDNEY OF CIS-INJECTED MICE

Treated groups	GSH (µg/gm protein)	MDA (µmol/gm protein)	TNF-α (pg/100 mg tissue)
Control	1.108 ± 0.324	0.874 ± 0.169	28.230 ± 6.658
RA 200 mg/Kg	1.692 ± 0.203 *	0.524 ± 0.134	19.840 ± 3.118 *
Cis 14mg/Kg	0.357 ± 0.091 *	3.300 ± 0.765 *	62.110 ± 5.242*
RA+ Cis 200 mg/Kg	0.887 ± 0.206#	1.949 ± 0.186 *,#	50.020 ± 4.681*,#

Values are expressed as Mean ± SD (n=7), *: Significantly different from control, #: Significantly different from Cis group, respectively, $p < 0.05$ using ANOVA followed by Tukey-Kramer as post-hoc test, GSH: Reduced glutathione, MDA: Malondialdehyde, TNF-α: Tumor necrosis factor-α.

Effect of RA on Apoptotic Markers: To investigate the anti-apoptotic effects of RA in kidney of cis -intoxicated mice, Bax and caspase-9 levels were measured. As shown in **Fig. 5**, cis (3.5 mg/kg, i.p.) induced a 5.82- and 4.1-fold increase in Bax and caspase-9 levels, respectively compared to the control group. However, Apoptotic markers were significantly improved by pre-treatment of cis -injected mice with RA (200 mg/kg, p.o) as evidenced by the significant decrease of Bax and caspases-9 levels by 51.96 and 25.64%, respectively as compared to the cis group.

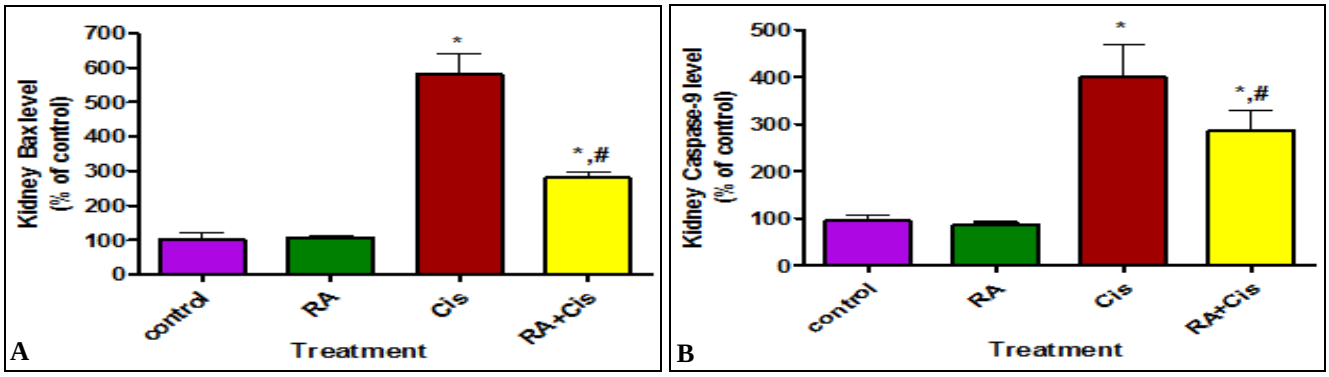

FIG. 5: EFFECTS OF PRE-TREATMENT WITH RA (200 mg/kg, p.o.) FOR 7 DAYS ON (A) BAX AND (B) CASPASE-9 LEVELS IN KIDNEYS OF CIS-INJECTED MICE. Values are expressed as Mean ± SD of 7 mice in each group, *: Significantly different from control, #: Significantly different from Cis group, respectively, $p < 0.05$ using ANOVA followed by Tukey-Kramer as post-hoc test.

