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Phytochemical screening and radical scavenging activity of green coffee bean extract: Multiple-protective efficacy against Bisphenol A

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ABSTRACT: In this study, phytochemical characterization, radical scavenging activity and the protective role of green coffee bean extract (Gcbe) against Bisphenol-A (BpA) were evaluated. In phytochemical analysis total phenolic, flavonoid and tannin contents were investigated and major components were determined by HPLC. The amerolative effects of Gcbe against BpA-induced toxicity were studied in albino mice. For this purpose, mice were separated into six groups which treated with tap-water, BpA and two doses of Gcbe. To determine BpA toxicity and the protective role of Gcbe, changes in some biochemical parameters, micronucleus (MN) frequency and DNA fragmentation were investigated. As a result, the phenolic compounds in the Gcbe content were more intense than the other components. In BpA-treated group, serum parameters increased and the antioxidant/oxidant dynamics were impaired. Gcbe treatment decreased the induced serum parameters and caused an improvement in antioxidant-oxidant dynamics and also a decrease in the DNA-tail percentage and MN frequency.

Keywords *– Bisphenol A, Comet, Green coffee bean extract, Phytochemical characterization, Radical scavenging activity*

1. Introduction

Consumption of natural supportive nutrients has an important role in reducing the toxic effects of harmful chemicals. Coffee is one of the most widely consumed beverages around the world and has positive effects on health. These positive effects are mostly associated with the phytochemicals in the coffee composition. Coffee beans are highly enriched in chlorogenic acid ingredient, which has a high antioxidant capacity. The roasting process reduces the chlorogenic acid content of the coffee beans, and therefore its antioxidant properties are also reduced (Del Castillo et al., 2002). Since the green coffee bean are not subjected to the roasting process, so chlorogenic acids, soluble fiber, trigonelline, caffeine and diterpenes are mostly found. The presence of some active minor phenolics such as anthocyanins, lignans, theobromine, theophylline in green coffee has been reported. Anthocyanins, theophylline and lignans have been shown to exhibit high antioxidant activity. Haile and Kang (2019) determined intense phenolic content as well as flavonoid and tannin content in the phytochemical analysis of green coffee extract. In HPLC analyzes of green coffee, chlorogenic acid, caffeoylquinic acid dimer, feruloylquinic acid dimer, 5-caffeoylquinic acid peaks were identified and it was reported that chlorogenic acid content was more dominant in general (Navarra et al., 2017; Vinson et al., 2019). Various studies have indicated that chlorogenic acid and polyphenolic compounds found in green coffee can

diminish the risk of different diseases. Salamat et al. (2019) have shown that green coffee bean extract (Gcbe) reduced low density lipoprotein-oxidation and increased the antioxidant capacity in patients with dyslipidemia. Yukawa et al. (2004) reported that MDA and thiobarbituric acid levels, which are indicators of lipid peroxidation, decreased after one week of green coffee consumption. These protective effects were associated with the active ingredients of the coffee bean. In this study, phytochemical characterization, HPLC analysis, radical scavenging activity and the protective role of Gcbe against Bisphenol A (BpA) induced toxicity were evaluated.

BpA is widely used in the production of flame retardants, polycarbonates, epoxy, polysulfone and polyacrylate resins. From these materials containing BpA, frequently used products such as milk and beverage bottles, water bottles, water pipes, feed containers and baby bottles are produced. The presence of BpA in these products, which are frequently used in daily life, makes contamination into organisms inevitable. After contamination to organisms, BpA enters the digestive system and it is distributed to other tissues. In the liver, it is highly conjugated to form glucuronide conjugates so that it can be excreted from the body (Fisher et al., 2011). The frequency of exposure to BpA in the world is increasing day by day and an average of 2.5-3.0 % increase is observed annually. BpA toxicity occurs mostly in the endocrine system, and this effect is due to the high affinity and binding capacity of BpA to estrogen receptors (Melzer et al., 2012). Liver and kidney tissues are also important organs affected by BpA toxicity in humans and animals. Damages in liver tissue due to oxidative stress have been reported in male rats treated with BpA (Bindhumol et al., 2003). In another study, Hoque et al. (2019) reported significant changes in biochemical parameters, decreases in high density lipoprotein and triglyceride levels and an increase in liver enzymes in mice exposed to 100 mg/kg BpA. BpA is known to have a genotoxic effect in addition to the toxicity in the endocrine system and biochemical metabolism. Anet et al. (2019) reported that BpA exposure showed a genotoxic effect due to oxidative stress in *Drosophila melanogaster*. Studies on BpA in the literature generally focus on its effects on the endocrine system. Multidisciplinary approach is very rare in studies examining other effects of BpA.

In this study, phytochemical characterization, radical scavenging activity and the protective role of Gcbe against BpA toxicity were investigated. In phytochemical analysis tannin, total phenolic and total flavonoid contents were investigated and major components were determined by HPLC analysis. Radical scavenging effect of Gcbe was tested against hydrogen peroxide (H_2O_2), superoxide and 1,1-difenil-2-pikrilhidrazil (DPPH). The amerolative effect of Gcbe against BpA-induced cytogenetic and biochemical toxicity were studied in Swiss albino mice.

2. Material and Methods

2.1. Materials

Powder form of *Coffea arabica* bean was obtained from Sepe Natural (930mgx60 capsules). Alanine aminotransferase, aspartate aminotransferase, blood urea nitrogen and creatinine test kits were purchased from Teco Diagnostics. All other reagents and chemicals were obtained from Sigma-Aldrich.

2.2. Phytochemical analysis of Gcbe

100 mg powder form of *Coffea arabica* beans were extracted with 200 mL of 80% methanol on a shaker for 24 h, the solvent evaporated and the extract was used for further analysis. For the phytochemical analysis of Gcbe tannin, total phenolic and total flavonoid contents were investigated. Total phenolic content of Gcbe was measured using the protocol described by Singleton and Orthofer (1999). 160 µL of Gcbe and 10 µL of Folin-Ciocalteu's reagent was mixed and kept for 8 min. 30 µL of Na_2CO_3 solution was added to the mixture and then incubated for 2 h in a dark room. Distilled water and gallic acid (GA) were used as a control and standard, respectively. Final absorbance was measured at 765 nm and the results were presented as mg GA equivalent (GAE)/mL extract.

Total flavonoid content of Gcbe was evaluated by using the method proposed by Dewanto et al. (2002). 250 µL of Gcbe, 1 mL of dH_2O and 75 µL of $NaNO_2$ were mixed and after 5 min, 150 µL of $AlCl_3$. $5H_2O$ solution was added. 500 µL of NaOH was added to final mixture and incubated for 11 min. Distilled water and quercetin were used as a control and standard, respectively. After incubation the absorbance was measured at 510 nm. Total flavonoid content of Gcbe was presented as mg quercetin equivalent (QE)/mL extract.

Total tannin content was analyzed by using the modified Folin-Ciocalteu method, with minor modifications. 0.1 mL of Gcbe, 7.5 mL of dH_2O, 0.5 mL of Folin-Ciocalteu reagent and 1 mL of Na_2CO_3 were mixed and completed to 10 mL with distilled water. After incubation for 30 min the absorbance of solution was recorded at 700 nm. Distilled water and tannic acid (TA) were used as a control and standard, respectively. The tannin content was expressed in terms of mgTA/mL extract.

