

## ***In vivo* examination of the effects of hydroxycinnamic acid on xenobiotic metabolizing and antioxidant enzymes**

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**Abstract:** In the last decade, hydroxycinnamic acids (HCA) have gained increasing attention from researchers due to their antioxidant potential. The aim of this study was to examine in detail the impact of dietary HCA on particular types of P450 and also selected phase II and antioxidant enzymes in Wistar rat. HCA (10 µM/kg/day, i.p.) was administered for ten continuous days. Examination of the activities and mRNA and protein levels revealed that CYP2B, 2C6 and 3A enzyme activities were not altered significantly, with Western blot and qRT-PCR results corroborating this result. While treatment with HCA led to a significant reduction in CYP1A1/CYP1A2-associated enzyme activities, CYP1A1 protein, and mRNA levels were found to be unchanged. Aromatase (CYP19) activity, as well as protein and mRNA levels, were significantly reduced with HCA treatment. On the other hand, the NAD(P)H:quinone oxidoreductase 1 (NQO1), catalase (CAT), glutathione peroxidase (GPx) and glutathione S-transferases (GSTs) activities were increased significantly. Also, HCA treatment significantly increased the GST-mu and GST-theta mRNA levels. These observations may be of importance given the potential use of HCA as a chemopreventive and as an anticancer agent

**Key words:** hydroxycinnamic acids; drug-metabolizing enzymes; antioxidant enzymes; chemoprevention

### **INTRODUCTION**

Dietary polyphenols have received significant consideration in last decades. They constitute a heterogeneous group of compounds, including phenolic acids, flavonoids, tannins, coumarins and lignans, among others [1]. Prolonged utilization of a polyphenol-rich diet has been demonstrated to be protective against cardiovascular and neurodegenerative ailments, diabetes and cancer [2-6]. Phenolic acids have antioxidant action as chelators and free radical scavengers with specific effect over hydroxyl and peroxy radicals, superoxide anions and peroxy nitrites [6]. They are made up of hydroxycinnamic and hydroxybenzoic acids.

Hydroxycinnamic acids (HCAs) are a subgroup of phenolic acids with a widespread distribution in plants. They are abundant in tea leaves, coffee, differ-

ent organic products, vegetables and whole grains [1]. Cytochrome P450s (CYP450), which are driving phase I enzymes, have an essential part in the metabolic activation or detoxification of medications in addition to dietary constituents and environmental compounds [7-9]. Accordingly, the balance of this protein framework can impact the metabolism of xenobiotics, creating impacts of pharmacological and toxicological significance. Various natural flavonoids have been demonstrated to alter the CYP450 framework, including the induction of particular CYP isozymes, and the activation or inhibition of these catalysts [10,11]. Among these P450s, the CYP1A, CYP2B, CYP2C and CYP3A subfamilies have received much consideration due to their capacity to metabolize different pharmaceuticals and their role in chemical carcinogenesis [12-14]. CYP19 (aromatase) is the pivotal catalyst of

estrogen biosynthesis and increased expression of aromatase has been seen in breast cancer [15,16].

Phase II biotransformation responses for the most part serve as a detoxifying step in xenobiotic metabolism. Thus, another conceivable system for the chemoprotective and cell reinforcement impacts of phenolic acids may include the prompting of phase II and antioxidant enzymes [17-20]. Glutathione S-transferases (GSTs) are a complex multigene family of enzymes [21] that assume a critical function in detoxification by conjugating glutathione with an extensive number of electrophilic metabolites formed from a diverse set of xenobiotics, including cancer-causing agents, toxins, and medications. NAD(P)H:quinone oxidoreductase 1 (NQO1) forestalls quinone redox cycling and lowers the levels of electrophilic quinones [22]. Therefore, the induction of GST and NQO1 by phenolic acids is potentially connected with tumor chemopreventive impacts.

Recently, we have reported on the modulatory activity of HCA on carcinogen activating proteins in HepG2 cell lines [23]. Therefore, the present study attempted to examine further the general synchronous changes following *in vivo* treatment of rodents with HCA, for this reason, changes in the activity, mRNA, and protein levels of particular hepatic P450s, phase II, and antioxidant enzymes were studied. To our knowledge, this is the first report of the *in vivo* impact of HCA on drug-metabolizing cytochrome P450 enzymes, examined by studying the changes in their mRNA and protein levels and activities in the rat.