Histopathological Studies: Kidney specimens from the control mice showed normal histological structure of the glomeruli and renal tubules at the cortex **Fig. 6A**. Treatment of mice with cis (3.5 mg/kg, i.p.) as a single dose induced degeneration and coagulation necrosis in the cortical tubules. Also, multiple eosinophilic casts in the lumen of the tubules at the medullary portion were observed **Fig. 6B**. Nevertheless, pre-treatment of cis-injected mice with RA (200 mg/kg, p.o.) ameliorated the pathological changes induced by cis injection, where this group showed only degeneration in the lining epithelium of the cortical tubules **Fig. 6C**. Regarding kidney specimens from mice treated with only RA (200 mg/Kg, p.o), no histo-pathological alternations were detected **Fig. 6D**.

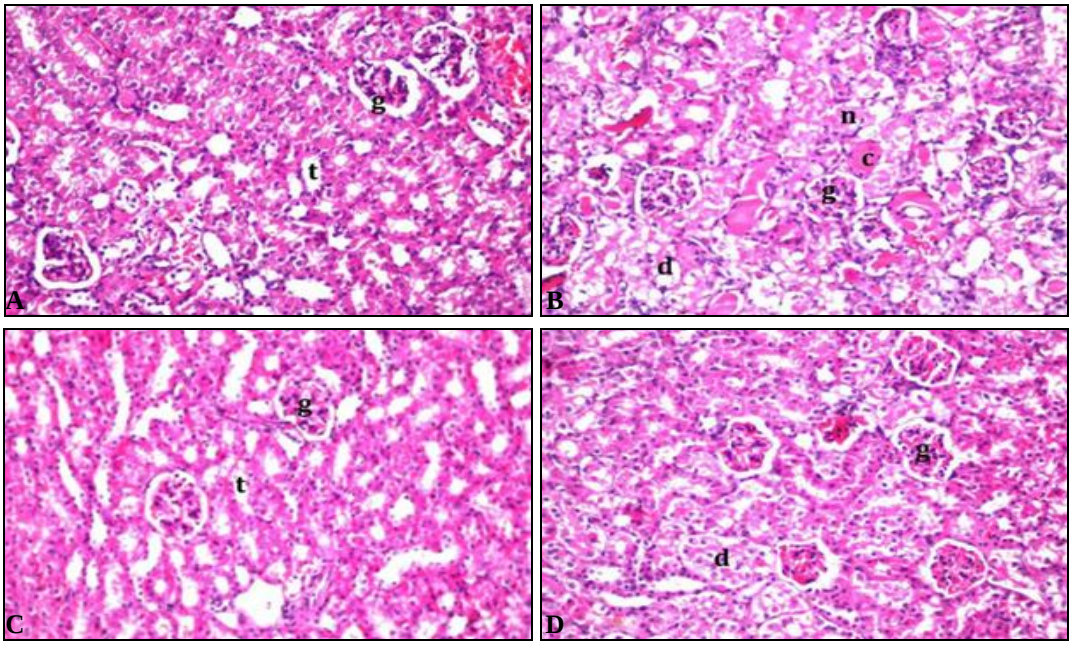

FIG. 6(A-D): REPRESENTATIVE PHOTOMICROGRAPHS OF KIDNEY SECTIONS STAINED WITH HEMATOXYLIN-EOSIN (MAGNIFICATION X 40): (A): SECTION OF KIDNEYS OF CONTROL MALE ALBINO MICE AND SHOW NORMAL HISTOLOGICAL STRUCTURE OF THE GLOMERULI (G) AND TUBULE (T) AT THE CORTEX PORTION. (B): SECTION OF KIDNEYS OF MALE ALBINO MICE TREATED WITH CIS (3.5 mg.kg, i.p.) AS SINGLE DOSE SHOWING DEGENERATION (D), COAGULATION NECROSIS (N) AND CASTS FORMATION (C) IN THE CORTICAL TUBULES. (C): SECTION OF KIDNEYS OF MALE ALBINO MICE TREATED WITH RA (200 mg/Kg, p.o.) FOR 7 DAYS SHOWING NORMAL HISTOLOGICAL STRUCTURE. (D): SECTION OF KIDNEYS OF MALE ALBINO MICE CO-TREATED WITH RA AND CIS SHOWING DEGENERATION IN THE LINING EPITHELIUM OF THE CORTICAL TUBULES (D)

DISCUSSION: Several studies have attempted to improve platinum-based therapy efficacy by incorporating newer cytotoxic drugs. A promising strategy is to use natural products having anticancer effects in combination with platinum-based drugs. Natural products are generally characterized by their low toxicity compared with standard chemotherapy drugs. Combinations of natural compounds with conventional chemotherapy drugs may exert additive or synergistic effects on killing cancer cells, thereby allowing lower and safer doses of the more toxic drug to be used [31].

