WAXY FLOWER (*HOYA CARNOSA* R. BR.): STUDIES ON ANTIOXIDANT ACTIVITY, PHENOLIC COMPOSITION, AND BIOCHEMICAL EFFECTS ON RATS

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ABSTRACT

Hoya carnosa R. Br. is a waxy flower that possesses a highly characteristic smell, unique taste and is commonly used as a houseplant. This study was designed to evaluate its phenolic composition and antioxidant activity, furthermore biochemical effects on rats were also studied. Seventeen different phenolic compounds were determined using reverse phase-high performance liquid chromatography (RP-HPLC). The major phenolic components present in the extract were chlorogenic acid (368.77 mg/100 g FW) followed by benzoic acid (207.06 mg/100 g FW), rutin (49.61 mg/100 g FW) and epicatechin (47.80 mg/100 g FW). Total phenolic content (TPC) (454.5.02 mg GAE/100 g FW), Ferric ion reducing antioxidant power (FRAP) (79.80 µM Trolox/100 g FW) and 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity (SC_{50}: 26.501 µg/mL) were also used as antioxidant determinants.

Keywords: Antioxidant, *Hoya carnosa,* phenolics, rats, biochemical parameter

No: of Tables 3 **No: of Figures: 3** **No: of References: 27**

Introduction

Hoya carnosa (L.f.) R.Br. , the porcelain flower or wax plant, is an Asclepiadoideae species of flowering plant in the dogbane family of Apocynaceae, tribe of Marsdenieae and genus Hoya. *Hoya carnosa*R. Br., is usuallycultivated as a garden plant or houseplant for its attractive waxy foliage and sweetly scented flowers. It is borne in clusters that look like tiny wax miniatures, attractive star-shaped, pink-white blossoms, **Fig. 1[1].**One umbel has about thirty to forty blossoms, they look indeed as if made of wax hence the names Waxflower of Porcelain Flower. The leaves of the plant are thick and succulent, are around 12 cm long and 5 cm at their widest point. **[2]**. The occurrence of Hoya carnosa is mainly as wild in Japan, China and Taiwan, but it is mostly indoor plant as its tolerance to dry conditions are not high**[3]**. Very little studies are done on the chemical constituents of the plant for example, Abe F. *et. al.* Studied and isolated its steroidal constituents, "Eleven pregnanes were isolated from the hydrolysate of the $CHCl_3$ from the causes of *Hoya carnosa*" **[4]**. Furthermore Rolf Altenburger *et. al.* investigated its volatile contents where they extracted 6 compounds from single flower by gas-liquid chromatography those were Linalool, 1,8-cineole, alfa and beta pinene, iso-pentanol and methyl salicyclatectivities, As of many plants *Hoya carnosa* also emit methyl salicylate from its flower **[5]**. Two oligosaccharides with lactone rings also extracted. Still, very little studies are done for its chemical constituents and almost and its extract's antioxidant or other properties were remained untouched.

Hoya carnosa R. Br. Plants are capable of synthesizing limitless aromatic compounds. These agents constitute a large part of the polyphenols (phenolic acids, flavonoids, triterpenols and their esters as well as other derivatives) The acid moieties of triterpene esters found were mainly acetate, isovalerate and cinnamate as reported**[6,7].** It has also been reported earlier that the phenolic composition of plants depends on their species and geographical origin, environmental factors, as well as post-harvest processing and storage conditions. Furthermore, they are synthesized by pentose phosphate, shikimate, and phenylpropanoid pathways **[6,8,9]**. Polyphenols are considered as the largest class of secondary metabolites of the plants, which mostly serve as plant's defense mechanisms to counteract reactive oxygen species in order of its survival and prevent molecular damage which could be caused by micro-organisms, insects, and herbivores. Phenolic agents serve as a source of antioxidants **[10]**, which play an important role in human health by combating oxidative stress, which causes degenerative and pathological disorders, such as cancer, aging, coronary heart disease (CHD), arthritis, ischemia and immune system decline, central nervous system injury, gastritis, cataracts and diabetes **[11,12]**. Reports confirmed that the antioxidant activity of phenolics is associated with a number of different mechanisms, such as free radical scavenging, hydrogen donation, singlet oxygen quenching, metal ion chelation and acting as a substrate for superoxide and hydroxy radicals **[13]**. There is, therefore, considerable interest has developed for the food industry to use

natural antioxidants, isolated from botanical sources especially medicinal and aromatic plants in order to prevent and combat with the different disease such as diabetes, hypercholesterolemia, malaria, and anemia **[14,15]**Plants have been used as medicines since ancient times. This is of great importance, because plants can provide drugs to widen the therapeutic arsenal. However, during the past decade, traditional systems of medicine have become increasingly important in terms of safety. Research is therefore carried out in order to determine the toxicity of medicinal plants. Current estimates suggest that, in many developing countries, a large part of the population relies heavily on traditional practitioners and medicinal plants to meet primary health care needs **[16]**.

