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Alpha Tocopherol Outperforms Melatonin and Silymarin Antioxidant Defense Against Cyclophosphamide Induced Cardiorenal Toxicity

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ABSTRACT

Introduction: Cyclophosphamide induces cardio-renal oxidative stress by its metabolite, acrolein. Alpha tocopherol, silymarin and melatonin are predetermined antioxidants, but comparative data on their capability to conserve tissue integrity is limited. This study addresses this gap by conducting detailed comparative analysis on multiple sera and tissue markers.

Aims and Objectives: To compare the protective potential of alpha-tocopherol, silymarin and melatonin against cyclophosphamide induced cardio-renal toxicity and elevated interleukin-23 levels, using sera, oxidative stress and histopathologic markers, to suggest most effective antioxidant for optimizing cancer chemotherapy.

Place and Duration of Study: Eight-month laboratory-based randomized control trial, conducted in Army Medical College, Rawalpindi following ethical approval ERC-ID11/2024/435.

Materials & Methods: Homogeneous population of 50 Sprague Dawley rats were selected via stratified random sampling and divided into five groups by lottery method. Experimental groups received alpha-tocopherol, silymarin and melatonin intraperitoneally for five days followed by cyclophosphamide to compare with untreated and cyclophosphamide groups. Analyses included cardiac (CK-MB, LDH), renal (urea, creatinine, electrolytes) and oxidative stress parameters (GSH, MDA), IL-23, and histology. Statistical analysis utilized Graph-pad Prism version 5 with Shapiro-Wilk and Levene's test for data distribution and variance. Quantitative variables were compared through one-way analysis of variance with Tukey test and qualitative variables via Chi-square test. $p \leq 0.05$ considered significant.

Results: Cyclophosphamide elevated LDH (3049.33±89.33IU/L), CK-MB (964.16±39.89IU/L), urea (78±5.6 mg/dL), creatinine (1.8±0.1mg/dL), potassium (5.9±0.2mEq/L) and decreased sodium (132±1.9mEq/L) than controls ($p \leq 0.001$). Tocopherol reduced these values the most, (LDH: 1547.16±21.92IU/L; CK-MB:302.16±36.03IU/L, urea:59±3.2mg/dL, creatinine:1.2±0.1mg/dL, potassium:4.7±0.3mEq/L, sodium:140±2.3mEq/L, $p \leq 0.001$), and oxidative stress markers (MDA cardiac:1.976±0.005, renal:2.01±0.01 nmol/ml; GSH cardiac:0.941±0.09, renal:1.22±0.16ng/ml, $p \leq 0.001$). Tocopherol showed superiority over silymarin and melatonin, in reducing IL-23 and histologic damage ($p \leq 0.001$).

Conclusion: This study suggests alpha-tocopherol as superior antioxidant than melatonin and silymarin against CP-induced oxidative damage advocating its co-supplementation with cyclophosphamide.

Keywords: Cyclophosphamide, alpha tocopherol, melatonin, silymarin, oxidative stress, IL-23

INTRODUCTION

Cyclophosphamide (CP) has an end metabolic product acrolein which generates reactive oxygen species (ROS) altering tissue's molecular and histologic integrity[1]. This has prompted researchers towards exploration of ant-oxidants. ROS damage to cardio-renal tissue results in elevated inflammatory marker interleukin-23 (IL-23), which is responsive to administrable antioxidants[2-4]. Alpha tocopherol (TOH), transforms into α-tocopheroxyl radical, guarding against ROS-induced lipid peroxidation and myocardial cytotoxicity conserving troponin and creatine kinase (CK-MB) simultaneously inhibiting caspase-3 and restoring long-chain polyunsaturated fatty acids in epithelial membranes reducing renal tubular cell death[4,5]. Melatonin (MEL), safeguards mitochondrial integrity and decreases cellular calcium overload by triggering sarcoplasmic ATPase in cardiomyocytes[6,7]. It offers nephroprotection by inhibiting lipid peroxidation by radical adduct formation and down-regulating NF-κB, conserving mitochondrial homeostasis and decreasing caspase-3-dependent apoptosis. MEL