In an effort to enhance the effectiveness of cis, the current study investigated the anticancer effect of RA, both alone and in combination with cis.

To determine whether RA is cytotoxic to HepG2 cells, this study firstly examined the effect of RA on cell viability. The results of the present study showed that RA inhibits the proliferation of HepG2 cell line. Similarly, many studies reported the anticancer activity of RA against a wide range of human cell lines such as hepatic stellate cell, MCF-7/Adr cell line, AGS (human gastric carcinoma) and KYSE30 (human esophageal squamous cell carcinoma) [32 - 34].

To identify the mechanism of inhibition of cell growth by RA, the influence of RA on the cell cycle distribution was measured. The results of the present study indicated that 1/2 IC_{50} of RA induced G_0/G_1 phase cell cycle arrest while IC_{50} of RA induced S phase cell cycle arrest of HepG2 cells 48 h after treatment. In accordance with the present data, RA induced G_1 arrest through down regulation of Cyclin D1 partly through the STAT3

signalling pathway in HSC-T6 [35]. Moreover, RA blocked cell cycle progression at G1/S phase in Jurkat cells due to down regulation of cyclin D3 and p21Waf1/Cip1 and up-regulation of p27Kip1 [36].

To further elucidate the possible mechanisms underlying the cytotoxic effect of RA, caspase-3 level was measured in cell lysate as an apoptotic marker. The present data showed that 1/2 IC_{50} and IC_{50} of RA induced caspase-3 in HepG2 cells 24 h after treatment. It has been reported that RA induced apoptosis in various cancerous cells such as HepG2 cell line, Jurket and peripheral T cell, human HL-60 promyelocytic leukemia cells, and acute lymphoblastic leukemia cells [37 - 40]. Lin *et al*., [37] reported that RA up-regulated caspase-8 expression, Bax and down-regulated Bcl-2 expression in HepG2 cells. Also, RA induced cytochrome c release and procaspase-9 activation as well as mitochondrial membrane depolarization, decrease of Bcl-2 expression and activation of caspase-3 and caspase-8 in Jurkat and peripheral T cells [38].

The antiangiogenic activity was the third mechanism to be studied. Results of the present study revealed that 1/2 IC_{50} and IC_{50} of RA inhibited the VEGF concentration in HepG2 cells 48 h after treatment. The antiangiogenic activity of RA is in accordance with a previous study conducted by Huang and Zheng [18] who reported that RA inhibited angiogenic processes involving endothelial cell proliferation, migration, adhesion and tube formation in human umbilical vein endothelial cells. Also, RA was shown to have anti-angiogenic activity during wound healing in a rabbit model of glaucoma surgery *via* inhibiting VEGF [41].

The present study indicated that pre-treatment of HepG2 cells with IC_{50} of RA for 24 h significantly enhanced cis cytotoxic activity in HepG2 cell line. Taken together, these findings suggest that RA improved therapeutic index of cis. Tai *et al*., [42] reported that rosemary extract enhanced the antiproliferation effect with cis on both human ovarian cancer cells; A2780 and its cis resistant daughter cell line A2780CP70. Additionally, RA as one of active ingredients of rosemary extract has been showed to enhance the antiproliferation activity of cis on A2780 cell line.

The present study was further extended to address whether RA would modulate cis-induced nephrotoxicity. Cis-induced nephrotoxicity in mice was manifested by significant increase in serum creatinine and BUN levels. Profound studies reported that the levels of renal function markers significantly elevated during cis-induced nephrotoxicity [43, 44].

