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Honeybee Propolis Phenol, Caffeic Acid Phenethyl Ester, Attenuates Cisplatin-Induced Kidney Damage – a Multitarget Approach

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Abstract: Cisplatin utilization is known to be limited due to numerous side effects, especially to the ones related to kidney tissue injury. The application of antioxidants, as potential supportive drugs, to prevent oxidative cisplatin tissue damage appears intuitive. In this work, we explored the usage of caffeic acid phenethyl ester (CAPE) in this respect through the analysis of standard serum biochemical parameters and the ones related to oxidative tissue damage, the changes in arginine metabolism, and apoptosis occurrence that follow cisplatin application in rat kidneys. Additionally, pathohistological analysis and a specific TUNEL staining for the detection of apoptosis was studied. Cisplatin produced marked changes in serum parameters that reflect kidney tissue function, while at the same time produced extensive tissue oxidative damage, increased arginase activity, and depleted reduced glutathione. Moreover, both biochemical and micromorphological analyses indicated significant tubular cell apoptosis. The application of CAPE together with cisplatin prevented the disturbance in serum biochemical and tissue oxidative stress parameters, without affecting arginase activity. Interestingly, CAPE inhibited different enzymes involved in the glutathione metabolism and the apoptotic process. The results of the present study indicate that CAPE could be used as supportive therapy for oncological patients receiving cisplatin since it attenuates the nephrotoxicity of this chemotherapeutic.

Keywords: Caffeic acid phenethyl ester; kidney; cisplatin; oxidative damage; arginase; apoptosis.

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1. Introduction

Cisplatin (CP) is a potential antineoplastic drug used for the treatment of various solid tumors such as ovarian, testicular, lung, bladder, breast, and cervical cancers [1]. One of the mechanisms underlying the cytotoxicity of CP is cell apoptosis resulting from DNA damage in both cancer and non-cancer cells [2]. The disturbance in cellular oxidative capacity, e.g. depletion of reduced glutathione (GSH), further leads to an excessive reactive oxygen species (ROS) production which is responsible for the cell damage [3-6]. Apart from DNA damage and apoptosis induction the produced ROS, which depends on the concentration and length of exposure to cisplatin, this drug affects mitochondrial membrane potential and induces lipid peroxidation, protein oxidative modification, and autophagy [2,3]. Increased production of superoxide and hydroxy radicals stimulates proinflammatory cytokine and enzyme production, which consequentially lead to various histopathological changes such as interstitial edema and inflammatory cell infiltration [7]. Cisplatin exerts its side effects through the disturbance in the function of different tissues, up to know the most common side effects related to CP includes myelosuppression, ototoxicity, nephrotoxicity, cardiotoxicity, etc. [8].

The exact mechanism of CP-induced nephrotoxicity has not been precisely defined, however, it is known that CP penetrates through the proximal tubule epithelial cells by passive diffusion and exerts its toxicity there, particularly in the S3 segment of the tubular cells. Cisplatin-induced nephrotoxicity is followed by a disturbance in glomerular filtration, reduced levels of potassium, and increased concentration of serum creatinine and urea [9,10]. Thus, nephrotoxicity makes the usage of CP limited, especially in the higher doses [11]. Although the primary target for CP are the cells undergoing division (cancer cells) and not nonproliferating/quiescent ones, the toxicity towards proximal tubule cells is highly specific [12]. The additional mechanism which is associated with CP nephrotoxicity is the conjugation of CP with GSH by brush border enzymes, making this an initial step in the toxicity of CP [12].

Caffeic acid phenethyl ester (CAPE) is a flavonoid extracted from honeybee propolis, with potential anti-inflammatory, antioxidant, antidiabetic, antiproliferative and antineoplastic features [13-15]. Propolis is used in the traditional medicine of many nations, especially in Asia for more than a thousand years [13]. Among the honeybee product associated flavonoids, CAPE is among the most toxic ones, however, it is estimated that a daily intake of around 70 mg is safe for human use [13]. The antioxidant effects of CAPE are based on neutralizing the oxidative stress-related parameters, inhibition of enzymes (e.g. lipoxygenase), suppression of lipid peroxidation, and activation of antioxidant enzymes [14,16]. The effects of CAPE on CP-induced kidney damage were evaluated on a single previous occasion [13,16] where the protective action is associated with ROS scavenging.

This study aims to investigate the potential protective effect of CAPE on CP-induced kidney damage in a rat model by analyzing the biochemical and histopathological changes. There are several proposed pathways through which the nephrotoxicity of CP could be targeted by a potential nephroprotective agent [17], and in the present study, we addressed four of them, namely oxidative stress, CP metabolizing one, inflammatory, and apoptotic.

2. Materials and Methods

2.1. Drugs and Chemicals

The CAPE (≥97%, powder, Sigma Aldrich, USA) was prepared fresh on a daily basis (dissolved in 0.1% ethanol in 0.9% NaCl) and injected intraperitoneally (i.p.) to animals in a dose of 10 μmol/kg/day throughout the experiment [18]. Cisplatin was purchased from TEVA (Actavis D.O.O., Serbia) in a form of a solution (50 mg/100 mL) and was applied once to animals via i.p. route in a dose of 8 mg/kg [7,19]. All other chemicals and reagents used in this research were purchased from Sigma Aldrich (USA) or Carl Roth (Germany) and were of the highest purity. A general anesthetic used for anesthetizing the animals was ketamine, purchased from Richter Pharma AG (Wels, Austria).

2.2. Animals and Housing

Male Wistar rats weighing from 200 to 250 g, 7 weeks old, were obtained from the Vivarium of the Institute of Biomedical Research, Faculty of Medicine, Niš, Serbia. Animals were housed in plexiglas cages in a temperature-controlled room (22 ± 2°C) with 12 h light-dark cycle. Rats were fed with standard food and tap water provided *ad libitum*. A week before the commencement of the experiment animals were allowed to acclimatize to the laboratory environment and cage distribution. The experiment was conducted in accord to ethical principles of the European Union (EU Directive of 2010; 2010/63/EU), and the Guide for the Care and Use of Laboratory Animals (8th edition, National Academies Press), and ones given by laws of the Republic of Serbia (approval No. 323-07-01762/2019-05).

2.3. Experimental Design

For this experiment, animals were divided into four identical groups consisting of 6 animals each. All animals were treated daily for 5 days by an i.p. injection, as follows:

I Group - Vehicle group – daily treated with an injection of 0.1% ethanol in 0.9% NaCl 1 mL/kg;

II Group – CAPE treated group – daily treated with an injection of CAPE (10 μmoL/kg/day) for five days;

III Group - CP treated group – treated with a single CP injection (8 mg/kg) on the 3rd day of the experiment;

IV Group –CP and CAPE treated group – treated with CAPE for five days (10 μmoL/kg/day), and a single injection of CP (8 mg/kg) on the 3rd day.