2.3. HPLC analysis

Samples were ground with a mortar and 30-50 mg sample were extracted with 100 mL of methanol on a shaker for 25 min and then centrifuged for 10 min. After centrifugation, the supernatants were filtered with 0.45 µm filters (Whatman) and then analyzed. HPLC analysis was carried out with a XDB-C_8 analytical column (150 mm*4.6 mm, 5 µm) at 25^oC. A gradient of (A): acetonitrile and (B): 1 % glacial acetic acid were used as mobile phase. Flow rate and injection volume were 1.0 mL/min and 10 µl, respectively. Before the injection, the extract dissolved in methanol at a concentration of 1 mg/mL. Caffeine, chlorogenic acid and caffeic acid were used as reference standards in the analyzes performed at 280 nm.

2.4. Scavenging activity of Gcbe

In this study, radical scavenging effect of Gcbe was tested against H_2O_2, superoxide and DPPH, which have strong oxidizing properties. The superoxide, DPPH and H_2O_2 radical scavenging activities of Gcbe were studied according to the modified method proposed by Gülçin (2010). Light induction of the reaction mixture was carried out using a fluorescent lamp (20 W). Riboflavin (1.33×10^{-5} M), methionine (4.46×10^{-5} M) and nitroblue tetrazolium (8.15×10^{-8} M) were prepared and illuminated at 25 °C for 40 min. The un-illuminated mixture was used as a blank. Different concentrations of Gcbe (0.125-1.0 mg/mL) were added to the reaction medium and the absorbance of all mixtures was measured at 560 nm.

The reduced absorption of the reaction mixture indicates increased scavenging activity. The percentage of superoxide scavenging activity was calculated using Eq. (1).

Superoxide scavenging (%) = $[1-(A_1/A_2)] \times 100$ (1)
where A_1 is the absorbance of Gcbe or standard solution, A_2 is the absorbance of the control. The experiment was repeated three times at each concentration.

To determine the H_2O_2 scavenging activity; a solution containing different concentrations of Gcbe (3.4 mL, 0.125-1.0 mg/mL) and 0.6 mL H_2O_2 (40 mM) was prepared. The absorbance of the reaction mixture was measured spectrophotometrically at 230 nm. Sodium phosphate buffer, which does not contain H_2O_2, was accepted as blank. The scavenging activity was determined by monitoring the decrease in H_2O_2 absorbance. The absorbance of H_2O_2 and the scavenging activity of Gcbe were calculated using Eq. (2) and (3), respectively.

Absorbance (λ_{230}) = $0.505 \times [H_2O_2]$ (2)
H_2O_2 scavenging activity (%) = $[1-(A_1/A_2)] \times 100$ (3)
A_1 is the absorbance of solution in the presence of Gcbe, A_2 is the absorbance of the control. The experiment was repeated three times at each concentration.

DPPH scavenging activity analysis is based on the principle of de-coloring DPPH in methanol solution. DPPH creates a purple color in the methanol solution and turns yellow in the presence of antioxidant substances. 2.4 mL DPPH (0.1 mM) solution in methanol was mixed with different concentrations of 1.6 mL Gcbe (0.125-1.0 mg/mL). The absorbance of the solution left in the dark for 30 min was measured at 517 nm. Butylated hydroxytoluene (BHT) was used as standard and DPPH radical scavenging activity was calculated using Eq. (4).

% DPPH radical scavenging activity = $(A_0-A_1)/A_0 \times 100$ (4)
A_0 is the absorbance of the control and A_1 is the absorbance of the Gcbe or standard solution. The experiment was repeated three times at each concentration.

2.5. Protective effect of Gcbe against BpA-induced toxicity

2.5.1. Animals and experimental protocol

The experiments were performed with 12-14 weeks old 36 male Swiss albino mice (*Mus musculus* var. *albinos*) (25–30 g). The mice were kept under controlled conditions at a 12 h light/dark cycle, 55±5 % relative humidity, temperature of 22±3 °C and kept in stainless steel cages during the experiment. In the experimental procedure, albino mice were separated into six groups which treated with tap water (control), 100 mg/kg bw Gcbe, 400 mg/kg bw Gcbe, 50 mg/kg bw BpA, 50 mg/kg bw BpA+100 mg/kg bw Gcbe, 50 mg/kg bw BpA+400 mg/kg bw Gcbe. In this study, a dose of 50 mg/kg bw BpA was used, in which the toxic effect was observed in our previous literature study (İştar et al., 2016). In the dose selection of Gcbe, the doses have preferred in which a clear protective effect against different toxicities was observed (Alristina et al., 2018). Gcbe administration was initiated seven days prior to BpA exposure so that the protective effect of Gcbe can occur immediately when the cells encounter the toxic agent. The techniques and methods were conducted as stated by the principles set by the ethical standards of the Giresun University

Experimental Animals Local Ethics Committee (Protocol date: 25.03.2015, Decision number: 2015/05) and the World Health Organization (Geneva, Switzerland).

2.5.2. Oxidant/antioxidant dynamic

The effects of BpA and Gcbe treatment on oxidant and antioxidant dynamic were investigated by Malondialdehide (MDA) and Glutathione (GSH) analysis in liver and kidney. At the end of the BpA and Gcbe treatment sacrification of mice was performed under halothane anesthesia. Kidney and liver tissues of each animal were taken and cleaned. The tissues were homogenized in 0.15 M KCI (*ice-cold*) by a homogenizer (Ultra-Turrax T25-B) for 3 min at 16000 rpm. The homogenates were centrifuged for 1 h at 5000 rpm at 4 ^{o}C. The supernatants were used for MDA and GSH analysis. MDA and GSH contents were measured colorimetrically as described by Yalçın et al. (2020).

2.5.3. Serum parameters

Blood samples were obtained by cardiac perforation when the animals were in light halothane anesthesia. Samples were transported to vacutainer plain tubes, centrifuged at 1200 rpm at 4 ºC for 10 min and maintained at -20 ºC until analyzed. Aspartate aminotransferase (AST-Teco Diagnostics, A559-150), alanine aminotransferase (ALT-Teco Diagnostics, A524-150), blood urea nitrogen (BUN-Teco Diagnostics, B549-150) and creatinine (Teco Diagnostics, C513-480) levels were determined by using commercially test kits with a Medispec 99 M Chemistry auto-analyser.

2.5.4. Histopathological analysis

For the detection of histopathological changes, routine histopathological preparation and Hematoxylin-eosin (H-E) staining were performed. The kidney and liver tissues were fixed in neutral phosphate buffer 10 % formalin solution, dehydrated with alcohol for routine processing embedded in paraffin wax, sectioned at 5 µ and stained with H-E. Histopathological evaluation was performed semi-quantitatively and photographed under a research microscope (Irmeco IM-450 TI model).