## MATERIALS AND METHODS

### Animals and treatment

Male Wistar rats, around 12 weeks old and weighing 200-250 g, were supplied by the University Animal House. They were housed in small confines at an encompassing temperature of  $22\pm 1$  °C, with a 12 h light/dark cycle, and a standard pellet diet and refined water were accessible without restriction. Every single trial with the animals was performed under suitable administrations with veterinary services within the authorized projects.

After being acclimatized for a week, the rats were randomized and divided into two groups: HCA was

administered to 15 rats at a dose of 10  $\mu\text{M}/\text{kg}$  (dissolved in 10% dimethyl sulfoxide (DMSO)); the control (10) rats received the vehicle (10% DMSO). The animals were treated intraperitoneally (i.p.) for 10 consecutive days. At the end of the experimental period and following 16 h of fasting, blood was collected by heart puncture, and the rats were killed. The livers were removed, rinsed with cold physiological saline and stored at  $-80^\circ\text{C}$  until analyzed.

### Preparations of S1.5, cytosolic and microsomal fractions

Tissues were homogenized in homogenization solution (1.15% potassium chloride containing 3 mM ethylenediaminetetraacetic acid, 0.5 mM 4-aminophenylmethyl-sulphonyl fluoride, 0.3 mM  $\epsilon$ -aminocaproic acid, 0.15 mM butylated hydroxytoluene, 0.025% Triton X-100) using a tissue homogenizer with a Teflon pestle at  $4^\circ\text{C}$ . Subcellular fractions (S1.5, cytosolic and microsomal) of rat tissues were prepared by standard differential centrifugation with calcium aggregation as described [24]. The amount of protein in individual fractions was measured using bicinchoninic acid [25] with bovine serum albumin as the standard.

### Enzyme assays

Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities were determined with an autoanalyzer using audit diagnostic reagents for AST and ALT. The microsomal cytochrome P450-dependent aminopyrene N-demethylase (APND), erythromycin N-demethylase (ERND), caffeine N-demethylase (C3ND), ethylmorphine N-demethylase (EmND) and benzphetamine N-demethylase (BPND) activities were determined by measuring the quantity of formaldehyde formed according to the method of Nash [26] and modified by Cochin and Axelrod [27]. Ethoxyresorufin-O-deethylase (EROD), methoxyresorufin O-demethylase (MROD), benzyloxyresorufin O-dealkylase (BROD), and pentyloxyresorufin O-dealkylase (PROD) activities were assayed as described by Sen and Arinc [28]. Dibenzylfluorescein O-debenzylase (DBFOD) activity was assayed according to the method of Kragie [29] as optimized by Celik et al. [16]. GST activities were assayed as described

bz Habig et al. [30]. Catalase (CAT) and glutathione peroxidase (GPx) activities were determined by the methods of Abei [31] and Paglia and Valentine [32], respectively. NQO1 enzyme activity was determined according to the method of Ernster [33] as modified by Karakurt and Adali [34].

### Gel electrophoresis and Western blotting

SDS-PAGE and Western blotting were performed as described [28]. Briefly, 120- $\mu$ g protein samples were separated on 8.5% polyacrylamide gels using the discontinuous buffer system of Laemmli [35]. Proteins were transferred to a nitrocellulose membrane by the iBlot dry blotting system (20 V, 12 min), using iBlot gel transfer stacks. Following transfer, the membranes were blocked using 5% non-fat dry milk in TBST (20 mM Tris-HCl, pH 7.4, 400 mM NaCl and 0.1% (v/v) Tween 20) for 60 min and incubated with mouse polyclonal anti-rat CYP1A1, CYP2B, CYP2C6, CYP3A1 or CYP19 antibodies (diluted 1:1000 in blocking solution) for 120 min at room temperature. The membranes were then washed with TBST (Tris-buffered saline with Tween 20) (3 x 5 min), incubated with the secondary antibody (HRP-conjugated anti-rabbit IgG at a 1:5000 or 1:10000 dilution) for 60 min and again washed with TBST (3 x 5 min). Proteins were detected using SuperSignal<sup>®</sup> West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, USA), and bands were visualized and recorded using GelQuant Image Analysis Software in a DNR LightBIS Pro Image Analysis System (DNR Bio-Imaging Systems Ltd. Jerusalem, Israel). Protein bands were quantified using Scion Image Version Beta 4.0.2 software.