All animals, at almost 9 weeks of age, were sacrificed by ketamine overdose, five days after the experiment beginning. Blood was taken by cardiac puncture, and renal tissue was dissected for biochemical (frozen and stored at −80 °C) and histopathological (fixated in 10% formalin) analyses.

2.4. Serum Biochemical Analyses

From each animal, blood was taken by a cardiac puncture using a Vacutainer system and was left to clot at room temperature before centrifugation (635 x g, 15 min at 4 °C). The serum obtained by centrifugation was used for the determination of urea, creatinine, sodium, potassium, and calcium levels using Olympus AU680 Chemistry-Immuno Analyzer.

2.5. Tissue Biochemical Analyses

The frozen kidney tissue sample was cut into fine pieces and homogenized (RJ 22713-00, IKA® Works de Brasil Ltda Taquara, RJ, Brazil) in ice-cold distilled water (10% homogenate, w/v). After homogenization samples were centrifuged at 8008xg for 10 min at 4 °C in order to obtain clear supernatants which were further used for the determination of tissue biochemical parameters. Protein homogenate concentrations were determined following standard Lowry's protocol [20] using a bovine serum albumin standard curve.

2.5.1. Catalase (CAT) and Peroxidase (POD) Activity Determination

Tissue catalase (CAT) and POD activity were determined by the spectrophotometric method described by Goth [21] and Chance and Maehly [22], respectively. The activity of CAT was estimated through the enzyme's ability to dissolve the substrate (H_2O_2), whereby enzymatic reaction is stopped by the addition of ammonium molybdate. The yellow complex of molybdate and the remaining H_2O_2 was measured at 405 nm using an automated microplate reader (Multiscan Ascent, Labsystems, Helsinki, Finland). Enzyme activity was expressed in catalytic units per gram of protein (kU/g). The activity of POD was determined in a reaction mixture consisted of kidney tissue homogenate, H_2O_2, pyrogallol,

and phosphate buffer (pH 6.0). The end product, purpurogallin, was measured at 420 nm using an automated microplate reader (Multiscan Ascent, Labsystems, Helsinki, Finland), and the activity was expressed as nmol/g of proteins.

2.5.2. Tissue Oxidative Damage Parameters Determination

Xanthine oxidase (XO) activity was determined in a reaction medium containing kidney tissue homogenate and xanthine (0.6 mM) [23]. The production of uric acid in a reaction mediated by XO was determined spectrophotometrically (V-1800 Shimadzu spectrophotometer (Japan)) and the amounts were calculated using a standard curve constructed with uric acid. The results are expressed as U/g of kidney tissue proteins.

The amount of lipid peroxidation was estimated through the levels of malondialdehyde (MDA) using a standard method that utilizes thiobarbituric acid [24]. The concentration of MDA in kidney tissue was calculated using a standard curve and the results obtained after measuring the sample absorbance using an automated microplate reader (Multiscan Ascent, Labsystems, Helsinki, Finland) are expressed as nmol of MDA per mg of tissue proteins.

The kidney tissue homogenate concentration of H_2O_2 was determined following a previously described method [22], which is based on the oxidation of phenol red arising from the degradation of H_2O_2 in the presence of horseradish peroxidase. The colored product intensity was measured at 610 nm using an automated microplate reader (Multiscan Ascent, Labsystems, Helsinki, Finland), and the concentration was expressed as μmol/mg of proteins.

Tissue total oxidative status (TOS) was measured following a previously described method [25]. Tissue homogenates and reagents I (xylenol orange) and II (o-dianisidine) were shortly incubated (3 min), after which the absorbance of the mixture was measured at 560 nm using an automated microplate reader (Multiscan Ascent, Labsystems, Helsinki, Finland). The obtained results are expressed as μmol/mg of tissue proteins.

2.5.3. Determination of Arginine Metabolism-Related Parameters

Total nitrite and nitrate (denoted as NO) concentrations were determined using Griess reagent following the previously described method [26] using an automated microplate reader (Multiscan Ascent, Labsystems, Helsinki, Finland). The amounts of NO were expressed as μmol/g of tissue proteins.

The amount of citrulline in heart tissue homogenate was determined according to a standard method that is based on a reaction with diacetyl monoxime and thiosemicarbazide [24]. The absorbance of the developed color was measured at 530 nm using an automated microplate reader (Multiscan Ascent, Labsystems, Helsinki, Finland), and the results were presented as mmol/g of proteins.

Kidney tissue arginase activity was determined using a biochemical method based on a reaction between ornithine and ninhydrin. The absorbance of the reaction mixture was measured at 515 nm using an automated microplate reader (Multiscan Ascent, Labsystems, Helsinki, Finland), and the obtained values were corrected with those obtained from the control [24]. The results are expressed as mU per gram of kidney tissue proteins.

2.5.4. Glutathione and Glutathione-Related Enzymes Determination

Tissue GSH concentrations were estimated using a standard method previously described by Ellman [27], where after short incubation of supernatants with DTNB reagent the absorbance of the reaction mixture was measured at 412 nm using an automated microplate reader (Multiscan Ascent, Labsystems, Helsinki, Finland). The amount of GSH was determined based on the standard curve of GSH and expressed as μmol of GSH/mg of proteins.

The activity of glutathione S-transferase (GST) was determined in a reaction mixture (at 310 nm using an automated microplate reader (Multiscan Ascent, Labsystems, Helsinki, Finland)) containing

GSH, 4-nitrobenzyl chloride according to the previously described method [27]. The enzyme activity in kidney tissue homogenate was expressed as nmol/L/mg of protein.

The activity of glutathione reductase (GR) was determined following the method described in detail [22]. The reaction mixture consisted of oxidized glutathione (2 mmol/L), kidney tissue homogenate, DTNB (3 mM), and NADPH (2 mM). The change in the absorbance was determined spectrophotometrically at 412 nm using an automated microplate reader (Multiscan Ascent, Labsystems, Helsinki, Finland). The activity of GR was expressed in nmol/L/min/g of tissue proteins.

2.5.5. Tissue DNAase Activity Determination

Kidney tissue alkaline and acidic DNAas activity were determined following the previously described methods by [28]. A substrate used for the reaction was calf thymus DNA (10% w/v). In the case of alkaline DNAse, the reaction was carried out in a Tris-HCl buffer with pH set at 7.4, while in the case of acidic DNAse the reaction was carried out in acetate buffer at an optimum pH of 5.0. After the incubation, the amount of remaining DNA was determined spectrophotometrically at 260 nm using V-1800 Shimadzu spectrophotometer (Japan). Enzyme activity was calculated in units (U) and final results were expressed as U/mg of tissue proteins.