2.5.5. Leukocyte MN assay

In order to determine the genotoxic effect of BpA and protective effects of Gcbe, the Leucocyte MN test was performed. For experimental procedure, halothane anesthesia was applied to the mice and then blood samples were collected. The samples were centrifuged at 5000 rpm for 10 min, the upper clear part was discarded, 5 mL of 0.075 M KCl solution was added to the remaining residue and left at room temperature for 20 min. After centrifugation for 10 min, the upper part was removed again, and 5 mL of the washing solution consisting of methanol (3 volumes) and glacial acetic acid (1 volume) mixture was added. Mixture was left in the deep freeze for 30 min. At the end of the period, leukocyte cells were spread on sterile slides, stained with Giemsa (5 %). For each group, 1000 cells were counted to evaluate the frequency of MN.

2.5.6. Comet assay

The protocol of Tice et al. (2000) was performed for alkaline single cell gel electrophoresis with slight modifications. Slides were dipped in 1 % normal melting point agarose for coating and allowed to dry at 37 °C. 10 µL of peripheral blood was added to 120 µL of low-melting point agarose at 37 °C, covered with a coverslip and left at 4 °C for 5 min. Slides were immersed into a solution containing 2.5 M NaCl, 100 mL Na_2EDTA, 10 mM Tris-HCl buffer, pH 10, 1 % Triton X-100 for 1 h and then the slides were transferred to a horizontal gel electrophoresis tank with cooled alkaline buffer. After a 20 min DNA unwinding period, electrophoresed at 0.86 V/cm (20 V, 300 mA) for 20 min. Slides stained using ethidium bromide staining solution after carefully flushing three times with Tris-buffer (0.4 M Tris, pH 7.5) for 5 min. The preparations were washed with cold water to remove excess stain and covered with a coverslip. To prevent DNA damage, all steps were performed in low light and analyzed by fluorescence microscopy. Cells appearing as comets were evaluated by Comet assay software version 1.2.3b with the parameters of tail DNA percentage, tail moment, and olive tail moment and 300 cells were calculated for each group.

2.6. Protective efficacy of Gcbe

Protective efficacy (PE) of Gcbe against BpA induced toxicity was calculated by using Eq. (5). In determining PE, data belonging to Group VI, where Gcbe provided the highest protection, and data from Group IV, in which BpA was administered alone, and data of control group were used. PE values for all tested parameters were calculated separately.

$$PE\ (\%) = [(D_1-D_2)\ /(D_3-D_2)] \times 100 \quad (5)$$

D_1: data of BpA+Gcbe treated group, D_2: Data of BpA treated group, D_3: data of control.

2.7. Statistical analysis

The statistical evaluation was performed using SPSS (v.22.0) statistical software. Results were analyzed using “one-way”ANOVA followed by Duncan’s test. The data are reported as means ± standard deviation (SD) values, and values of $p < 0.05$ are considered statistically significant.

3. Results and Discussion

3.1. Phytochemical analysis of Gcbe

The results obtained by the phytochemical analysis of Gcbe are given in Figure 1. Total phenolic, flavonoid and tannin contents of Gcbe were 0.91±0.11 mgGAE/mL, 0.76±0.05 mgQE/mL and 0.65±0.07 mgTA/mL extract, respectively. It was observed that phenolic compounds were more intense in Gcbe content than other components. The percentages of total phenolic content, total flavonoid content and tannin content were found to be 39 %, 33 % and 23 %,respectively. Briefly, Gcbe exhibits an order according to the content ratio as phenolic > flavonoid > tannin. Similarly, Haile and Kang (2019) determined intense phenolic content as well as flavonoid and tannin content in the phytochemical analysis of green coffee extract. Mehari et al. (2021) performed phytochemical analysis in various commercially available coffee types and determined phenolic content in the range of 21.8-

43.6 mg GAE/g and flavonoid content in the range of 3.3-6.2 mg catechin equivalents/g. Mussatto et al. (2011) reported a total polyphenol constituent of Gcbe as 16.00 mg GAE/g dry sample.

Phenolic compounds are secondary metabolites with various biological roles in plants. Due to its electron donor ability, they have activities to scavenge free radicals and prevent lipid peroxidation. Flavonoids are hydroxylated phenolic compounds synthesized against biotic and abiotic stress in plants. Flavonoids have many biological properties such as antioxidant, antimicrobial, anti-inflammatory, hepatoprotective, anti-viral. The tannins analyzed in the Gcbe content are secondary polyphenolic metabolites of higher plants. Tannins may exert different biological effects such as antioxidant, antimicrobial, radical scavenging, antimutagenic and antiviral, anti-diabetic and antiobesity effects (Haile and Kang, 2019). The rich biological activities of phenolic, flavonoid and tannin compounds detected in phytochemical analysis enable Gcbe to have a multi-biological role.

3.2. HPLC chromatogram of Gcbe

The chromatogram obtained by HPLC analysis of Gcbe is given in Figure 1. Chlorogenic acid, caffeine and caffeic acid are clearly defined in the chromatogram. Considering the peak areas, it shows a ranking as caffeine > chlorogenic acid > caffeic acid according to the ratios of Gcbe content. Analysis of phenolic compounds in green coffee has been extensively studied in the literature. Vinson et al. (2019) identified active components such as chlorogenic acid, caffeoylquinic acid dimer and feruloylquinic acid dimer in green coffee by HPLC analysis. They reported that 5-caffeoylquinic acid was found at the highest rate among the active ingredients. Navarra et al. (2017) analyzed Arabica and Robusta coffees by HPLC and obtained intense caffeine and chlorogenic acid peaks and reported that the chlorogenic acid content was more dominant in both species. Similarly, Mussatto et al. (2011) reported that Gcbe contains large amount of chlorogenic acid. Chlorogenic acid is one of the most abundant polyphenol compounds and is a secondary metabolite produced by many plant species. The highest polyphenolic compound found in green coffee is chlorogenic acid. Chlorogenic acid is an ester of cinnamic acids and quinic acid, and its most common form is 5-caffeoylquinic acid. Convincing results from scientific researches have shown that 5-CQA is a phenolic compound that protects against oxidative stress, inflammatory stress, hepatotoxicity, nephrotoxicity, cardiovascular disease and cancer. These protective properties are closely related to the multi-biological properties of chlorogenic acid. Chlorogenic acid has many biological properties such as antioxidant, antibacterial, anti-inflammatory, antimutagenic, anticarcinogenic, hypoglycemic and hypolipidemic (Navarra et al., 2017; Vinson et al., 2019). With the antioxidant role of chlorogenic acid, oxidative stress in cells is neutralized and abnormalities that may occur in the cell can be prevented. It is known that caffeine, another compound detected in Gcbe content, has important biological activities and especially reduces oxidative stress and increases antioxidant enzyme activities. Despite its name, caffeic acid is structurally unrelated to caffeine but has similar biological activities. Caffeic acid detected in Gcbe content shows antioxidant activity by increasing the expression of antioxidant enzymes GPx and GR, which reduces oxidative stress (Jamali et al., 2019). As a result of the cumulative effects of the biological properties of caffeine, caffeic acid and chlorogenic acid, Gcbe also gains a strong protective effect.