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stimulates glutathione peroxidase with increased metabolism of ROS. It also mitigates nitric oxide synthetase (NOS) and NO associated oxidants to preserve renal physiology, lessen tubular pathologic index and better prognosis[8]. Silymarin (SIL), scavenges ROS by inducing transcription factors like NF-κB[9]. It protects mitochondrial electron transport chain and cellular ATP. It synthesizes protective molecules encoding glutathione, thioredoxin, and sirtuins complexes and lessens endothelial dysfunction by reducing asymmetric di-methyl arginine and NO[10]. It inflicts cardioprotecton by antagonizing angiotensin II which relaxes smooth muscle and promotes renal clearance supporting shrinkage of myocardial infarct[11]. In addition, via blocking tumor necrosing factor induced kinases and apoptosis, it plays strategic role in renal protection[9,11]. Considering its additional role in tumor growth arrest, SIL nanoparticles are under trials to enhance endurance against chemotherapy induced toxicity[9,10]. Despite pre-established protective role of TOH, MEL and SIL; determination of most effective agent remains challenging. This study compares their protective potential against CP induced cardiorenal toxicity and IL-23 regulation. This novel comparison will conclude single best agent for future human trials to help improve cancer chemotherapy outcomes.

MATERIALS AND METHODS

A laboratory-based randomized controlled trial was conducted at Pharmacology Department, Army Medical College, Rawalpindi over eight months following ethical approval ERC-ID11/2024/435. CP was acquired from Pharmedic Laboratories, Pakistan. Pharmaceutical-grade 95% TOH and MEL from Sigma-Aldrich, USA and 98% silymarin by Global Pharmaceuticals, Pakistan. The drug dosage protocol (Table 1), had 5 days of experimentation for which homogeneous population of fifty Sprague Dawley rats was selected via stratified randomization sampling and divided into five equal groups (n=10) by lottery method; including males and non-pregnant females aged 5-8 weeks and weighed 200-250 gram; and kept under standard housing conditions[12]. Each group had dosages intraperitoneally (IP). Twenty-four hours after intervention animals were euthanized following Institutional Animal Care guidelines, (United Stated). Procedure was done in bio-safety cabinet with minimal human exposure. For anesthesia drop jar method with 30% v/v isoflurane in propylene-glycol was used[13]. Cardiac puncture was done for blood sampling. Cardiac and renal tissues were excised for biochemical and histopathologic assessment.

Table 1: Dosage Protocol

Experimental Groups	Drugs administered	Regimens
Group-I	None	Control.
Group-II	Cyclophosphamide	Single IP injection of CP [200 mg/kg][3]
Group-III	Alpha-tocopherol & Cyclophosphamide	Once daily dose of TOH [100 mg/kg IP] for 5 consecutive days followed by single dose of CP [200 mg/kg, IP] one hour after last dose[5,6]
Group-IV	Silymarin & Cyclophosphamide	Once daily SIL [100 mg/kg IP] for 5 days followed by CP as above[11]
Group-V	Melatonin & Cyclophosphamide	Once daily MEL [20 mg/kg IP] for 5 days followed CP as above[7].

Lactate dehydrogenase (LDH), CK-MB, creatinine and urea were analyzed using Selectra-XL analyzer and serum electrolytes via ion-selective electrode method. Polymerase chain reaction (PCR) assessed expression of IL-23. RNA extraction was done using TRIZOL and quantified by nanodrop, followed by complementary DNA synthesis by kit. Primer-3 software was used for IL-23 primer designing (Table 2).

Table 2: Primer characteristics for IL-23

Gene	Primer Sequence	Size
IL-23	**Forward** 5'GTCATCCTGCTCTTCTTTCTCG3' **Reverse** 5'TGTGGTGTTCTTCGTTGCTGT3'	**198bp**
GAPDH	**Forward** 5'AGAGACAGCCGCATCTTCTTATA3' **Reverse** 5'TGCATTGCTACAATCTTGATATA3'	**390bp**