2.6. Pathohistological Examination

Dissected kidney tissue samples were fixed in 10% solution of neutral formalin, processed routinely, and molded into paraffin. The paraffin blocks were sectioned at a thickness of 4 μm, then deparaffinized, rehydrated, and stained with hematoxylin and eosin (H&E) following by standard protocols. Kidney sections taken from each animal were examined using a light microscope Olympus BX50 (Olympus, Japan) connected with a digital camera Leica DFC 295 (Leica Microsystems, Germany). Pathohistological analysis of kidney tissue [29] was based on the estimation of changes like degree of tubular degeneration, necrosis, inflammation, hyaline casts occurrence, vascular congestion, and dilatation of Bowman's capsule. All kidney samples were observed for studied parameters with a detailed examination of the entire H&E section for each animal by a pathologist unaware of the treatment protocol. The pathohistological changes were graded as follows: absent (-), mild (+), moderate (++), and severe (+++).

2.7. TUNEL Staining

Analysis of terminal deoxynucleotidyl transferase (TdT) deoxyuridine triphosphate nick end labeling (TUNEL) stained tissue sections was performed using in situ cell death detection kit (TUNEL Assay Kit - HRP-DAB (ab206386)) according to the manufacturer's instructions. Evaluation of apoptosis was performed using a light microscope Olympus BX50 (Olympus, Japan) on TUNEL stained samples from each study group. The TUNEL stained apoptotic cells appeared as brown-colored nuclei, while nuclei of normal, intact cells appeared blue/green. The occurrence of TUNEL positive apoptotic in the tubule epithelium cells was examined by a pathologist unaware of the treatment protocol (magnification × 200).

2.8. Statistical Analysis

Results were expressed as the mean ± SD. Statistically significant differences were determined by one-way analysis of variance (ANOVA) followed by Tukey's post hoc test for multiple comparisons (GraphPad Prism version 5.03, San Diego, CA, USA). Probability values (p) less or equal to 0.05 were considered to be statistically significant.

3. Results and Discussion

Five-day treatment with CAPE (10 μmol/day) had no significant impact on the studied serum kidney damage-related parameters (Figures 1A-F) which were found to be almost identical to the ones measured in the control group (I group). Cisplatin applied on its own (group III) caused a statistically significant disturbance in serum urea, creatinine, and sodium levels (Figures 1A, B, and D), while the levels of uric acid, potassium, and calcium remained unaffected (Figures 1C, E and F). Obtained serum biochemical parameters reflecting kidney function revealed that the application of CAPE in combination with CP (group IV) prevented an increase in urea and creatinine (Figures 1A and B), while it had no effects on other studied serum parameters.

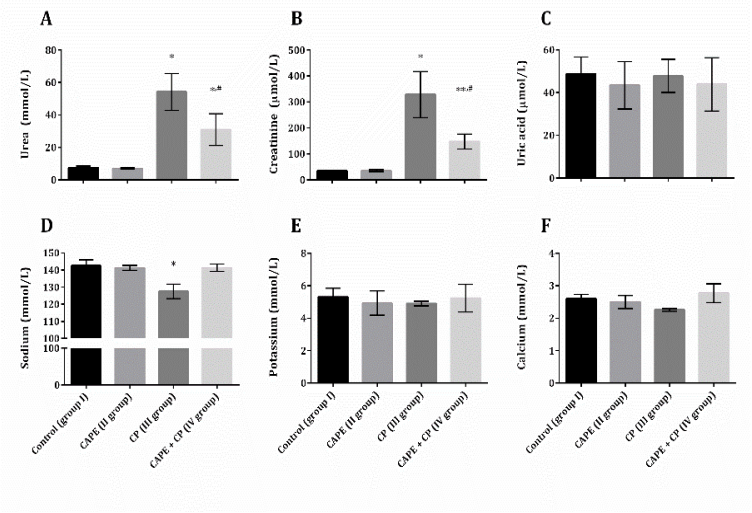

Figure 1. Serum biochemical parameters related to kidney function obtained from animals belonging to different experimental groups: (A) urea, (B) creatinine, (C) uric acid, (D) sodium, (E) potassium and (F) calcium levels. Data are given as mean ± SD, ANOVA followed by Tuckey's post hoc test. *p<0.001, **p<0.001 vs. Control (Group I); $^{\#}p$<0.001 vs CP (Group III)

Studied antioxidant parameters, CAT and POD, were found to be significantly decreased in animals treated with CP (group III) compared to the untreated animals (group I) (Table 1). After the application of CAPE in CP-treated animals (group IV), the CAT activity was found to be significantly higher than in the group treated only with CP (group III). On the other hand, in the group treated with CAPE and CP (group IV) the level of POD was significantly lower compared to the untreated animals (Table 1).

When the cisplatin was applied (group III) the activity of XO and the concentrations of MDA, H_2O_2, and TOS showed a statistically significant increase compared to the group of healthy rats (group I). Besides that, the level of TOS was found to be significantly lower in the group treated with CAPE only (group II) compared to the untreated animals (Table 1). The protective effects of CAPE on CP induced kidney changes were observed through all studied oxidative stress-related parameters (XO activity and the concentration of MDA, H_2O_2, and TOS) which were significantly lower in the group treated with both substances (group IV) compared to the group treated with CP only (Table 1). The group of animals treated with CAPE and CP had significantly lower activity of the XO, while the concentrations of MDA and H_2O_2 were higher when compared to the group of untreated animals (Table 1).

Interestingly, the application of a single dose of CAPE (group II) caused a significant decrease in GST activity compared to the control group, without affecting the GSH and GR activity (Table 1). The concentration of GSH, as well as the activity of both metabolizing enzymes, GST and GR, was found to be significantly lower in CP treated group (group III) than in the group of healthy animals. After the CAPE treatment in CP treated rat's kidney levels of GSH, and the activity of GST and GR were significantly decreased compared to the control group. The same was true for the levels of GST

when they are compared between groups IV and III. In the same group (group IV) the levels of GSH and the activity of GR were found to be higher than the group treated with CP only (Table 1).

Table 1. Kidney tissue damage parameters obtained from different experimental groups

Parameter/Group	Group I (Vehicle)	Group II (CAPE 10 µmol/kg)	Group III (CP 8 mg/kg)	Group IV (CP 8 mg/kg + CAPE 10 µmol/kg)
Tissue antioxidant parameters				
CAT (U/g of prot.)	349.5 ± 10.2	366.3 ± 16.7	258.8 ± 42.5*	326.7 ± 24.1$^{\#}$
POD (nmol/g of prot.)	146.9 ± 27.5	166.6 ± 17.5	61.4 ± 7.9*	59.4 ± 8.2*
Tissue oxidative stress-related parameters				
XO (U/g of prot.)	439.1 ± 8	466.4 ± 17.1	672.4 ± 8.5*	369 ± 11.1$^{\#,*}$
MDA (nmol/mg of prot.)	12.4 ± 1.1	13.1 ± 1.7	20.5 ± 0.4*	15.8 ± 1.3$^{\#,**}$
H_2O_2 (µmol/mg of prot.)	76.7 ± 7.9	69.9 ± 9.8	152.1 ± 12.2*	133.5 ± 4.8$^{\#,**}$
TOS (µmol/g of prot.)	14.8 ± 3.8	12.7 ± 0.9***	31.4 ± 1.2*	15.1 ± 1.6$^{\#}$
GSH and GST metabolizing enzymes				
GSH (µmol/g of prot.)	1.18 ± 0.2	1.33 ± 0.1	0.21 ± 0.1*	0.6 ± 0.04$^{\#\#,*}$
GST (nmol/g of prot.)	185.8 ± 26.5	134.5 ± 24.1***	121.4 ± 7.2*	97.3 ± 20.1$^{\#\#,*}$
GR (nmol/g of prot.)	49.9 ± 0.9	41.5 ± 3.9***	26.9 ± 6.7*	29.4 ± 2.1*

Data are presented as mean ± SD, n = 6; ANOVA, Tuckey's post hoc test *p<0.001; **p<0.01; ***p<0.05 vs. Control group (Vehicle animals); $^{\#}p$<0.001; $^{\#\#}$ p<0.01 vs. CP treated group.