### RNA isolation and qRT-PCR of CYP mRNAs

Total RNA was extracted from 100 mg of rat liver using Trizol reagent. Extracted RNA was quantified spectrophotometrically at 260/280 nm, and the integrity was checked using 1% agarose gel. For cDNA synthesis, 2.5  $\mu$ g of RNA was incubated at 70°C for 10 min with 0.5  $\mu$ g of oligo(dT). After 5 min on ice, 50 U Moloney murine leukemia virus reverse transcriptase, 1 mM dNTPs and 5X reaction buffer were added to the previous mixture and incubated at 42°C for 60 min. The reaction was stopped by heating at

70°C for 10 min, and the cDNA was stored at -80°C for further analysis.

The qRT-PCR assay was performed using gene-specific primers. The oligo sequences used as forward and reverse primers for rat CYP450 isozymes were based on those reported in Agus et al. [36]. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as a housekeeping gene. PCR amplification was performed using Power SYBR Green PCR master mix (Roche Applied Science, Basel, Switzerland) and 500 nmol/L of forward and reverse primers for each gene for which the final primer concentration was 125 nmol/L each. Quantitative PCR was done using a Light Cycler 1.5 Instrument (Roche Applied Science, Basel, Switzerland). The PCR conditions were as follows: DNA polymerase activation at 95°C for 15 min, followed by 45 cycles at 95°C for 10 s, 54-57°C annealing (depending on the gene) for 5 s and 72°C for 30 s. All gene analyses were done at least three times with triplicates.

### Statistical analysis

Statistical analyses were carried out utilizing the Minitab 13 statistical software bundle. (Minitab, Inc., State College, PA, USA). All outcomes were expressed as means including their standard error of means (SEMs). Following normalization, the data can be used for determining the magnitude of effect due to the treatment. In the current literature this has typically been achieved by applying a Student's t-test, and  $P < 0.05$  was chosen as the level of statistical significance.

## RESULTS

Control and treated rats demonstrated no critical contrasts in alimenting or body weight (data not given) after i.p. treatment with HCA. As indicated in Table 1, blood serum AST and ALT activities were not changed when compared with the control rats.

As can be seen in Table 2, hepatic 1A1-associated EROD and 1A2-associated MROD and C3ND activities were decreased by 23%, 13% and 13% in the HCA-treated rats, respectively when compared to the control rats. CYP2B-associated BPND, EmND, BROD and PROD activities were not changed significantly in the HCA-treated rats as compared to the control ani-

**Table 1.** Blood serum AST and ALT enzyme activities in control and HCA-treated rats.

	<b>Aspartate</b>		<b>Alanine</b>	
	<b>Aminotransferase</b>	<b>Change</b>	<b>Aminotransferase</b>	<b>Change</b>
	(AST) Unit/min/mg protein	(Fold)	(ALT) Unit/min/mg protein	(Fold)
Control	1.55±0.09	--	1.47±0.29	--
10 µM/kg HCA	1.57±0.11	--	1.64±0.22	--

**Table 2.** The effects of HCA on xenobiotic metabolizing and antioxidant enzyme activities in rat liver microsomes.

	<b>Control</b>	<b>10 µM/kg HCA</b>	<b>Change (%)</b>
EROD (pmol resorufin/min/mg prot.)	8.10± 1.89	6.26±2.03*	23 ↓
MROD (pmol resorufin/min/mg prot.)	9.59± 3.75	8.39±2.23*	13 ↓
C3ND (nmol HCHO/min/mg prot.)	0.46±0.06	0.40±0.02*	13 ↓
EmND (nmol HCHO/min/mg prot.)	1.93±0.25	1.98±0.12	--
BPND (nmol HCHO/min/mg prot.)	1.46±0.30	1.49±0.31	--
BROD (pmol resorufin/min/mg prot.)	2.61±0.87	2.55±0.71	--
PROD (pmol resorufin/min/mg prot.)	6.50±0.99	6.59±0.90	--
APND (nmol HCHO/min/mg prot.)	0.339±0.03	0.343±0.01	--
ERND (nmol HCHO/min/mg prot.)	0.106±0.02	0.110±0.02	--
DBFOD (nmol fluorescein/min/mg prot.)	6.60±0.12	5.55±0.27*	16 ↓
NQO1 (nmol/min/mg prot.)	187±11.35	254±16.32*	35 ↑
GST-CDNB (nmol/min/mg prot.)	539±34.21	651±97.12*	20 ↑
GST-DCNB (nmol/min/mg prot.)	13.89± 4.29	20.30±3.25*	46 ↑
GST- EA (nmol/min/mg prot.)	13.45±2.61	21.32±2.21*	59 ↑
CAT (nmol/min/mg prot.)	10.33±0.38	14.65±0.63*	42 ↑
GPX (nmol/min/mg prot.)	0.038±0.003	0.05±0.009*	32 ↑