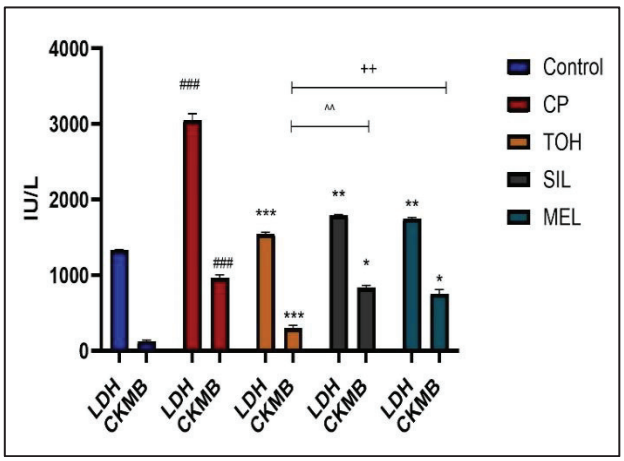
Glutathione [GSH] and malondialdehyde [MDA] were assessed by Sandwich ELISA. H&E staining was used for histopathology to assess hemorrhage, tubular degeneration, fibrosis, apoptosis, necrosis and inflammatory cell count[28]. Data were expressed as mean ± standard error of mean and analyzed on GraphPad Prism version-5. Shapiro-Wilk test confirmed normal distribution ($W \approx 1$, $p>0.05$), and Levene's showed variance equality ($p=0.62$), Independent quantitative variables were compared

through one way analysis of variance [ANOVA] followed by Tukey test. Qualitative variables, presented by frequency were compared via Chi-square test. $p \leq 0.05$ = statistically significant.

RESULTS

Group I displayed normal LDH and CK-MB at 105.7±7.3 U/L and 88.3±5.1 U/L. CP elevated LDH to 178.9±8.6 U/L and CK-MB to 159.4±10.2 U/L ($p \leq 0.001$). Group III LDH and CK-MB values were lowest at 110.5±6.4 U/L and 92.8±5.7 U/L ($p \leq 0.001$). Groups IV and V demonstrated reductions, with LDH at 125.4±7.1 U/L and 132.2±6.8 U/L, and CK-MB levels at 100.1±6.2 U/L and 105.9±6.4 U/L ($p \leq 0.05$) (Figure 1).

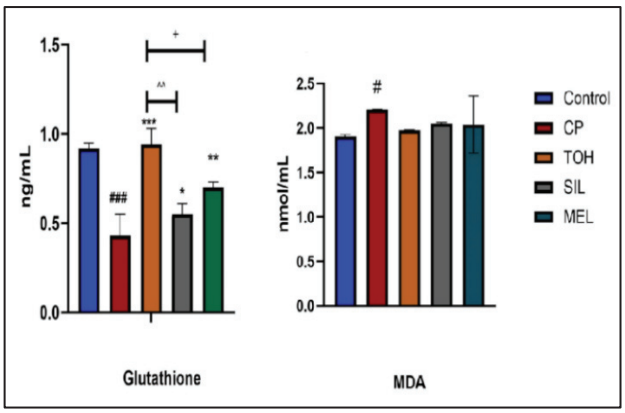
Figure 1: Comparisons of serum LDH and CK-MB values.



In all graphs presented # shows significant difference from normal control.* shows significant difference from CP group. ^ shows significant difference between group III and IV + shows significant difference between group III and V.

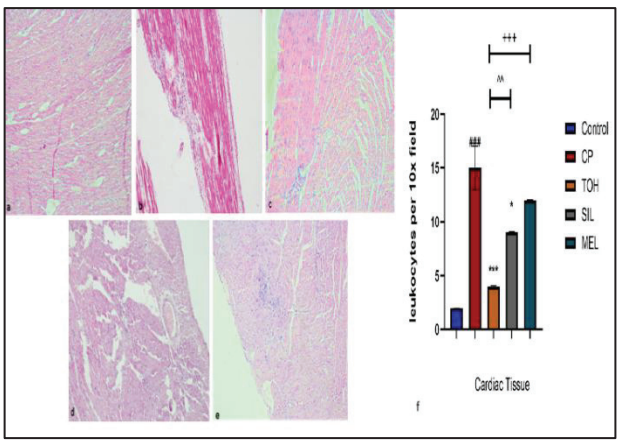
CP decreased GSH to 3.1 ± 0.2 nmol/mg compared to control's 7.3 ± 0.4 nmol/mg ($p \leq 0.001$). Group III restored it at 7.6±0.3 nmol/mg ($p \leq 0.001$), followed by group IV at 6.9±0.4 nmol/mg ($p \leq 0.05$) and V at 6.2±0.3 nmol/mg ($p \leq 0.01$). MDA increased in group II to 6.2±0.3 nmol/mg versus 2.5±0.2 nmol/mg ($p \leq 0.05$). Group III showed most substantial decrease at 2.7±0.3 nmol/mg ($p \leq 0.05$). Group IV at 3.1±0.3 nmol/mg and group V at 3.4±0.4 nmol/mg had lesser effects (Figure 2).