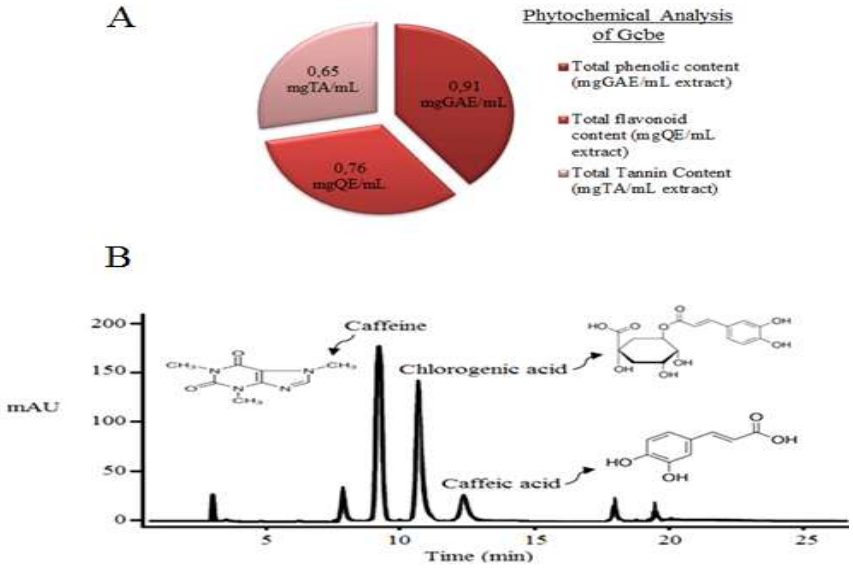
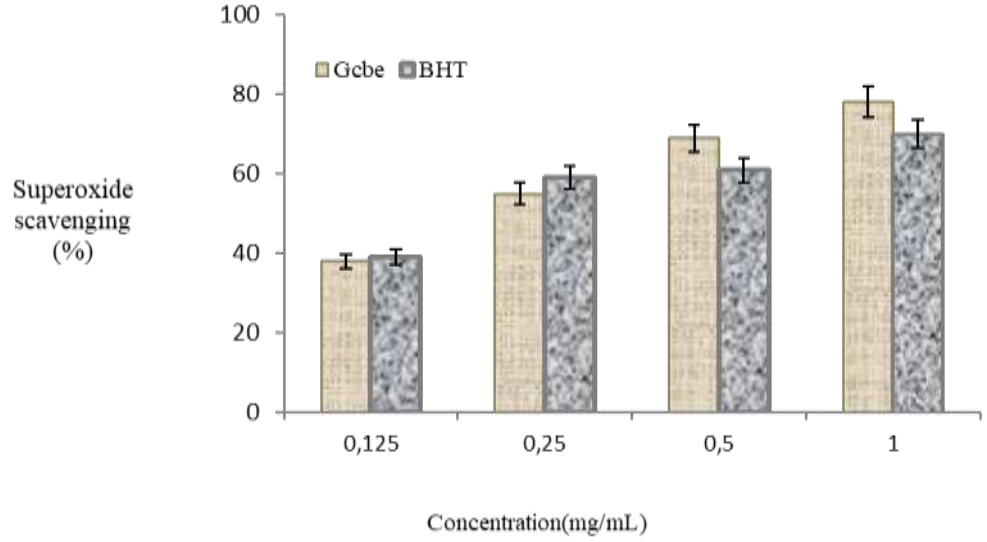


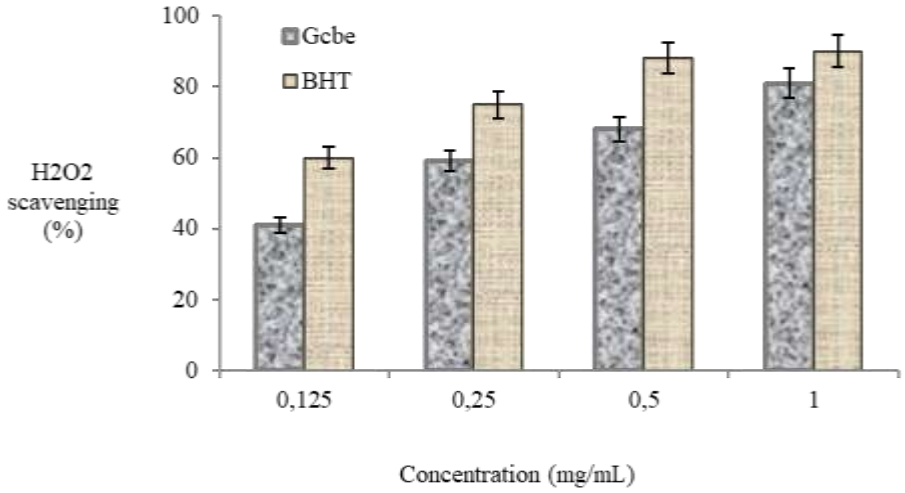
Figure 1. Total phenolic, flavonoid and tannin contents of Gcbe (A), HPLC chromatogram of Gcbe (B).

3.3. Scavenging activity of Gcbe

There is a close relationship between the protective role of Gcbe and its antioxidant activity. Radical scavenging activity plays an important role in antioxidant activity mechanism. The radical scavenging property of Gcbe was tested against superoxide, DPPH and H_2O_2 and the results are given in Figure 2. The scavenging effect of Gcbe also increased with the increasing dose and the highest scavenging effect was achieved against DPPH (86 %). 1 mg/mL Gcbe and BHT exhibited 86% and 80% DPPH scavenging activity, respectively. Among free radicals, superoxide is a strong oxidizing agent and higher superoxide removal activity of Gcbe than BHT was detected at all doses tested except the 0.125 mg/mL. H_2O_2, itself is not very reactive; however, it can sometimes be toxic to cells as it mediates the formation of hydroxyl radical in cells. Gcbe showed a dose-dependent H_2O_2 scavenging activity and the peroxide scavenging activity of 1 mg/mL Gcbe and BHT were 81 % and 90 %, respectively. The strong radical scavenging effect of Gcbe can be attributed to the chlorogenic acid, caffeic acid and caffeine it contains. Chlorogenic acids have an important role in the radical scavenging effect of Gcbe, as they are strong reactive oxygen species (ROS) scavengers. Chlorogenic acid, a phenolic compound, has antioxidant activity and the ability to trap superoxide anions and hydroxyl radicals. In vitro free radical scavenging property of chlorogenic acid provides many beneficial effects on health by preventing the spread of the oxidative process (Naidu et al., 2008). Liu et al. (2010) reported that the chlorogenic acid isolated from *Folium eucommiae* exhibited a strong DPPH and OH inhibition effect depending on the concentration. Caffeic acid and caffeine, which are the major components in green coffee, also strengthen the radical scavenging activity of Gcbe. Nadanasabapathi et al. (2013) stated that caffeine, caffeic acid and caffeine+caffeic acid combination have strong free radical scavenging activity, reduce lipid peroxidation and can be used in the treatment of free radical-mediated diseases. The strong radical inhibiting

activities of phytochemical compounds in green coffee were reflected in the radical scavenging power of Gcbe. Naidu et al. (2008) stated that green coffee extract exhibited hydroxyl radical scavenging activity in the range of 80 % - 92 % and DPPH scavenging activity in the range of 76-92 %.