Application of CAPE caused a significant increase in kidney tissue citrulline concentrations (compared to the control group), while it had no significant impact on arginase activity nor NO^{2+}/NO^{3+} concentrations (Figure 2). When administered CP led to a significant increase in arginase activity, followed by a decrease in NO^{2+}/NO^{3+} concentrations, with the concentrations of citrulline remaining unchanged (Figure 2). When the CAPE was applied as a cotreatment even higher increase in arginase activity, significantly higher than both in the control and CP-treated group, was found. Interestingly no changes in citrulline concentrations were noted in group IV, however, a slight increase in its concentrations could be observed, even though this increase was not statistically significant. As in the case of rats treated with CP only the levels of NO^{2+}/NO^{3+} in the group that received CP and CAPE were significantly decreased when compared to the control group (Figure 2).

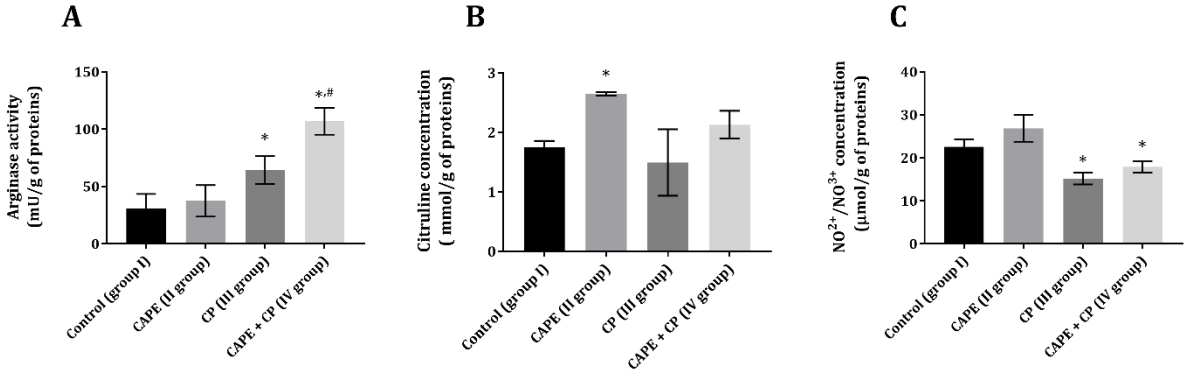

Figure 2. Arginase activity (A), citrulline (B) and nitrate/nitrite concentrations (C) measured in rat kidney tissue. Data are given as mean ± SD, ANOVA followed by Tuckey's post hoc test. *p<0.001 vs. Control (Group I); $^{\#}p$<0.001 vs. CP (Group III)

The application of CP leads to a statistically significant increase in both acidic and alkaline DNAase activity in the rat kidney tissue (Figure 3). When the CAPE was applied as a protective agent an increase in the activity of DNAases was partially modulated. In the case of alkaline DNAase the

treatment regimen with CAPE, prior and after CP, significantly prevent its increase, while at the same time had only a moderate impact on acidic DNAase (Figure 3). In the case when CAPE was administered on its own no changes in the activity of the studied DNAases were noted (Figure 3).

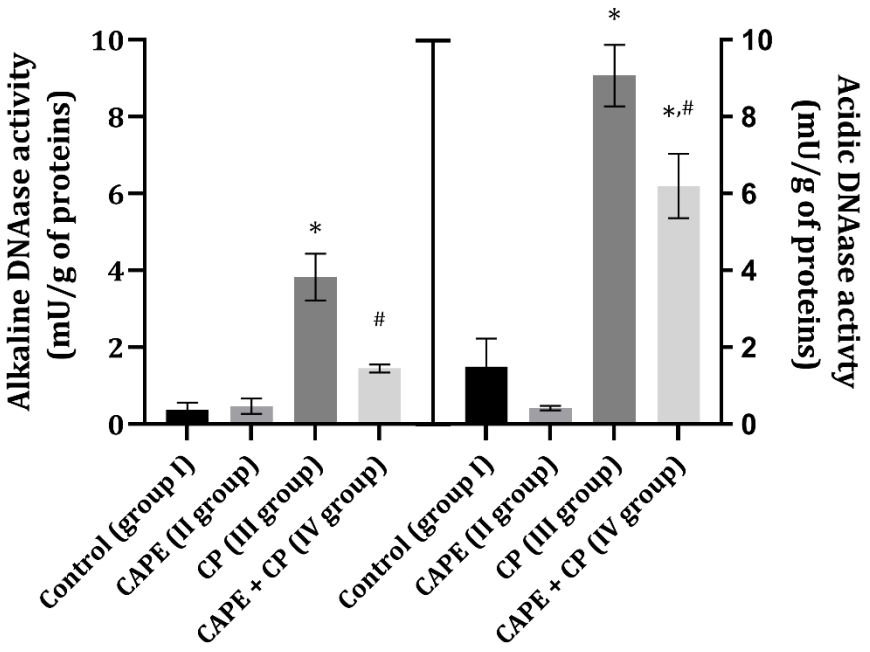

Figure 3. Alkaline (left) and acidic (right) DNAase activity in kidney tissue of rats obtained from different experimental groups. Data are given as mean ± SD, ANOVA followed by Tuckey's post hoc test. *p<0.001 vs. Control (Group I); #p<0.001 vs. CP (Group III)

Rat kidney tissue obtained from the control group and CAPE treated group showed a normal histomorphology, without any noticeable changes in glomerular and tubular structures (Table 2, Figure 4A and B). On the other hand, treatment of cisplatin-induced extensive lesions in rat kidney cortex manifested in a form of tubular degeneration and accumulation of hyaline casts predominantly in S1 and S2 segments of proximal tubules (Figures 4C and D). Also, Bowman's spaces were significantly larger compared to vehicle and CAPE treated groups, as well as with group treated with both cisplatin and CAPE (Figures C and D, Table). Kidney tubules and blood vessels were surrounded with mild mononuclear cell infiltration, which was present at a minimum after the treatment of CAPE (Figures 4C and D, Table 2). Congestion of blood vessels was also observed in group III, however, it was still present in the group IV (Figures 4C and D, Table 2). The degree of cellular necrosis wasn't significant in any group receiving CP (Figures 4C and D, Table 2).