Although volatile compounds and some botanical properties of this plant have been studied previously to some extent **[3,17]** there are no reports of its effects on organisms. The purpose of this study was to evaluate the phenolic composition, including some phenolic acids and flavonoids, and *in vitro* antioxidant activities, and the effects on biochemical changes in experimental animals of aquatic flowers of *H. carnosa*. The results of the study will reveal any toxic effects of the plant used as a houseplant.

1. Material and Methods

Standard (purity > 99.0%) phenolic compounds for RP-HPLC-UV (reverse phase-high performance liquid chromatography with ultraviolet detection) analysis were as follows: gallic acid, protocatechuic acid, *p*-hydroxy benzoic acid, vanillic acid, caffeic acid, chlorogenic acid, syringic acid, epicatechin, *p*-coumaric acid, ferulic acid, benzoic acid, *o*-coumaric acid, *trans*-cinnamic acid, abscisic acid, catechin, rutin, quercetin and propylparaben as internal standards (IS) were supplied from Sigma-Aldrich (Sigma-Aldrich Chemie, Munich, Germany) and Merck (Merck, Darmstadt, Germany). The solvents of methanol, acetic acid, and acetonitrile used were obtained from Sigma-Aldrich (Sigma-Aldrich Chemie, Munich, Germany) and Merck (Merck, Darmstadt, Germany). Trolox (6-hydroxy–2, 5, 7, 8-tetramethylchroman–2-carboxylic acid), TPTZ (2, 4, 6-tripyridyl-s-triazine), DPPH (2, 2-diphenyl-1-picrylhydrazyl) and Folin-Ciocalteu's phenol reagent were obtained from Fluka (Fluka Chemie *GmbH*, Buchs, Switzerland). A spectrophotometer(Spectro UV-Vis Double Beam PC LaboMed Inc., Los Angeles, CA, USA) was used in all absorbance measurements. All solutions were prepared with deionized water (Human, Zeneer Navi UP, Song Pa-Ku, Seoul, Korea).

1.1. Plants Sample

Samples of the waxy flowers were obtained from a florist shop from the city of Trabzon, Turkey, in July 2015.

2.2. Extract Preparation of Antioxidant Analysis

Approximately 10 g fresh of flower samples were blended with 100 mL methanol (98%) and then stirred with a shaker (Heidolph MR HEI-Standard, Schwabach, Germany) for 24 h at room temperature. After shaking, the mixture was sonicated using appropriate apparatus (Elma® Transsonic Digital, Germany) for 3h. After sonication, the

suspension was filtered and the raw filtrate was kept at 4 °C until used for antioxidant tests and feeding to rats.

2.3. Determination of Total Phenolic Contents (TPC)

Total phenolic content was analyzed by using Folin-Ciocalteu assay with the gallic acid which was taken as standard. For this purpose, 680 µL distilled water, 20 µL aquatic extracts and 400 µL of 0.2 N Folin-Ciocalteu were mixed and then vortexed. After 3 min, 400 µL Na_2CO_3 (7.5 %) was added and the mixture was incubated for 2 h at room temperature. Later on, absorbance was measured at 760 nm. The concentration of TPCs was calculated as mg gallic acid equivalents (GAE) per 100 g of fresh weight (FW), using a standard curve for gallic acid in the concentration range between 0.03 and 0.5 mg/mL (r^2 = 0.997).

2.4. Determination ferric reducing/antioxidant power (FRAP)

To determine total antioxidant capacity ferric tripyridyltriazine (Fe-III-TPTZ) complex was used (Benzie and Strain, 1999). FRAP reagent was obtained as required by mixing 25 mL acetate buffer (300 mM, pH 3.6), 2.5 mL of 10 mMTPTZ solution dissolved in 40 mM HCl and 2.5 mL of 20 mM $FeCl_3.6H_2O$ solution. After that, 100 µL of the sample was mixed with 3 mL of freshly prepared FRAP reagent and incubated for 5 min at 37 °C. Absorbance was measured at 595 nm against blank reagent containing distilled water. Trolox was used a positive control to construct a reference curve (15.625-500 µM, r^2=0.999), FRAP values were expressed as µM Trolox equivalent of 100 g.