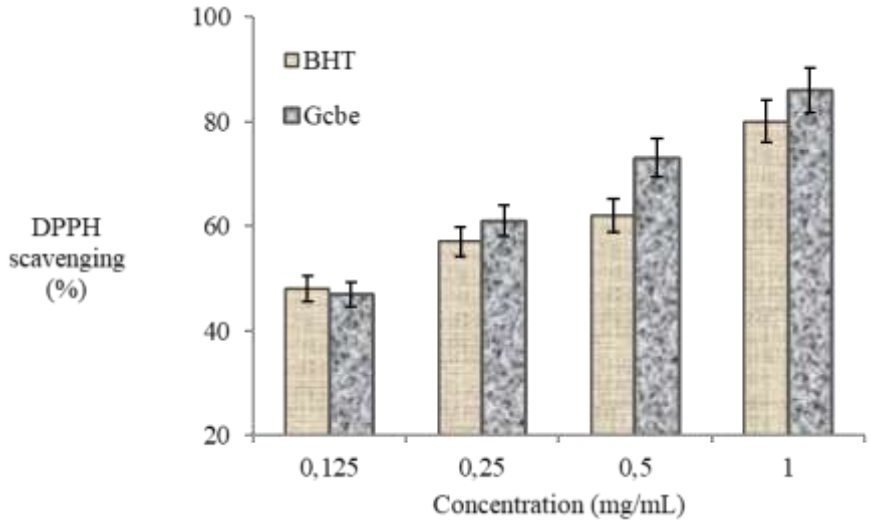


Figure 2. Superoxide, DPPH and H_2O_2 scavenging property of Gcbe and BHT. The experiment was repeated three times at each concentration. Each histogram is a decimal average.

3.4. Protective effect of Gcbe against BpA-induced toxicity

3.4.1. Oxidant/antioxidant dynamic

The effects of BpA and Gcbe treatment on MDA-GSH levels of the kidney and liver are shown in Table 1. The levels of MDA and GSH in kidney and liver showed similar levels in Group I, II and III. However, MDA and GSH levels showed significant changes after BpA exposure. Namely, BpA treatment caused an increase in MDA level but caused a decrease in GSH level. After 50 mg/kg BpA exposure, MDA levels of kidney and liver increased 1.65 and 1.84 times compared to control, respectively. GSH levels in liver and kidney decreased by 50% and 59%, respectively, compared to control after BpA treatment. The increase in MDA and decrease in GSH levels observed after BpA treatment indicate that antioxidant-oxidant dynamic was disrupted. The oxidant and antioxidant systems are in balance in cells and in the case of oxidative stress, the balance deteriorates and the antioxidant system is damaged. Toxicity of BpA observed in this study can be described by the oxidative damage and free radicals formation, and the deterioration of cell integrity due to these formations. Similarly, Eshak and Osman (2014) reported that BpA caused the free radical formation and this formation could lead to injuries in liver, kidney and brain tissues. Free radicals cause lipid peroxidation by acting on unsaturated fatty acids containing multiple double bonds. Lipid peroxidation products such as MDA often cause neurotoxic, hepatotoxic and nephrotoxic effects in mammals. GSH, which is in a reduced form in the cell, protects the cell against various damages induced by harmful substances such as MDA and is oxidized while neutralizing free radicals. This neutralization process decreases the reduced GSH level (Yalçın et al., 2020). Similar results supporting our data have been reported in the literature, and it has been mentioned that BpA disrupts oxidative homeostasis in organisms through indirect or direct pathway. Edres et al. (2018) reported that 50 mg/kg BpA application increased the level of MDA and decreased the total antioxidant capacity and GSH level in the kidney tissues of rats. In this study, it was determined that the oxidative homeostasis deteriorated after BpA application started to improve with Gcbe application. It is clear that the 100 mg/kg bw and 400 mg/kg bw Gcbe treatment with BpA significantly ameliorated the changes in oxidative parameters. To confirm this hypothesis, it can be said that BpA treatment, which caused a 2.44 times

decrease in the GSH level of the kidney compared to the control, caused a decrease of 1.48 times when administered with 400 mg/kg bw Gcbe. A similar ameliorative effect was observed at MDA levels, and these results showed that Gcbe treatment reduced BpA toxicity and had a protective effect in oxidant-antioxidant balance. In literature Moawed et al. (2020) reported that green coffee significantly decreased the MDA level and increased the GPx level in rats with induced acute liver toxicity.

3.4.2. Serum parameters

The levels of ALT and AST were examined as liver injury indicators in serum and also BUN and creatinine levels were investigated as a marker of renal damage. The results of serum parameters in BpA and Gcbe treated groups are given in Table 1. The levels of indicator parameters showed a similar level in Group I, II and III and these results indicate that both doses of Gcbe treatment-alone do not cause any changes in related parameters. However, compared with the control group, statistically significant increases were observed in the group treated with 50 mg/kg bw BpA ($p< 0.05$). In Group IV, ALT and AST levels were 2.2 and 1.35 times higher than the levels obtained in the control group, respectively. This result showed that the ALT levels were more sensitive to BpA toxicity compared to AST. In 50 mg/kg bw BpA treated group, BUN and creatinine levels, which are the indicators of kidney damage, increased by 53.1 % and 38.0 % compared to the control group, respectively. It has been proved by previous analysis of antioxidant/oxidant dynamic in this study that BpA causes oxidative damage in liver and kidney tissue. The increase in AST, ALT, BUN and creatinine levels supports these results and strengthens the hypothesis that BpA causes damage to the liver and kidneys. Briefly, the results obtained from oxidative stress parameters and serum parameters support each other. Oxidative stress formation and lipid peroxidation in a cell can lead to arbitrary damage to biological molecules, loss of function, and even cell death. In particular, oxidation of lipids in the cell membrane can result in loss of function and deterioration in membrane integrity. In all these abnormalities, cell damage is inevitable and liver and kidney are the important organs affected by oxidative damage, where toxic agents are detoxified and excreted from the body. Through hepatotoxic effects, normally found enzymes in the cytoplasm of hepatocytes pass into the bloodstream and the levels in the blood are abnormally increased (Yalçın et al., 2020). Similarly, Hassan et al. (2012) reported the increased levels of ALP, ALT and total bilirubin after hepatoxicity induced by BpA in rats. Renal cell damage caused by oxidative stress can cause kidney function loss and failure. While BUN is formed as a result of protein metabolism, creatinine is formed as a result of creatine metabolism. Both metabolites are filtered by the kidney through glomerular filtration and excreted in the urine. The increased BUN and creatinine levels observed in this study indicate a glomerular injury and impaired kidney function induced by BpA. Similar to our results, Haroun et al. (2019) reported that BpA administration induced nephrotoxicity in albino rats and caused significant changes in uric acid and creatinine levels. In this study, it was observed that application of Gcbe with BpA caused an improvement in the abnormal increases observed in serum parameters. To confirm this result, it can be said that BpA treatment causing an increase as 2.20 times in ALT level according to control, but BpA treatment with Gcbe administration (400 mg/kg bw) caused an increase only 1.72 times in same level. This result indicates that Gcbe treatment reduced toxicity and Gcbe has a strong protective role against BpA toxicity. Shahmohammadi et al.(2017) reported that green coffee bean extract supplementation improved the serum AST, ALT, cholesterol, triglyceride levels in patients with non-alcoholic fatty liver disease. El-Deen et al. (2019) observed an important

reduction in serum urea, serum creatinine, and blood urea nitrogen levels in albino rats exposed to cisplatin toxicity.