\*Significantly different from the respective control value  $P < 0.05$

mals (Table 2). CYP19-associated DBFOD activities of the control and HCA-treated rats are also given in Table 2. DBFOD activities were decreased by 16% in HCA-treated animals ( $P < 0.05$ ). The effect of the HCA on CYP2C6-associated APND and CYP3A-associated ERND is presented in Table 2. HCA treatment did not change APND and ERND activities significantly as compared to the controls.

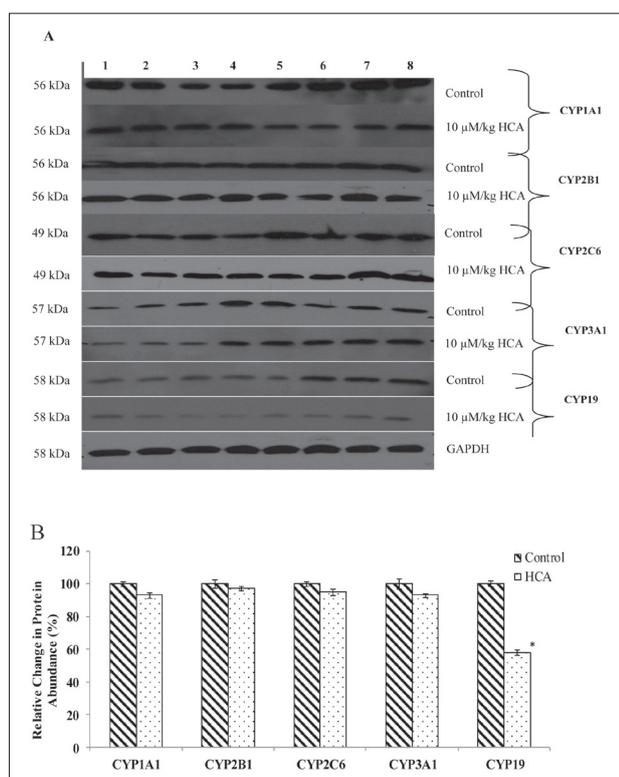
As can be seen in Table 2, GST-1-chloro-2,4-dinitrobenzene (GST-CDNB), GST-1,2-dichloro-4-nitrobenzene (GST-DCNB) and GST-ethacrynic acid (GST-EA) activities were increased by 20%, 46% and 59% ( $P < 0.05$ ), respectively. Furthermore, treatment of rats with HCA caused 35%, 42% and 32% increases in NQO1, CAT and GPX activities, respectively, when compared to the control values (Table 2).

Catalytic activities were by and large predictable with the protein levels of related CYP isoforms in rat liver microsomes that were prepared from control and HCA-treated rats (Fig. 1A). The densitometric scanning of Western blot results demonstrated that hepatic CYP1A, CYP2B, CYP2C and CYP3A were not significantly altered in the HCA-treated rats in respect to the control animals, while the CYP19 protein level was decreased (42%) (Fig. 1B).

The effect of HCA on the mRNA levels of CYP isozymes was also determined in this study. The relative CYP1A, CYP2B, CYP2C and CYP3A mRNA levels were not changed significantly in the HCA-treated rats compared to the control animals (Fig. 2). However, the CYP19 mRNA level was decreased by 47% in HCA-treated rats (Fig. 2). In addition, we established that HCA treatment significantly increased GST-mu and GST-theta mRNA levels in the liver (40% and 23%, respectively) (Fig. 2).