2.5. Free radical-scavenging activity of DPPH

The scavenging of DPPH radicals was assayed using the technique described by Molyneux [18]. This method is based on the fact that the DPPH radical, which is purple in color, decays in the presence of an antioxidant agent with the loss in color. The change in absorbance can then be monitored spectrophotometrically at 517 nm. Briefly, 0.75 mL of the aquatic solution was mixed with 0.75 mL of 0.1 mM DPPH (dissolved in methanol), and then mixed was vortexed and incubated for 50 min in the dark at room temperature until stable absorbance values were obtained. After the incubation period, the absorbance was recorded at 517 nm against a blank and control.

2.6.Determination of phenolic compounds using RP-HPLC-UV

Methanolic extracts were prepared for HPLC analysis by using dry materials. Twenty-gram flowers were dried at 40 °C and ground. The powder samples (5 g) were extracted with methanol solvent (200 mL) by using Soxhlet extractor for 24 h. The extracts of methanol were evaporated until dryness and concentrated using a rotary evaporator at 40 °C. The residue was dissolved in 10 mL acidified distilled water (pH 2). The reaction mixture was first extracted with diethyl ether (20 mL) and then with ethyl acetate (20 mL). The organic solvent was evaporated to dryness under reduced pressure in a rotary evaporator at 40 °C. The residue was dissolved in methanol for RP-HPLC-UV analysis (*Can et al.*, 2015). Before injection of HPLC, all solutions were filtered through a 0.2 µm membrane filter (Sartorius, Goettingen, Germany).

HPLC analysis was carried out on an Agilent 1100 series using a Zorbax Eclipse XDB-C18 column (4.6 mm x 150mm, 5 µm) and a gradient program with two solvent systems (A: 0.5% acetic acid in 50:50

acetonitrile: water; B: 2%acetic acid in water). A flow rate of 1.2 mL.min -1 and injection volume of 20 µL was used, and signals were detectedat 280. Propylparaben was the appropriate compound as an internal standard for this system.

2.7. Animal Groups and Feeding Procedure

Twenty-eight female Sprague-Dawley rats weighing 220-250 g were used. The rats were kept in cages at 22±2 °C, in a 12-h/12-h light/dark cycle, and received standard chow and water ad libitum. All of the experimental protocols described in this study were approved by the Karadeniz Technical University School of Medicine Animal Ethics Committee.

The rats were randomly allocated into four equal groups (n = 7). The experimental procedures were completed in three days.

Group 1: Control group
Group 2:*H. carnosa* extracts (200 mg/kg)
Group 3:*H. carnosa* extracts (400 mg/kg)
Group 4:*H. carnosa* extracts (600 mg/kg)

While 0.8 mL/kg saline (SF) was administered by gavage for three days in the control group, 200, 400 or 600 mg/kg of the extracts were administered by gavage for three days in groups 2, 3 and 4, respectively. After gavage feeding of all rats for three days, the experiment was terminated on the 4th day. The rats were sacrificed by decapitation, and blood was obtained from a trunk vessel for biochemical analysis.

Glucose, urea, creatine, sodium, potassium, chloride, aspartate transaminase (AST), alanine transaminase (ALT), lactate dehydrogenase (LDH), creatine kinase (CK), total protein (TP) and triglycerides (TG) were measured in the serum samples to determine biochemical changes caused by the aquatic *H. carnosa* solution, using a Siemens Advai 2400 autoanalyzer (Modular System, GmbH, Mannheim, Germany).

2.8. Statistical analysis

The assay results were expressed as mean values and standard deviations (mean ± S.D.). Rat groups were examined for normal distribution. Kruskal-Wallis variance analysis and the Mann-Whitney *U* test were used to compare biochemical parameters, *p* values of less than 0.05 were considered significant.

2. Results and Discussion

Sample TPCs were measured in aquatic extracts of *H. carnosa* flowers using the Folin Ciocalteu method. The value obtained was 454.07±5.02 mg GAE/100 g (**Table 1**). To date, there has been no information in the literature about the phenolic composition and antioxidant properties of H. carnosa. Only the antioxidant property of *H. carnosa* was investigated as a test of biological activity in this study. For this purpose, the two most widely used different techniques in the literature, FRAP and DPPH, were employed. The FRAP and DPPH value results are also given in **Table 1**.Detection in the range of 315–280 nm is the most generally used wavelength for separation of mixtures of phenolic acids. The spectra were recorded from the peak fractions separated by HPLC and were identified by comparison of retention times (peak normalization, PN). The chromatogram of the standard phenolic compound is presented in **Fig. 2**, and the profiles and quantities of phenolic compounds as mg/100 g FW are given in **Table 2**.