Figure 2: Comparison of cardiac tissue GSH and MDA values.



Histology of control group exhibited normal myocardial fibers (Figure 3a). Group II showed myocyte disfigurements, interstitial edema, hemorrhagic foci and aggressive inflammatory infiltrates (Figure 3b). Group III, showed minimal myofibrillar changes and dispersed leukocytes (Figure 3c) Group IV, showed diffused inflammation damaging myofibrils (Figure 3d). Group V had mild to moderate changes with diffuse aggressive leukocyte encroachment, cardiac ultrastructure was intact (Figure 3e).

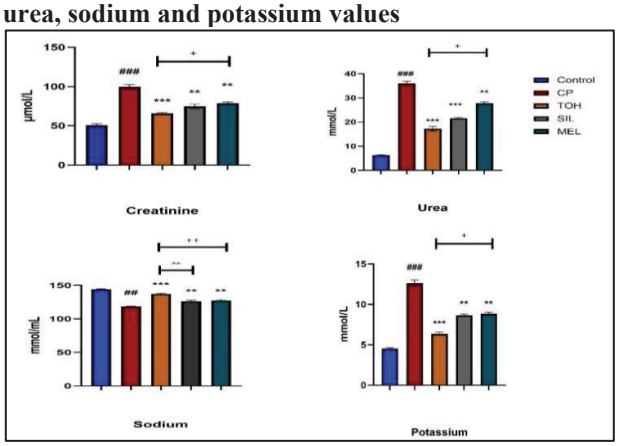
Figure 3: Cardiac Tissues: a) Group I [untreated control] b) Group II (CP) c) Group III (TOH+CP) d) Group IV (SIL+CP) e) Group V (MEL+CP) f) Comparisons of Leukocyte count



CP raised creatinine to 1.8±0.1 mg/dL versus control 0.9±0.05 mg/dL and urea to 56.4±3.2 mg/dL versus 28.6±2.1 mg/dL ($p \leq 0.001$). TOH decreased it to 1.0±0.05 mg/dL and urea to 30.2±1.9 mg/dL ($p \leq 0.001$), while SIL lowered to 1.2±0.08 mg/dL and 35.6±2.5 mg/dL, and MEL to 1.3±0.1 mg/dL and 38.7±2.8 mg/dL respectively ($p \leq 0.01$). CP dropped sodium to 128.5±2.5 mmol/L versus control 140.3± 3.1 mmol/L ($p \leq 0.01$) and increased

potassium to 5.8±0.3 mmol/L from 4.5±0.2 mmol/L ($p \leq 0.001$). Sodium was restored in group III at 139.8±2.8 mmol/L and potassium at 4.6± 0.3mmol/L ($p \leq 0.001$), SIL and MEL showed limited improvement ($p \leq 0.01$) indicating strongest restorative effects by TOH.

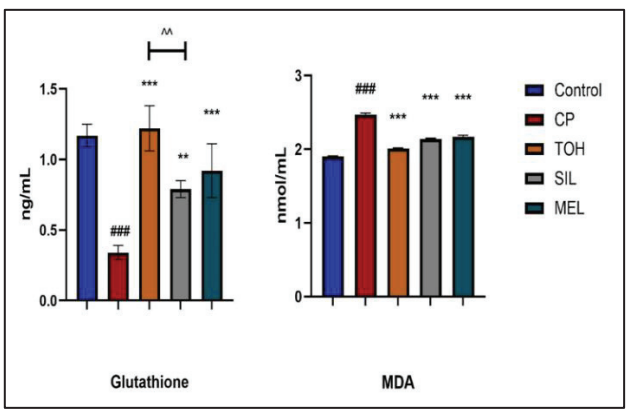
Figure 4: Multiple comparisons of serum creatinine, urea, sodium and potassium values



In group I, renal GSH was 7.3±0.4 nmol/mg reduced by CP to 3.1±0.2 nmol/mg ($p \leq 0.001$). TOH regained it at 7.6±0.3 nmol/mg, SIL 6.9±0.4 nmol/mg and MEL 6.2±0.3 nmol/mg ($p \leq 0.001$).