## DISCUSSION

Studies have revealed that HCAs can be used for preventive and/or complementary therapeutic purposes in several diseases related to oxidative stress, such as atherosclerosis, inflammation, cancer and cardiovascular diseases [1]. However, there is no detailed information regarding the *in vivo* effect of HCA on phase I- and II-drug metabolizing enzymes that are important in drug-phytochemical interactions. Thus, the present study aimed to examine in detail the impact of dietary HCAs on specific forms of P450 and

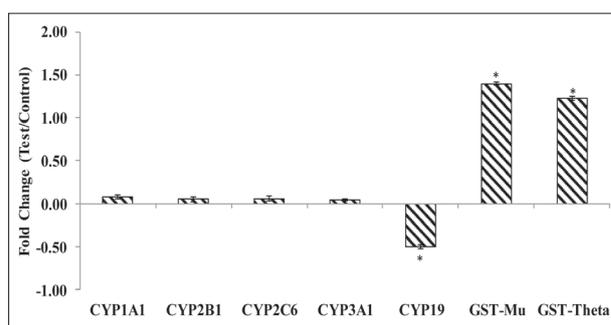


**Fig. 1. (A)** The expression levels of CYP1A1, CYP2B1, CYP2C6, CYP3A1 and CYP19 proteins in control and HCA-treated rats. Treatments were carried out as described in Materials and Methods. Representative immunoblot analysis of liver microsomal CYP1A1, CYP2B1, CYP2C6, CYP3A1 and CYP19 proteins in experimental groups, using rabbit anti-rat CYP1A1, CYP2B1, CYP2C6, CYP3A1 and CYP19 IgG. All lanes contain equal amounts of microsomal protein, i.e. 100  $\mu$ g. **(B)** Semiquantitative analysis of Western blot data for all samples. Results are presented as the mean from three independent experiments and expressed as relative mean  $\pm$  standard deviation. Relative control values are taken as 100%. \*  $P < 0.05$  compared with the control group.

also on selected phase II and antioxidant enzymes in Wistar rat.

The range of the reported concentrations of HCA in fruits and vegetables varies between 0.03 and 14.4 mg per 100 g food weight [37,38]. Ten  $\mu$ M of HCA was used throughout this study, based on our previous cell culture experiences with HCA and predictions [39] since there are no any related reports on HCA treatment of rat in the literature.

The activities of AST and ALT, alone or together, serve as indicators of hepatocellular damage in rodents and non-rodents in non-clinical studies. They are precise markers for medication-prompted liver



**Fig. 2.** The expression levels of CYP1A1, CYP2B1, CYP2C6, CYP3A1, CYP19, GST-mu and GST-theta mRNAs in control and HCA-treated rats. Treatments were carried out as described in the Materials and Methods. Normalization of the real-time quantitative RT-PCR data was carried out using housekeeping (GAPDH) genes. The bar graph represents the mean fold differences calculated by 2DDCt method using normalized Ct values obtained from qRT-PCR analysis. Results are presented as the mean from three independent experiments performed in triplicate. \*  $P < 0.05$  compared with the control group.

damage, and elevated activities of these markers in plasma point to cellular leakage and loss of functional integrity of the liver [40]. The activities of these markers in plasma were not changed in HCA-treated rats as compared to the control group. Hence, HCA, at the present measurements, can be utilized as a part of preventive or complementary medicine with wellbeing safety measures and within the scope of the methods employed in public health.

Among all cytochrome P450 isoforms, CYP1A holds priority due to its role in the metabolism of carcinogens, mutagens and environmental pollutants [41]. In this study, CYP1A1/CYP1A2-associated enzyme activities (EROD, MROD and C3ND) were decreased as a result of HCA treatment. Although HCA treatment led to a significant reduction in CYP1A1/CYP1A2-associated enzyme activities, CYP1A1 protein and mRNA levels were unchanged by HCA treatment. These data suggest that HCA directly inhibits these P450 enzymes without an alteration of its gene and protein expression. There are contradictory results between cell lines and animal studies regarding the HCA effect on CYP1A enzymes [11,23]. One of the reasons might be the differences in the metabolism of HCA in different species, as well as experimental conducts such as doses, treatments, etc. More studies are needed to provide clear evidence for this discrepancy of its effects in different species.