The analysis of the TUNEL kidney samples obtained from the control group and group only treated with CAPE showed rare TUNEL positive cells (Figures 5A and B). Group treated with CP showed a significant increase in the number of positive TUNEL cells, indicating the toxic effect of CP on kidney tubule cells (Figure 5C). On the other side, treatment with CP and CAPE led to a significant reduction in TUNEL positive cells and only occasional apoptotic cells could be seen (Figure 5D) compared to group III (Figure 5C).

Table 2. Pathohistological semiquantitative score of kidney tissue samples obtained from different experimental group of animals

Group/Parameter	Group I (Vehicle treated animals)	Group II (CAPE treated animals)	Group III (CP treated animals)	Group IV (CP and CAPE treated animals)
Tubular degeneration	-	-	+++	++
Necrosis	-	-	+	+
Inflammation	-	-	+	- /+
Hyaline casts	-	-	+++	++
Dilatation of Bowman's capsule	-	-	+++	++
Vascular congestion	-	-	++	+

Degree of changes in kidney tissue: absent (-), minimal (-/+), mild (+), moderate (++), severe (+++)

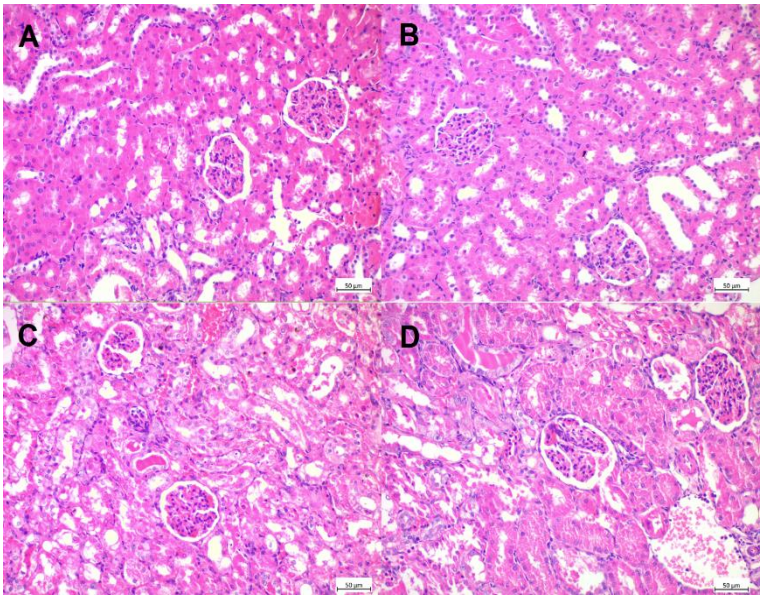

Figure 4. Histopathological findings in kidney tissue specimens obtained from each group of animals: group I and II are animals with a normal histological kidney tissue appearance, without any pathological substrate (**A** and **B**), while group treated with cisplatin (group III) showed extensive tubular degeneration, dilatation of Bowman's capsule and hyaline casts inside of tubules (**C**); persistent tubular degeneration with focally intratubular hyaline casts and vascular congestion was observed in group IV (**D**) (H&E, magnification ×200).

Kidney function was evaluated by analyzing serum levels of urea, creatinine, sodium, potassium, and calcium, all representing useful biomarkers of acute kidney damage in routine clinical practice (Figure 1). In the present research, treatment with a single dose of CP induced a significant increase of serum levels of urea, creatinine, and sodium, while values of potassium and calcium were unaffected. These findings could collaborate with pathomorphological changes associated with acute kidney tissue injury that follow CP application, which include extensive tubular degeneration, necrosis, vascular congestion, and mild peritubular infiltration [30]. Also, the presence of hyaline casts in the lumen of S1 and S2 segment tubules causing their obstruction and disturbance of regular diffusion and secretion processes occurring at the tubule level [30]. On the other hand, we found that treatment with CAPE (group IV) has protective effects, since it ameliorated the disturbance in the serum urea and creatinine levels (Figure 1A and B), while it had no results on other biochemical parameters. Interestingly, in a study dealing with the effects of CAPE in the glycerol-induced acute kidney model, it was found that

CAPE has no significant effect on the here studied biochemical parameters [31]. Even opposite, CAPE application caused an increase in urea and creatinine levels, suggesting that this molecule damages kidney tissue [31].

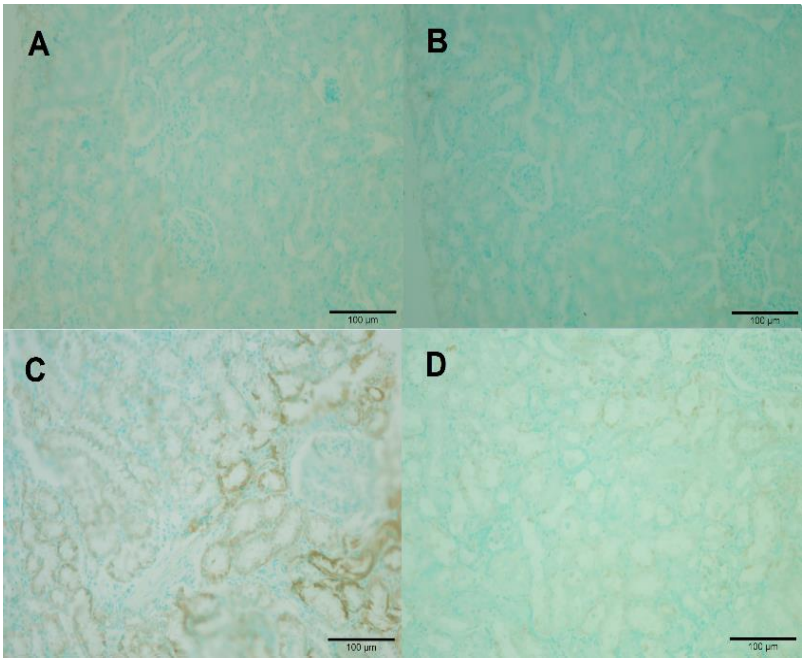

Figure 5. TUNEL stained sections from each of the study groups (magnification × 200). Control group (**A**) and CAPE treated group (**B**) with rare TUNEL positive apoptotic cells; CP treated group (**C**) with a high number of TUNEL positive (brown colored) apoptotic cells in proximal and distal tubules; CAPE and CP treated group with occasional presence of TUNEL positive cells (**D**).