Table 1.The effects of BpA and Gcbe treatment on some biochemical parameters of mice.

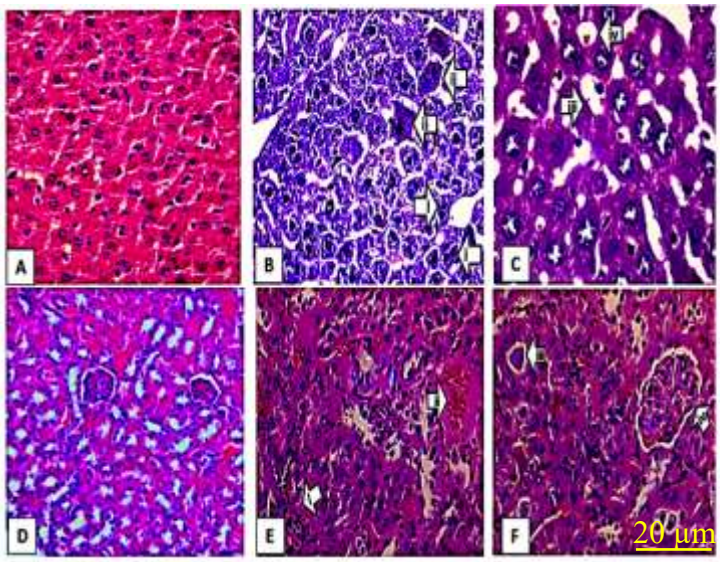
Parameters	Group I	Group II	Group III	Group IV	Group V	Group VI
GSH_{liver}	0.48 ± 0.07^{a}	0.47 ± 0.09^{a}	0.44 ± 0.08^{a}	0.24 ± 0.03^{b}	0.27 ± 0.04^{b}	0.31 ± 0.03^{b}
GSH_{kidney}	0.61 ± 0.06^{a}	0.60 ± 0.04^{a}	0.60 ± 0.06^{a}	0.25 ± 0.04^{d}	0.33 ± 0.06^{c}	0.41 ± 0.05^{b}
MDA_{liver}	2.30 ± 0.41^{c}	2.39 ± 0.51^{c}	2.40 ± 0.47^{c}	4.24 ± 0.33^{a}	3.85 ± 0.46^{ab}	3.37 ± 0.51^{b}
MDA_{kidney}	2.72 ± 0.31^{d}	2.77 ± 0.35^{d}	2.79 ± 0.32^{d}	4.49 ± 0.38^{a}	3.91 ± 0.42^{b}	3.47 ± 0.32^{c}
AST	55.17 ± 6.34^{c}	54.50 ± 5.13^{c}	54.83 ± 6.79^{c}	74.67 ± 7.26^{a}	67.67 ± 7.12^{ab}	60.33 ± 6.02^{bc}
ALT	26.17 ± 5.60^{c}	25.50 ± 7.74^{c}	26.00 ± 7.72^{c}	57.67 ± 5.82^{a}	52.83 ± 6.77^{ab}	45.00 ± 6.45^{b}
BUN	47.67 ± 6.09^{c}	46.83 ± 8.35^{c}	47.00 ± 8.17^{c}	73.00 ± 6.87^{a}	66.17 ± 7.76^{ab}	57.83 ± 8.23^{b}
Creatinine	6.36 ± 0.87^{bc}	6.22 ± 0.74^{c}	6.28 ± 0.70^{bc}	8.78 ± 0.82^{a}	8.35 ± 0.73^{a}	7.16 ± 0.46^{b}

[a-d] Within a line, means not sharing the same letter are significantly different ($p < 0.05$). Group I: control, Group II: 100 mg/kg bw Gcbe, Group III: 400 mg/kg bw Gcbe, Group IV: 50 mg/kg bw BpA, Group V: 50 mg/kg bw BpA+100 mg/kg bw Gcbe, Group VI: 50 mg/kg bw BpA+400 mg/kg bw Gcbe. GSH: mg/g tissue; MDA: nmol/g tissue; AST, ALT: U/L; BUN, creatinine: mg/L

3.4.3. Histopathological analysis

Histopathological evaluation of tissues treated with BpA and Gcbe are given in Figure 3. In histological examinations, there was no change in kidney and liver histology of Group I, II and III. In BpA treated liver binucleated cell, karyomegaly, stellate cell and proteinous deposits were observed. Stellate cells are important major cells that develop in liver fibrosis in response to liver damage. Inflammation is related to liver injury, which is thought to quicken liver disease progression. Inflammatory or damaged liver cells leak enzymes into the blood flow and cause high liver enzyme levels in blood analysis. So, elevations in ALT and AST and histopathological findings support each other, and both changes confirm liver damage caused by BpA. Inflammation, hemorrhagic foci, hyaline materials and basement membrane irregularity were observed in kidney tissue of 50 mg/kg BpA treated group. In the kidneys, the accumulation of hyaline (amorphous, homogeneous material) can cause disruption of the kidney's natural structure. Depending on the degree of hyalinization, fibrosis tissue or renal failure may occur. The inflammation, basement membrane irregularity and hemorrhagic foci in the kidney may reduce the ability of the kidney to filter and retain the molecules, and this decrease may cause nephrotic syndrome. Nephrotic syndrome causes increases in blood levels of creatinine and BUN, which are two biochemical indicators of kidney damage (Ebert and Nagart, 2008). As a result of serum analysis, observation of BUN and creatinine increases in the group treated with BpA confirms this hypothesis. There are studies in the literature reporting that BpA exposure causes anomalies in the liver and kidney. Poormoosavi et al. (2018) reported the formation of histopathological damage in the liver and kidney in rats treated with BpA. While inflammatory areas, dilated and congested veins were detected in liver, they found acute tubular necrosis and urinary tubules in kidney. In previous analyzes, it was determined that Gcbe application together with BpA caused an improvement in serum parameters and oxidative stress parameters. This improvement was also observed in histopathological findings and it was found that the application of Gcbe together with BpA caused a decrease in the frequency of histopathological findings. In addition, the most significant improvement was observed in the 400 mg/kg bw Gcbe+BpA treated group. Similarly, Rabey et al. (2021) reported that induced fatty infiltrating and degenerated hepatocytes,

congested hepatic vein and sinusoidal degenerated bile duct and bridging necrotic cells had almost normal histology after green coffee application in rats. Abdelaal et al. (2019) found that there was a significant improvement in pathological findings observed in liver vessels and heptocyte cells treated with green coffee combinations.