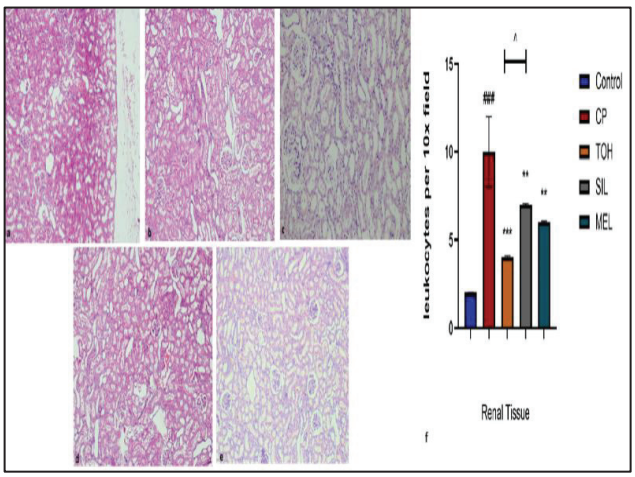
Renal MDA was raised by CP to 6.2±0.3 nmol/mg from 2.5±0.2 nmol/mg ($p \leq 0.001$). TOH showed most significant reduction at 2.7±0.3 nmol/mg ($p \leq 0.001$) as compared to SIL 3.1±0.3 nmol/mg ($p \leq 0.001$), and MEL 3.4±0.4 nmol/mg ($p \leq 0.001$).

Figure 5: Comparison of renal GSH and MDA.



The control group slides had no inflammatory cells (Figure 6a). CP caused inflammation with sparse hemorrhagic foci (Figure 6b). TOH showed minimal changes and few inflammatory cells in parenchyma (Figure 6c). SIL had few inflammatory cells and hemorrhagic foci at perivascular area (Figure 6d). Some of group V specimens fell into grade 2 with single layered inflammatory cell rings, others had multiple dense leukocytes rings (Figure 6e).

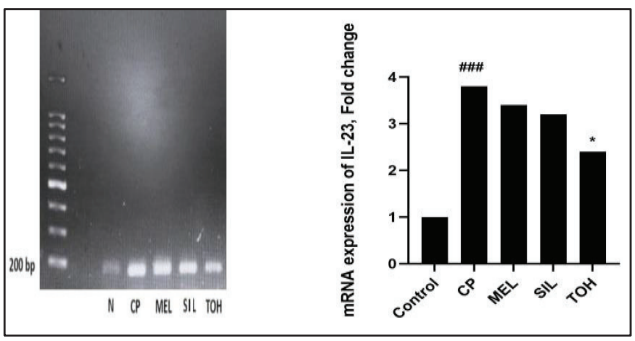
Figure 6: Renal tissue: a) Group I [untreated control] b) Group II (CP) c) Group III (TOH+CP) d) Group IV (SIL+CP) e) Group V (MEL+CP) f) Comparisons of Leukocyte count



IL-23 expression

The expression of IL-23 was significantly increased in CP group. Both MEL and SIL had no effect, but it was significantly reduced with TOH($p<0.05$) (Figure 7).

Figure 7: Fold change of IL-23. ### shows significant difference from normal ($p < 0.001$) while * shows significant difference from CP treated group ($p<0.05$)



DISCUSSION

The aforementioned antioxidants are under extensive trials for their protective potential against antineoplastics. This study compares their role against CP toxic byproduct acrolein, which breaches oxidant-antioxidant equilibrium by demolishing endogenous defense enzymes and triggering ROS[1].
CP is extensively associated with acute cardiotoxicity and nephrotoxicity hampering its clinical utility. Multiple studies recommend adding predetermined exogenous antioxidants to

chemotherapeutic regimens to counter adverse reactions[14-16] notably TOH, SIL and MEL[6,13-17]. However, no single agent with superlative protective potential has been identified till yet. Present study is uniquely designed to bridge this gap by comparing cardio- and nephroprotective potential of these agents aiming to nominate the best one.

Single dose CP [200 mg/kg] was used to generate cardiorenal toxicity. After 23 hours of CP, CK-MB and LDH elevated indicating acute myocardial injury while altered levels of urea, creatinine, potassium and sodium confirmed renal injury, further alterations in GSH and MDA correlated with cardiac histopathology i.e. myofibrillar mutilation, hemorrhage, edema and inflammatory infiltrates. Similar findings were reported with chrysin against CP induced alteration in urea, creatinine, CK-MB, LDH, and MDA[18]. Combination of direct endothelial and oxidative damage triggers vicious cycle resulting in fibrin deposition, micro-thrombi and effusions. Identical ultrastructure have been reported previously with same dosage[19].