Table 1. Antioxidant properties of *H. carnosa*

Plant	Total phenolics (mg GAE/100 g)	FRAP (µmol Trolox/100 g)	DPPH SC_{50} (mg/mL)
H. carnosa	454.070±5.020	79.800±1.100	26.500±0.220
Trolox	-	-	0.070±0.000

FRAP: Ferric ion reducing antioxidant power
GAE: Gallic acid equivalents
DPPH: 2,2-diphenyl-1-picrylhydrazyl
SC_{50}:Radical scavenging activity

Table 2.Phenolic composition of *Hoya carnosa*of determined by RP-HPLC-UV

Phenolic compounds	(mg phenolic compound/100 g FW)
Gallic acid	0.07
Protocatechuic acid	1.68
p-OH benzoic acid	1.36
Catechin	3.03
Chlorogenic acid	368.77
Vanillic acid	0.65
Caffeic acid	6.05
Syringic acid	0.13
Epicatechin	47.80
p-coumaric acid	0.73
Ferulic acid	12.36
Benzoic acid	207.06
Rutin	49.61
o-coumaric acid	0.42
Abscisic acid	n.d.
t-cinnamic acid	0.78
Quercetin	1.03

n.d.: not detected

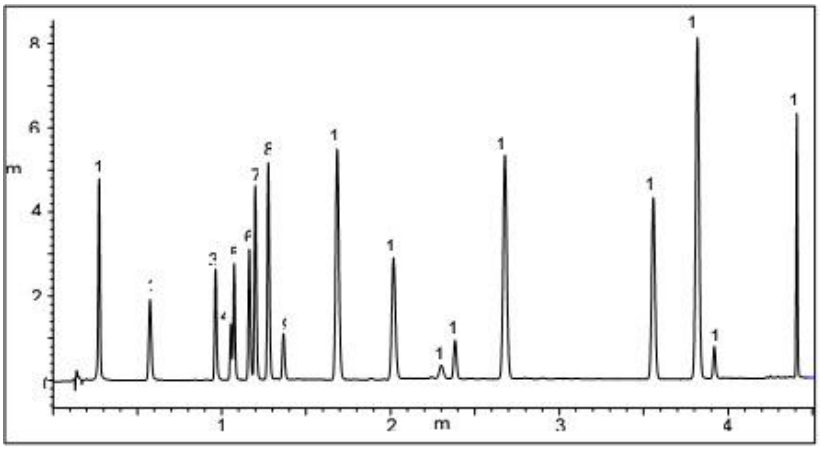

Fig. 2. A RP-HPLC-UV detection procedure for separating 17 standard phenolic compounds. Peak identification: (1)gallic acid, (2) protocatechuic acid, (3) *p*-OH benzoic acid, (4) catechin, (5) chlorogenic acid, (6) vanillic acid, (7) caffeic acid, (8) syringic acid, (9) epicatechin, (10) *p*-coumaric acid, (11) ferulic acid, (12) benzoic acid, (13) rutin, (14) *o*-coumaric acid, (15) *cis, trans*- abscisic acid, (16) *trans*-cinnamic acid, (17) quercetin, and (18) propilparaben (IS).

Sample TPCs the value obtained was 454.07±5.02 mg GAE/100 g (**Table 1**). This made it impossible to compare our results. However, for better evaluation, we were able to compare the TPC with some known flower species. For example, TPC has been reported at 431.2 mg GAE/100 gin Helichrysum plicatum DC. Subspecies **[19]**, 233.54 mg GAE/gFW in Rosa damascena Mill. **[20]**, 373.8 mg GAE/100 gFW in Mentha piperita (mint), 365 mg GAE/100 g in Rosmarinus officinalis **[21]**, and 173.72 mg/100 gin Tilia rubra subsp. Caucasica **[22]**, Compared with these values reported in the literature, H. carnosa has a high level of phenolic content. Many scientific studies have shown that antioxidant capacity rises in line with phenolic material content. Amount of TPC is a reflection of biological activity, and plants with a high phenolic content always have antioxidant, antibacterial, anti-tumor, anti-viral and anti-inflammatory capacities **[10,20,23]**. Therefore, H. carnosa or wax plant aquatic extracts high a high biological activity potential.