	Findings	BpA	BpA + 100 Gcbe	BpA + 400 Gcbe
Liver	Stellate cell	++	++	+
	Proteinaceous deposits	+++	++	+
	Karyomegaly	++++	+++	++
	Binucleated cell	++	+	+
	Inflamation	++++	+++	++
Kidney	Hemoragic foci	++	++	+
	Hyaline materials	+++	++	+
	Basement membrane irregularity	++	+	+

(-): No change, (+): mild change, (++): moderate change, (+++): severe change, (++++): hard change

Figure 3. Histopathology of tissues in BpA treated group. Liver tissue of control group (A), i. binucletaed cell, ii. Karyomegaly (B), iii. stellate cell, iv. proteinous deposits in BpA treated liver (C), kidney tissue of control group (D), i. inflamation, ii. hemoragic foci (E), iii. hyaline materials, iv. basement membrane irregularity in BpA treated kidney (F).

3.4.4. MN frequency in leukocyte

MN is an indicator of cytotoxicity that results from mitotic spindle damages or chromosome breaks. BpA induced MN frequency in leukocyte cells is shown in Figure 4. In this study, MN was not observed in the control group, Group II and Group III. This result indicates that Gcbe application alone does not have a genotoxic effect. It was determined that MN frequency increased after BpA treatment and the highest MN frequency was determined as 64.33±3.39 in Group IV. MN formation with high frequency after BpA application points to the genotoxic and cytotoxic effects in the cell. MNs are defined as formations that arise during the mitosis of the cell, are not included in the main nucleus, and originate from full chromosome or asymmetric chromosome fragments. MNs usually result from deficiencies in cell cycle genes, abnormalities in the mitotic spindle, kinetochore or mitotic device and chromosomal damage. The increase in the level of MN is considered as an indicator of the structural and numerical chromosome irregularities caused by various agents in the cells. Therefore, the formation of MN and increase in MN frequency are considered as an indicator of genomic instability in somatic cells (Yalçın et al., 2019). So, high frequency of MN in leukocytes observed in this study indicates the genotoxic and cytotoxic effects of BpA. In literature, BpA application has been reported to induce MN formations, modify the microtubule regulation centers of the mitotic spindles and cause abnormalities in meiotic double-strand break repair mechanisms of mammals (Allard and Colaiacovo, 2010).

Similarly, Santovito et al. (2018) reported that BpA exposure induced significant MN formations and chromosomal abnormalities such as chromosome break, gap, dicentric chromosome, ring and fragments in human cultured lymphocytes. In this study, it was observed that the frequency of MN decreased in groups treated with Gcbe+BpA compared to BpA treated group. In 400 mg/kg bw Gcbe+BpA treated group MN frequency in leukocyte cells decreased by 49.5 % compared to only BpA treated group and this decrease was statistically significant ($p < 0.05$). Ansari et al. (2021) reported that induced MN formations in bone marrow cells of mice were reduced after green coffee consumption.

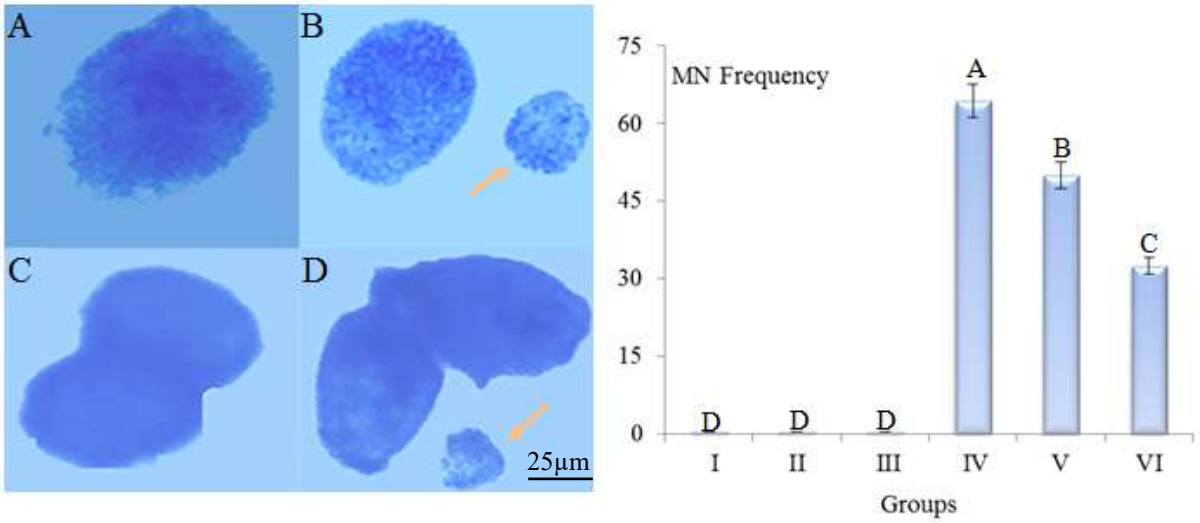


Figure 4. MN appearance and frequency in leukocyte cells. Lymphocyte in control group (A), lymphocyte with MN in Group IV (B), basophil in control group (C), basophil with MN in Group IV (D). Each histogram is a decimal average. The vertical lines on the bars indicate the standard error. Different letters$^{(A-D)}$ indicated averages $p < 0.05$ is important.

3.4.5. DNA damage in leukocyte

DNA fragmentation induced by BpA in leukocyte cells and the protective role of Gbce against this damage were evaluated using single cell gel electrophoresis with tail DNA (%), tail moment and olive tail moment (Figure 5). No statistically significant differences were found in tail DNA percentage, tail moment and olive tail moment formation in control group (Group I) and Gbce-alone application groups (Group II and III) ($p > 0.05$). In Group IV, where BpA has applied alone at a dose of 50 mg/kg bw, the tail DNA percentage was 64.52 %, the tail moment was 68.65, and the olive tail moment was 33.68, and these values were the highest values among the groups. In Group V where 100 mg/kg bw Gcbe was applied as a combination to BpA (50 mg/kg bw), tail DNA percentage decreased to 50.54, and the tail moment and olive tail moment decreased to 38.24 and 25.76, respectively. In Group VI, with the application of 400 mg/kg bw Gcbe in combination with BpA, tail DNA percentage decreased to 36.22, tail moment to 32.87 and olive tail moment to 15.25. Application of Gcbe in combination with BpA showed a healing effect and caused a decrease in tail DNA (%), tail moment and olive tail moment parameters. Although the values in the control group could not be reached, Gcbe application showed a dose-dependent protective effect by partially inhibiting the DNA damage caused by BpA. There

are also other studies reporting that BpA exposure causes DNA damage. Similarly, in the study carried out by Yuan et al. (2019), different doses of BpA were administered to the Marc-145 cell line, resulting in a dose-dependent increase in DNA tail size and a simultaneous decrease in head DNA. In a study conducted by Kose et al. (2020), BpA was applied to the RWPE-1 cell line, as a result, it was reported that BpA administration produced high levels of DNA damage by causing higher tail densities compared to the control group, and changed expressions of DNA repair enzymes. In another study, Ansari et al. (2021) reported that the percentages of DNA tail and fragmented nuclei in peripheral blood samples of mice decreased after green coffee consumption.