Five-day treatment with CAPE significantly reflected on CAT activity disturbance following the exposure to CP, while having a modest impact on POD activity (Table 1). The effects of CAPE on CAT activity are debatable [7], and some studies indicate that CAPE can increase CAT activity [32]. The impact of CAPE might be at the transcriptional and/or translational levels of this antioxidant enzyme. However, it also could be associated with the prevention of enzyme utilization by ROS, since CAPE could also decrease ROS [32]. Although CAPE prevented the decrease in CAT and POD induced by CP, the amounts of H_2O_2 remained significantly increased compared to the untreated animals, suggesting that there must be some significant source for the generation of ROS that CAPE is not targeting (e.g. superoxide dismutase). Also, one might speculate that a complete prevention of ROS formation that follows CP application would not be an ideal mechanism of a protective drug, since the absence of ROS would potentially mean an absence of CP antineoplastic activity.

One of the prime mechanisms associated with CP toxicity is the generation of ROS which includes lipid peroxidation, different enzyme inactivation, disturbance in the cellular non-enzymatic and enzymatic antioxidant system [33]. An increase in kidney XO activity that follows CP application, which could be the consequence of several mechanisms, is a good source for ROS which in turn inhibits some of the antioxidant enzymes, resulting in lipid peroxidation (e.g. MDA) and TOS increase. This scenario was observed in the present study as well (Table 1), and the results of the present study suggest that CAPE could play a significant role in preventing the disturbance in kidney antioxidant status that follows CP application. By inhibiting XO activity, as is shown previously in various models [7,32,34], CAPE could inhibit the propagation of oxidative damage induced by CP. With this in mind, the MDA and TOS results obtained from animals treated with CAPE and CP are completely understandable. On the other hand, CP could cause an increase in ROS production through other different enzymatic and non-enzymatic mechanisms which might not be under influence of CAPE.

Cellular GSH, as well as enzymes associated with its replenishment (GR) and metabolism (GST), are involved in the regulation of numerous biological functions. The GSH itself is one of the main non-enzymatic protector against ROS, since it prevents the oxidative coupling of SH-groups, inactivates peroxides, and detoxifies foreign substances [11]. The levels of this non-enzymatic antioxidant were found to be significantly decreased in rats treated with CP (group III and IV), which is logical since the GSH is associated with the toxicity of CP [12]. In order for CP to exert its toxicity towards kidney tubular cells, it has to react with GSH and then enter the cells. As a result of this one would expect a significant drop in GSH tissue concentrations, followed by marked tubular degeneration [12], which was the case in our study (Table 1 and 2, Figure 4C). The application of CAPE together with CP significantly prevented the decrease in GSH concentration (Table 1), which obviously repercussed to the tubular degeneration (Table 2, Figure 4D). However, it is important to mention that this effect was not absolute, but rather modest having in mind that the levels of GSH were still significantly below the values of the ones in the control group and that the tubular degeneration was still visible in group IV.
Regarding the enzymes involved in the GSH metabolism (GST) and replenishment (GR), it is known that their activity and function are tightly related to the toxicity of CP [35]. One of the GST forms, the so-called Pi form, is known to be a rate-determining enzyme in the conjugation of CP with GSH, and further toxicity of this CP conjugate [35]. The results of our study indicate that CAPE inhibits total GST activity, thus potentially preventing the conjugation step in the cascade of events leading to CP toxicity. These results might be explained through a direct GST inhibition by CAPE and metabolic products generated with CAPE in the presence of tyrosinase, such as CAPE-quinone [36]. The activity of GR in both CP and CAPE + CP groups was found to be decreased, and the treatment regimen had no impact on the GR activity. The activity of GR also reflects GSH levels, so it is not unusual that the GSH levels in both groups III and IV are decreased (Table 1). One should not expect the activity of GR to be maintained at normal, or to be returned to the values near the one found in the control group tissue, since the CAPE is also proven, both at molecular and *in silico* level, to be an inhibitor of this enzyme [37].

Arginase is an enzyme involved in the metabolism of arginine and by its action the amount of substrate for polyamine synthesis increases [38]. An increase in this enzyme's activity was noted in both groups receiving CP and was most prominent in the group that received CP and CAPE (Figure 2A). This increase in arginase activity was followed by a significant decrease in NO_2/NO_3 concentrations, which seems logical if one has in mind that NO is a negative regulator of arginase activity and/or expression [39]. Also, arginase and nitric oxide synthase are competing for the same substrate, arginine, and if the arginase utilizes almost all the substrate, this would decrease total NO levels (Figure 1C). A decrease in arginase activity was found to be associated with amelioration in kidney injury, positively correlating with a decrease in serum urea and creatinine, as well as with a decrease in tissue oxidative damage and inflammatory cell recruitment [40]. These statements do not completely overlap with pathohistological findings, since in group IV (CP + CAPE) event thought arginase activity is increased (Figure 2A) the degeneration score and inflammation infiltrate are lessened compared to group III (Table 2). This suggests that the inflammatory infiltrate that could be modulated by a number of cytokines and small molecules is not only NO-dependent. The activity of CAPE on the inflammatory infiltrate could be due to the inhibition of various cytokine production, such as tumor necrosis factor α and myeloperoxidase, as shown in the previous study [7].

The present study did not find any significant alteration in kidney tissue citrulline levels after the CP treatment (group III and IV, Figure 2B). Although kidney tissue is essential for balancing the amino acid pool in the blood, the toxicity associated with the application of CP is known only to increase plasma glutamine [38]. Apart from the mentioned, the kidney is a place where the *de novo* synthesis of arginine is conducted from citrulline [41], and the importance of increasing the level of citrulline is essential for keeping the metabolism running. The 5-day treatment with CAPE increased kidney citrulline levels (Figure 2B), through the mechanisms which should be further investigated more in-depth.

Apoptosis or programmed cell death is a multistage process which includes internucleosomal fragmentation of DNA molecule catalyzed by enzymes such as alkaline and acidic DNase, which activity is significantly increased during the process [42-44]. One of the mechanisms through which CP may cause DNA damage through the formation of crosslinks between neighboring guanine bases [45],

resulting in the induction of apoptosis within tubule epithelium cells. In the present study, after the treatment of CP levels of acidic and alkaline DNase were significantly increased, while treatment of CAPE, prior and after CP, showed some potential in preventing an increase in these enzymes' activities (Figures 3A and B), especially the activity of alkaline DNAase. The DNA fragmentation could also be assessed by the TUNEL method, which we performed in order to better corroborate the findings of the DNAase activities. The application of CP leads to a significant increase in the number of TUNEL positive cells (Figure 5C), which corresponds well with a statistically significant increase in both alkaline and acidic DNAase activities (Figures 3A and B). In a group treated with CAPE and CP only a mild increase in TUNEL positive cells (Figure 5D), followed by a slight disturbance in acidic DNAase (Figure 3B) was noted. In a model of methotrexate-induced kidney cell apoptosis, it was found that polyphenol-rich propolis extract prevents this process [46].