CYPs are an essential phase I drug-metabolizing enzyme framework and metabolize a mixed bag of medications [36]. The CYP3A subfamily comprises around 30% of the total P450 content in the human liver [42,43] and is included in the metabolism of more than half of the therapeutic ingredients [8]. The CYP2C subfamily is the second most abundant CYP in the human liver; it metabolizes a few regular medicines including ibuprofen, phenytoin and warfarin [43,44]. In a similar way, the CYP2B subfamily metabolizes clinical medications, misused medications (e.g. nicotine), toxicants and endogenous neurochemicals [45]. In humans, the CYP families examined in this study are in charge of 90% of the phase I-related metabolism of clinically utilized medications. HCA treatment did not change any CYP2B-associated EmND, BPND, BROD or PROD activities significantly nor CYP2B mRNA and protein levels. Similarly, HCA treatment did not change CYP2C6-associated APND and CYP3A-associated ERND activities significantly in the HCA-treated rats as compared to the control. These results showed that HCA did not cause any significant changes in the expression and activity levels of drug-metabolizing enzymes in rats. It could be speculated that HCA did not depict any interactions with transcriptional regulators, such as the aryl hydrocarbon receptor, constitutive androstane receptor, phenobarbital-responsive enhancer module and pregnane X receptor.

The aromatase enzyme, which transforms androgen to estrogen, assumes a key role, especially in breast carcinogenesis [46]. CYP19-associated DBFOD activity was decreased as a result of HCA treatment. Additionally, CYP19 protein and mRNA levels were reduced with HCA treatment. Hence, the observed inhibition resulting from HCA treatment could be either transcriptional or translational and this remains to be elucidated. The results of this study suggest that HCA administration may be a suitable strategy for cancer chemoprevention of some cancers.

In this study, HCA treatment caused an increase in CAT, GPx, GSTs and NQO1 activities in rat liver. In addition, HCA caused significant ( $P < 0.05$ ) elevations in the levels of hepatic GST-mu and -theta mRNA, which handle the metabolism of numerous xenobiotics and play a major cellular antioxidant role. The redox-sensitive transcription factor nuclear factor

erythroid 2-related factor 2 (Nrf2) plays a key role in the cellular defense against oxidative stress via transcriptional upregulation of phase II defense enzymes and antioxidant stress proteins [47]. The induction of detoxification/antioxidant enzymes (GPx, GST, SOD, CAT, PRX) by Nrf2 is regulated via the antioxidant response element (ARE) in the promoter region of target genes [48]. Polyphenols activate the Nrf2/ARE pathway and increase the expression of detoxification/antioxidant enzymes [48]. According to our results, the chemopreventive impact of HCA might be reflected in the activation of the Nrf2 pathway, stimulating the transcription of antioxidant enzymes.

Moreover, induction of GST-mu and -theta activity with HCA treatment is possibly associated with a potential chemoprotective ability of HCA, because GST-mu and GST-theta are GST isoforms that participate in the metabolism of a wide range of chemicals, including carcinogens [49]. A lack of these enzyme activities has been associated with cancer susceptibility [50]. Therefore, the results presented in this study may be of importance, given the possible use of HCA both as a potent anticancer agent as well as a chemopreventive agent. It is known that dietary polyphenols may apply their anticancer impacts through various mechanisms, for example, elimination of cancer-causing factors, adjustment of cancer cell signaling, reinforcement of antioxidant enzyme activities and the induction of apoptosis and cell-cycle arrest [5,51,52]. Some of these impacts may be connected to their indirect antioxidant activities. For instance, the induction of CAT, GPx, NQO1, GSTs and/or phase II enzymes by polyphenols could help the detoxification of cancer-causing factors [41]. Therefore, the induction of these enzyme activities by HCA could be attributed to its cancer chemopreventive impact.

In conclusion, the inhibition of CYP1A and CYP19 and the induction of CAT, GPx, GSTs and NQO1 enzymes reveal potential anticancer and chemopreventive roles for HCA, while the unchanged mRNA and protein levels of CYP2B, 2C and 3A indicate that there is little or no drug interaction after HCA application.

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**Authors' contribution:** Alaattin Sen and Orhan Adali coordinated the research. Asli Semiz did the practical research work and helped in manuscript preparation. Gurbet C. Turgut and Serdar Karakurt assisted in the experimental work. GCT prepared the graphics and Sevki Arslan performed the statistical analysis. Alaattin Sen, Orhan Adali and Hakan Akca analyzed the data and wrote the first draft. All authors commented on and edited the manuscript and agreed on its final version.

**Conflict of interest disclosure:** The authors declare that there is no conflict of interests regarding the publication of this article.

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