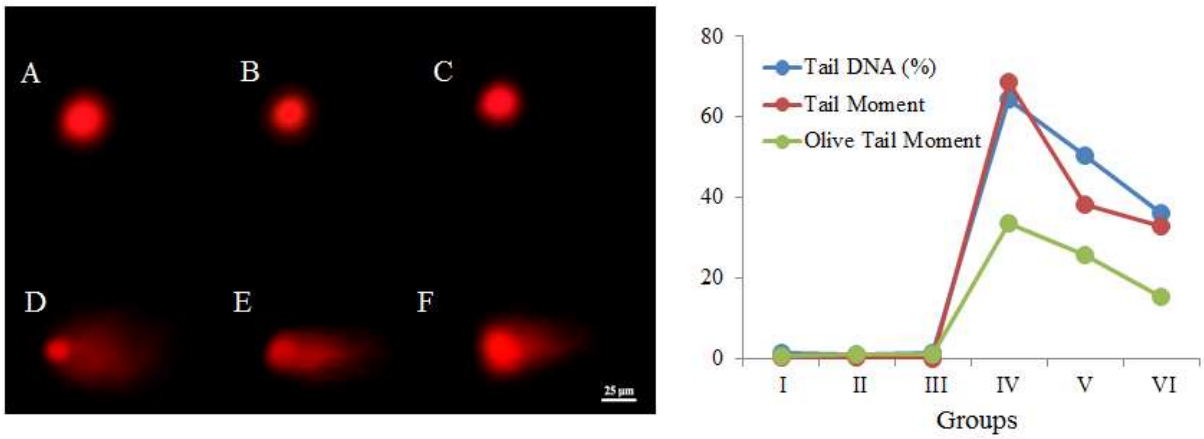


Figure 5. Comet assay in nuclei isolated from leukocyte cells. Control (A), 100 mg/kg bw gcbe (B), 400 mg/kg bw gcbe (C), 50 mg/kg BpA (D), 50 mg/kg bw BpA+100 mg/kg bw Gcbe (E), 50 mg/kg bw BpA+400 mg/kg bw Gcbe (F).

3.5. Protective effects of Gcbe

The protective efficacy of 400 mg/kg Gcbe with the highest protective effect against each parameter was calculated as a percentage. In terms of all parameters tested, no statistically significant differences were observed in Groups II and III treated with Gcbe compared to the control group. This result shows that Gcbe application does not have toxic effects on the tested doses. However, treatment of Gcbe with BpA has been found to cause an improvement in the toxic effects of BpA. It was observed that this amelioration was increased depending on the dose and an improvement in the tested parameters was observed in the range of 29.1% - 73.5% at the dose of 400 mg/kg bw Gcbe. It was found that BpA induced deterioration in antioxidant-oxidant dynamics improved and the increased MDA levels in liver and kidney after BpA administration decreased by 44.9% and 57.6%, respectively, after administration of Gcbe. 400 mg/kg Gcbe provided 44.4% and 29.1% protection against GSH levels of kidney and liver, which is an element of the antioxidant/oxdian dynamic. Gcbe also showed an improvement in the range of 40.2% - 73.5% against serum AST, ALT, BUN and creatinine levels, which showed a significant increase with BpA administration. A similar improvement was observed in genotoxic effects and the frequency of MN and DNA tails in leukocyte tends to decrease in Groups V and VI compared to 50 mg/kg bw BpA treated group. An important improvement in genotoxic effects was observed, and the MN frequency and DNA tails in leukocytes were reduced by 49.4% and 44.8%, respectively, after treatment with 400 mg/kg Gcbe.

Protective property of Gcbe can be explained by the active ingredients it contains. Coffee is a bioactive phytochemical source containing methylxanthines (eg caffeine), amino acids, phenolic acids and polyphenols. Antioxidant and anti-inflammatory activities of bioactive phytochemicals of coffee are known. The coffee roasting significantly damages phytochemicals which are thought to be responsible for biological activity. Green coffee beans are the beans that are not roasted and have more active ingredients compared to roasted coffee beans. Green coffee has a rich content in polysaccharides, lipids, sterols, polyphenols, free amino acids, phenolic acids, vitamins, proteins and minerals. Green coffee has many active properties due to its antioxidant compounds such as diterpenes, chlorogenic acid and trigonelline. Of these active compounds, chlorogenic acid is the main phenolic compound, and the protective properties of Gcbe have been specifically associated with this compound (Hosseinabadi et al., 2020). There are many studies in the literature on the protective properties of both chlorogenic acid and Gcbe. Nogaim et al. (2020) reported that 2000 mg/kg bw and 4000 mg/kg bw green coffee treatment improved the induced oxidative damage and antioxidant enzyme levels and ameliorated the histopathological findings in liver, brain and kidney. Shahmohammadi et al. (2017) have reported an important improvement in impaired ALT level, inflammation, oxidative stress, hyperlipidemia and liver function after Gcbe application in patients with fatty liver disease. In another study, İştar et al. (2016) reported that BpA increased the frequency of MN in erythrocytes and caused chromosomal abnormalities in the bone marrow, while these genotoxic effects decreased with 100 mg/kg bw and 400 mg/kg bw Gcbe treatment. This protective feature of Gcbe can be associated with the active properties of chlorogenic acid in its content. Chlorogenic acid has been reported to reduce free radicals, prevent lipid peroxidation and neutralize the effects of oxidative stress in the cell (Godos et al., 2014). In addition to neutralizing free radicals, chlorogenic acid is known to suppress inflammatory expression. In this study, this hypothesis is confirmed by the findings of decreased inflammation obtained in the histopathological findings. The decreased BpA-induced inflammation incidence in the liver after Gcbe treatment proves the anti-inflammatory property of Gcbe. In addition to chlorogenic acid, the other active compounds in the Gcbe content contribute to the protective biological activity. High antioxidant activity, anti-inflammatory activity, anticancer, antimutagenic activities of phenolic acids, polyphenols and terpenes found in Gcbe are known (Bhattacharya and Sil, 2018). These compounds neutralize the oxidative stress-induced in the cell by direct and indirect mechanisms. Briefly, as a result of the cumulative effects of these active compounds in Gcbe content, high protective activity occurs. The reduction of BpA-induced biochemical and genetic toxicity with the Gcbe application observed in this study confirms these propositions.

4. Conclusion

As a result of rapid industrialization, production of high amounts of chemicals and contamination of these chemicals to organisms has been inevitable. As a result of this contamination, various degrees of toxic effects on organisms and various diseases occur. For this reason, the effects of any chemical that may contaminate organisms must be examined with *in vivo* toxicity tests. In this study, the toxic effects of BpA, frequently used in the plastic industry, and the protective role of Gcbe against these toxic effects were investigated in albino mice and two main data were obtained. It has been determined that 50 mg/kg bw BpA causes genotoxic effects in albino mice, changes in oxidant-antioxidant

dynamics and damages in liver and kidney tissues. It was determined that lipid peroxidation occurred in the group treated with BpA and this result indicates the formation of oxidative damage. The fact that Gcbe application has protective effects against these damages is another important issue obtained in the study. Gcbe was observed to exhibit a dose-related improvement against BpA-induced toxicity. This protective feature has been associated with the active ingredients in the Gcbe content, and it has been observed that the BpA toxicity is reduced with the tested Gcbe doses but not completely neutralized. Since chemical contamination is inevitable in daily life, many studies are needed to investigate the protective effects of such natural foods. This study investigating the protective effects of Gcbe, a natural food source, will be a guide for the use of such foods.

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