4. Conclusions

From the results of our study, we could say that CAPE prevents nephrotoxicity induced by CP through several mechanisms. Primarily, CAPE partially prevents an increase in ROS levels thus acting as an antioxidant, by itself or through an increase in antioxidant enzymes activities. Moreover, CAPE might impact CP metabolism through the alterations in GSH metabolism, however, these claims need more in-depth studies. Also, we might speculate that the nephroprotective activity of CAPE in the cisplatin damage model is not associated with the arginase/NO pathway, but rather with some other pathway(s). Finally, a nephroprotection of CAPE might be mediated through the inhibition of the renal cell programmed death which might or might not be associated with a ROS scavenging activity of CAPE. In the end, the sum results of our study suggest that CAPE might find its application in an everyday clinical/oncological practice as a supplement during CP treatment

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References

[1] A. M. Florea and D. Busselberg (2011). Cisplatin as an anti-tumor drug: Cellular mechanisms of activity, drug resistance and induced side effects, *Cancers* **3,** 1351-1371.

[2] S. Dasari and P. B. Tchounwou (2014). Cisplatin in cancer therapy: Molecular mechanisms of action, *Eur. J. Pharmacol.* **740**, 364-378.

[3] L. Galluzzi, L. Senovilla, I. Vitale, J. Michels, I. Martins, O. Kepp, M. Castedo and G. Kroemer (2012). Molecular mechanisms of cisplatin resistance, *Oncogene* **31**, 1869-1883.

[4] H. Kızıltaş, A. C. Goren, Z. Bingol, S. H. Alwasel and I. Gulcin (2021). Anticholinergic, antidiabetic and antioxidant activities of *Ferula orientalis* L. determination of its polyphenol contents by LC-HRMS, *Rec. Nat. Prod*. **15**, 513-528.

[5] I. Gülçin (2012). Antioxidant activity of food constituents: an overview, *Arch. Toxicol*. **86**, 345-391.
[6] I. Gulcin (2020). Antioxidants and antioxidant methods: an updated overview, *Arch. Toxicol*. **94**, 651-715.
[7] B. Stošić, R. Janković, M. Stošić, D. Marković, I. Veselinović, I. Ilić and D. Sokolović (2020). Caffeic acid phenethyl ester attenuates changes in pancreatic tissue damage biomarkers induced by cisplatin, *Can. J. Physiol. Pharmacol*. **98**, 296-303.
[8] E. Yuluğa, S. Türedib, O. Yıldırıma, E.Yenilmeza, Y. Aliyazıcıoğluc, S. Demird, S. Ozer-Yaman and A. Mentese (2019). Biochemical and morphological evaluation of the effects of propolis on cisplatin induced kidney damage in rats, *Biotech. Histochem*. **94**, 204-213.
[9] N. A. G. Dos Santos, M. A. C. Rodrigues and N. M. Martins (2012). Cisplatin-induced nephrotoxicity and targets of nephroprotection: an update, *Arch. Toxicol*. **86**, 1233-1250.
[10] L. A. Peres and A. D. da Cunha Jr (2013). Acute nephrotoxicity of cisplatin: molecular mechanisms, *J. Bras. Nefrol*. **35**, 332-340.
[11] D. Appenroth and K. Winnefeld (1993). Role of glutathione for cisplatin nephrotoxicity in young and adult rats, *Ren. Fail*. **15**, 135-139.
[12] D. M. Townsend, M. Deng, L. Zhang, M. G. Lapus and M. H. Hanigan (2003). Metabolism of Cisplatin to a nephrotoxin in proximal tubule cells, *J. Am. Soc. Nephrol.* **14**, 1-10.
[13] S. Akyol, V. Ugurcu, A. Altuntas, R. Hasgul, O. Cakmak and O. Akyol (2014). Caffeic acid phenethyl ester as a protective agent against nephrotoxicity and/or oxidative kidney damage: A detailed systematic review, *Sci. World J.* **2014**, 561971.
[14] Y. Yordanov (2019). Caffeic acid phenethyl ester (CAPE): Pharmacodynamics and potential for therapeutic application, *Pharmacia* **66**, 107-114.
[15] H. Göçer and I. Gülçin (2011). Caffeic acid phenethyl ester (CAPE): correlation of structure and antioxidant properties, *Int. J. Food Sci. Nutr*. **62**, 821-825.
[16] S. Özen, O. Akyol, M. Iraz, S. Sögüt, F. Özugurlu, H. Özyurt, E. Odaci and Z. Yildirim (2014). Role of caffeic acid phenethyl ester, an active component of propolis, against cisplatin-induced nephrotoxicity in rats, *J. Appl. Toxicol.* **24**, 27-35.
[17] G. J. Dugbartey, L. J. Peppone and I. A. de Graaf (2016). An integrative view of cisplatin-induced renal and cardiac toxicities: Molecular mechanisms, current treatment challenges and potential protective measures, *Toxicology* **371**, 58-66.
[18] T. Mercantepe, D. Unal, L. Tümkaya and Z. A. Yazici (2018). Protective effects of amifostine, curcumin and caffeic acid phenethyl ester against cisplatin-induced testis tissue damage in rats, *Exp. Ther. Med*. **15**, 3404-3412.
[19] S. Bakır, U. C. Yazgan, I. İbiloğlu, B. Elbey, M. Kızıl and M. Kelle (2015). The protective effect of pomegranate extract against cisplatin toxicity in rat liver and kidney tissue, *Arch. Physiol. Biochem*. **121**, 152-156.
[20] O. H. Lowry, N. J. Rosenbrough, A. L. Farr and R. J. Randall (1951). Protein measurement with the Folin phenol reagent, *J. Biol. Chem.* **193**, 265-275.
[21] L. Goth (1991). A simple method for determination of serum catalase activity and revision reference range, *Clin. Chim. Acta*. **196**, 143-151.
[22] D. Popović, G. Kocić, V. Katić, Z. Jović, A. Zarubica, Lj. Janković-Veličković, V. Nikolić, A. Jović, B. Kundalić, V. Rakić, N. Poklar Ulrih, M. Skrt, D. Sokolović, Lj. Dinić, M. Stojanović, A. Milosavljević, F. Veličković and D. Sokolović (2019). Protective effects of anthocyanins from bilberry extract in rats exposed to nephrotoxic effects of carbon tetrachloride, *Chem. Biol. Interact.* **304**, 61–72.
[23] D. T. Sokolović, L. Lilić, V. Milenković, R. Stefanović, T. P. Ilić, B. Mekić, I. Ilić, N. M. Stojanović and I. R. Ilić (2018). Effects of melatonin on oxidative stress parameters and pathohistological changes in rat skeletal muscle tissue following carbon tetrachloride application, *Saudi. Pharm. J.* **26**, 1044-1050.
[24] M. Radović, L. Ristić, D. Krtinić, M. Rančić, V. Ničković, Z. N. Vujnović Živković, J. B. Živković, M. V. Mirković, D. R. Toškić and D. Sokolović (2019). Melatonin treatment prevents carbon tetrachloride-induced acute lung injury in rats, *Bratis. Med. J.* **120**, 527-531.
[25] D. Popović, G. Kocić, V. Katić, A. Zarubica, Lj. Janković Veličković, V. P. Ničković, A. Jović, A. Veljković, V. Petrović, V. Rakić, Z. Jović, N. Poklar Ulrih, D. Sokolović, M. Stojanović, M. Stanković, G. Radenković, G. R. Nikolić, A. Lukač, A. Milosavljević and D. Sokolović (2019). Anthocyanins protect hepatocytes against CCl4-induced acute liver injury in rats by inhibiting pro-inflammatory mediators, polyamine catabolism, lipocalin-2, and excessive proliferation of Kupffer cells, *Antioxidants* **8**, 451.
[26] N. M. Stojanović, P. J. Randjelović, M. Z. Mladenović, I. R. Ilić, V. Petrović, N. Stojiljković, S. Ilić and N. S. Radulović (2019). Toxic essential oils, part VI: Acute oral toxicity of lemon balm (*Melissa officinalis* L.) essential oil in BALB/c mice, *Food Chem. Toxicol*. **133**, 110794.