In the current study, histopathological examination emphasized the biochemical changes, where cis resulted indegenerative changes, coagulation necrosis with desquamation and formation of eosinophilic casts in the tubular lumen at the cortex. Administration of RA (200 mg/Kg, p.o) significantly improved kidney function. Histopathological examination has furthermore asserted the nephroprotective activity exerted by RA against cis nephrotoxic effects which manifested an improvement in the histology of kidney. These results are in agreement with previous studies [45, 46].

Previous studies showed that oxidative stress exerts a pivotal role in the pathogenesis of cis induced acute renal toxicity [47]. Results of the present study revealed that, using cis significantly increased MDA production in renal tissues. This result is in harmony with previous studies showing that administration of a single dose of cis resulted in lipid peroxidation in renal cell, determined by the significant increment of the MDA level when compared to the control group [48, 49]. This finding could be secondary to the increase of free radical generation induced by cis, GSH depletion and inhibition of the antioxidant enzymes activity in renal tissue [50, 51].

Co-treatment of cis-treated group with RA has been shown to counteract oxidative stress induced by cis nephrotoxic dose where MDA levels were significantly decreased. Similar results were observed in other studies [13, 52, 53].

In the present work, cis caused a marked decrease in GSH content in renal tissues. In tubular cells, cis is conjugated to glutathione and then metabolized to a reactive thiol, which is a potent nephrotoxin through a ɤ-glutamyltranspeptidase and cysteine-S conjugate β-lyase-dependent pathways [54]. These results are in accordance with previous studies [7, 55].

Additionally, administration of RA alone or in combination with cis significantly increased GSH content when compared to control group or cis treated group respectively; a finding which is in agreement with previous studies [13, 52]. It was reported that RA upregulated GSH as a result of free radicals scavenging [56]. Moreover, previous studies have shown that the polyphenol compounds from plant increased activity of Gamma-glutamate cysteine ligase (ɤ-GCL) which is a key enzyme of GSH synthesis [57].

In the present study, cis-treated group showed a marked pro-inflammatory response as evidenced by significant increase in kidney level of TNF-α. These findings were supported by previous studies [58, 59]. It was shown that the cis-induced free radical overproduction activated NF-κB which then induced the expression of several pro-inflammatory genes, including TNF-α [60]. Moreover, p38 MAPK (mitogen activated protein kinase) activated by hydroxyl radicals was reported to play a crucial role in acute renal injury and inflammation induced by cis, through the production of TNF-α [61].

Data of the present study showed that RA treatment exhibited anti-inflammatory effects as evidenced by a significant decrease in kidney level of pro-inflammatory marker TNF-α. Similar anti-inflammatory effects of RA were obtained in different models of inflammatory diseases [11, 62]. The anti-inflammatory properties of RA were proven to be attained through the inhibition of COX-2 and inhibition of production of inflammatory chemokines and cytokines [63].

Apoptosis is recognized as a main cause of cis-induced nephrotoxicity [64]. In our study, proapoptotic Bax level in the cis-treated group was significantly higher when compared with the control group. These results are in accordance with earlier studies [65, 66].

Previous studies have reported that both caspases and Cis-induced apoptosis are correlated. The results of the present study revealed that caspase-9 level was induced in cis-treated mice. These results are in agreement with other studies [67, 68]. A previous study reported that apoptotic cell death in renal tubule cell was caused by cis through the generation of reactive oxygen species, which activate the pro-apoptotic Bcl-2 family member Bax, which as a consequence induces mitochondrial permeability transition, causing the release of cytochrome c, caspase-9 activation, and entry into the apoptosis execution phase [69]. Pre-treatment with RA resulted in a significant decrease in the levels of pro-apoptotic Bax and caspase-9 compared to cis-treated mice.

In a previous study, RA was shown to have protective effects on hyperthermia-induced cellular apoptosis and damage of animal muscle cells by decreasing the expression of caspase-3 mRNA, Bax/Bcl-2 ratio and increasing intracellular antioxidant capability [70].

CONCLUSION: From the previous findings obtained from the current study, it could be concluded that RA possesses cytotoxic activity. In addition, RA enhances cis induced-cytotoxicity in HepG2 cells. Furthermore, RA alleviated cis-induced nephrotoxicity through attenuation of oxidative stress, inflammation and apoptosis.

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