Modern research focalize molecular basis of stress-injury and combating it by co-supplementation of antioxidants. In our study, TOH exhibited attenuation of CP induced injury. There was significant fall in CK-MB and LDH as compared to toxic group. Moreover it was the only group with Il-23 significantly reduced. Similar protective effects were reported earlier where myocardial infarct in rats was successfully attenuated by Shukla[20]. TOH successfully reversed acute kidney failure by lowering serum urea and creatinine. A comparable protective magnitude of TOH has been reported by Busari against cisplatin[21]. Selim also highlighted its nephroprotection in amikacin induced renal injury, referring to its capability to preserve collecting ducts[22]. Meanwhile, Stojakovic reported parallel outcomes by co-administering TOH with gentamicin for fourteen days, attributing to TOH shielding low-density lipoproteins and polyunsaturated fats from oxidation[23]. These studies are confined towards determination of antioxidant potential of TOH and do not address if other antioxidants provide equitable results. In SIL treated rats, 23 hours post CP injection, statistical fall in LDH and CK-MB were observed alongside restoration of endogenous antioxidants in heart, The findings were parallel to Zalat, where SIL's cardioprotection against anthracyclin on parameters of serum iron and ferritin and anticardiolipin IgG was measured[11]. Similar results have been seen against rat models of 5-fluorouracil-induced cardiotoxicity[24]. Lately, conclusive meta-analysis has emerged siding with us against doxorubicin validated by echocardiographic, biochemical and oxidative stress markers, it stressed on anti-neoplastic activity also for which another systemic review idealized formulation of lipid soluble nanoparticles[25]. In renal tissue, Ali and Esfandiar inferred protective aspects of SIL against diclofenac by attenuating histologic injuries and reduction of urea, creatinine and MDA[26]. Another study strengthened our results by concluding that SIL down regulates TNFα and NF Kβ while up-regulating IL-10[14]. Another research found SIL restoring GSH and reducing MDA levels by impeding cytokines induced inflammation[27].

MEL group also exhibited cardiotoxicity reversal. Earlier Arrino proved it against doxorubicin via reduced lipid peroxidation, inflammation, mitophagy, autophagy and apoptosis[28]. Our results also parallels Othman"s who found MEL improves not only urea and creatinine but also GSH with conserved renal histology against aluminum[29]. Project of Aygun also declared that MEL decreases blood urea nitrogen and creatinine against Adriamycin[30]. Song et al. presented that anti-oxidative properties of MEL preserved histoarchitecture of testes and sperm integrity in rats against acrolein emphasized by successful in-vitro fertilization on same group of rats where unlike CP alone group[8].

This study validates multi-organ protective potential of aforementioned antioxidants. As our main aim was to explore single best antioxidant agent to be incorporated in CP regimens we made a hierarchy of four diagnostic tests levels including biochemistry, molecular study of oxidative stress, mRNA expression of inflammatory gene and histopathologic findings. After detailed statistical intergroup comparisons, we nominate TOH as the most efficacious cardioprotective and nephroprotective agent against CP with most superlative antioxidant and anti-inflammatory properties versus SIL and MEL. TOH not only protected tissue integrity but also conserved antioxidant pools significantly higher than other antioxidants in comparison.

CONCLUSION

The antioxidants TOH, SIL, and MEL exhibited significant reduction in abnormally raised cardiac and renal parameters alongside restoration of tissue GSH and ultrastructure. However, the reduction in mRNA expression of IL-23 was done solely by TOH. Upon comparing outcomes of diagnostic parameters, cardio- and nephroprotective effects of TOH outperformed SIL and MEL, advocating its

potential co-supplementation in future cyclophosphamide-based regimens.

Further researches on similar model are suggested including other predetermined antioxidants to identify most efficient agent for human trials to optimize cancer chemotherapies with reduced multiorgan morbidness.

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MS: Conceived idea designed study; collected data; drafted manuscript.
NI: Drafted manuscript; analyzed data.
ZS: Drafted abstract and manuscript; interpreted data and results.
MN: Assisted in data management
S: Interpreted results; and contributed to the discussion section of manuscript.
II: Contributed to methodology and overall research framework