[27] V. P. Ničković, T. Novaković, S. Lazarević, L. Šulović, Z. Živković, J. Živković, B. Mladenović, N. M. Stojanović, V. Petrović and D. T. Sokolović (2018). Pre- vs. post-treatment with melatonin in CCl_4-induced liver damage: Oxidative stress inferred from biochemical and pathohistological studies, *Life Sci.* **202**, 28-34.

[28] D. Sokolović, B. Đorđević, G. Kocić, T. J. Stoimenov, Z. Stanojković, D. M. Sokolović, A. Veljković, G. Ristić, M. Despotović, D. Milosavljević, R. Janković and I. Binić (2015). The effects of Melatonin on oxidative stress parameters and DNA fragmentation in testicular tissue of rats exposed to microwave radiation, *Adv. Clin. Exp. Med*. **24**, 429-436.

[29] N. S. Radulović, P. J. Randjelović, N. M. Stojanović, I. R. Ilić, A. B. Miltojević, M. B. Stojković and M. Ilić (2015). Effect of two esters of N-methylanthranilic acid from Rutaceae species on impaired kidney morphology and function in rats caused by CCl_4, *Life Sci.* **135**, 110-117.

[30] X. Yao, K. Panichpisal, N. Kurtzman and K. Nugent (2007). Cisplatin nephrotoxity: A review, *Am. J. Med. Sci.* **334**, 115-124.

[31] N. Aydogdu, G. Atmaca, O. Yalcin, K. Batcioglu and K. Kaymak (2004). Effects of caffeic acid phenthyl ester on glycerol-induced acute renal failure in rats, *Clin. Exp. Pharmacol. Physiol*. **31,** 575-579.

[32] A. Altuntaş, H. R. Yılmaz, A. Altuntaş, E. Uz, M. Demir, A. Gökçimen, O. Aksu, D. S. Bayram and M. T. Sezer (2014). Caffeic acid phenethyl ester protects against amphotericin B induced nephrotoxicity in rat model, *Biomed. Res. Int.* **2014**, 702981.

[33] H. R. Yilmaz, E. Uz, N. Yucel, I. Altuntas and N. Ozcelik (2004). Protective effect of caffeic acid phenethyl ester (CAPE) on lipid peroxidation and antioxidant enzymes in diabetic rat liver, *J. Biochem. Mol. Toxicol.* **18**, 234-238.

[34] S. Turkyilmaz, E. Alhan, C. Ercin, B. Kural Vanizor, N. Kaklikkaya, B. Ates, S. Erdogan and S. Topaloglu (2008). Effects of caffeic acid phenethyl ester on pancreatitis in rats, *J. Surg. Res.* **145**, 19-24.

[35] D. M. Townsend, K. D. Tew, L. He, J. B. King and M. H. Hanigan (2009). Role of glutathione S-transferase Pi in cisplatin-induced nephrotoxicity, *Biomed. Pharmacother.* **63**, 79-85.

[36] S. K. Kudugunti, H. Thorsheim, M. S. Yousef, L. Guan and M. Y. Moridani (2011). The metabolic bioactivation of caffeic acid phenethyl ester (CAPE) mediated by tyrosinase selectively inhibits glutathione S-transferase, *Chem. Biol. Interact.* **192**, 243–256.

[37] R. E. Salmas, M. F. Gulhan, S. Durdagi, E. Sahna, H. I. Abdullah and Z. Selamoglu (2017). Effects of propolis, caffeic acid phenethyl ester, and pollen on renal injury in hypertensive rat: An experimental and theoretical approach, *Cell. Biochem. Funct*. **35**, 304-314.

[38] M. Cobo, M. A. Martín Gómez, M. A. Frutos and M. Benavides (2007). Glutamin concentrations in patients treated with cisplatin have a predicting value of renal failure development, *Nefrologia*. **27**, 23-29.

[39] W. Durante, F. K. Johnson and R. A. Johnson (2007). Arginase: a critical regulator of nitric oxide synthesis and vascular function, *Clin. Exp. Pharmacol. Physiol*. **34**, 906-911.

[40] H. You, T. Gao, T. K. Cooper, S. M. Morris and A. S. Awad (2013). Arginase inhibition mediates renal tissue protection in diabetic nephropathy by a nitric oxide synthase 3-dependent mechanism, *Kidney Int.* **84**, 1189-1197.

[41] S. N. Dhanakoti, M. E. Brosnan, G. R. Herzberg and J. T. Brosnan (1992). Cellular and subcellular localization of enzymes of arginine metabolism in rat kidney, *Biochem. J*. **282**, 369-375.

[42] R. J. Mogil, Y. Shi, R. P. Bissonnette, P. Bromley, I. Yamaguchi and D. R. Green (1994). Role of DNA fragmentation in T cell activation-induced apoptosis in vitro and in vivo, *J. Immunol*. **152**, 1674-1683.

[43] P. R. Walker and M. Sikorska (1997). New aspects of the mechanism of DNA fragmentation in apoptosis, *Biochem. Cell. Biol.* **75**, 287-299.

[44] G. Cao, W. Pei, J. Lan, R. A. Stetler, Y. Luo, T. Nagayama, S. H. Graham, X. M. Yin, R. P. Simon and J. Chen (2001). Caspase-activated DNase/DNA fragmentation factor 40 mediates apoptotic DNA fragmentation in transient cerebral ischemia and in neuronal cultures, *J. Neurosci.* **21**, 4678–4690.

[45] D. S. Goodsell (2006). The molecular perspective: Cisplatin, *Oncologist* **11**, 316-317.

[46] H. B. Ulusoy, I. Öztürk, and M. F. Sönmez (2016). Protective effect of propolis on methotrexate induced kidney injury in the rat, *Ren. Fail.* **38**, 744-750.