Only the antioxidant property of *H. carnosa* was investigated as FRAP and DPPH. The FRAP test measures the ability of components in a polar solution environment to convert the Fe-III-TPTZ complex into the Fe-II-TPTZ complex, and the emerging colored complex gives absorbance at 595 nm (Benzie and Strain, 1999). High absorbance shows high antioxidant activity. The FRAP value results are also given in **Table 1**. As with TPC, since there are no previous antioxidant studies involving *H. carnosa,* we had no means of comparing the FRAP values. However, *H. carnosa* has a high antioxidant capacity compared with other different plants or natural products in the literature **[20]**.

Many scientific studies have shown that antioxidant capacity rises in line with phenolic material content. The antioxidant properties of phenolic agents may result from the greater H-atom

donating ability of phenolic acids and flavonoids to several radicals, thus terminating the chain radical reaction **[24]**.

Plants possess the ability to synthesize countless phenolic compounds. To date, the presence of approximately 4000-6000 phenolic compounds has been described. However, it is impossible to elucidate all phenolic compounds in studies of the phenolic composition of natural products. It is possible, however, to establish levels of TPC using the Folin Ciocalteu technique. Since it is impossible to describe every phenolic compound in studies performed, only the major phenolic acids and some flavonoids that may be present in plants are clarified using chromatographic analysis **[25]**. For this reason, we measured only 17 phenolic substances using reversed phase high-performance liquid chromatography (RP-HPLC) in this study **Table 2**. A total of 17 peaks representing phenolic compounds were observed, but there were minor differences in this profile between flower extracts.The amount of phenolic acids in the samples varied widely, from 0.01 to 369 mg/100 g FW. The HPLC profile of *H. carnosa* extract indicates its complex composition with several peaks of varied retention times (**Fig. 3**). With the exception of abscisic acid, all phenolic compounds of the 17 phenolic standards investigated were detected in varying amounts in *H. carnosa*. Chlorogenic acid and benzoic acid were detected as the major phenolic acids and rutin and epicatechin as the major flavonoids in *H. carnosa*. Gallic acid, vanillic acid, syringic acid, *p*-coumaric and *o*-coumaric acids were detected in small concentrations, but no abscisic acid was detected.

Phenolic compounds are agents responsible for biological activity and are best known for their antioxidant activities. Their antioxidant capacities vary depending on the number and position of the hydroxyl (-OH) groups and methoxy (-OCH_3) groups in their structures

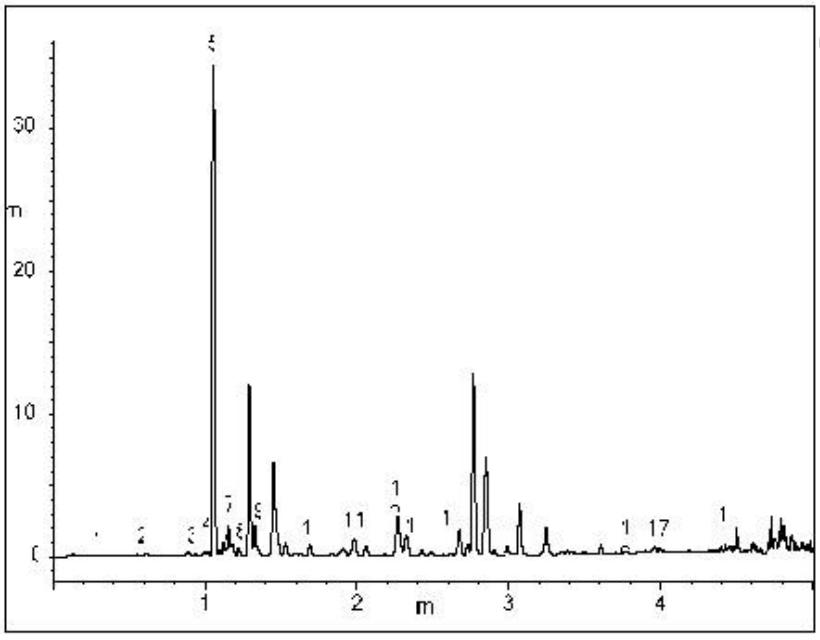

Fig. 3. A RP-HPLC-UV detection of *Hoya carnosa* flowers. (1) gallic acid, (2) protocatechuic acid, (3) *p*-OH benzoic acid, (4) catechin, (5)

[26]. It has been reported that the ortho-dihydroxy groups exhibit higher antioxidant activity than other positions. *H. carnosa* contains an important number of phenolic acids (chlorogenic acid, and benzoic acids) and flavonoids (rutin) known to have high antioxidant and other biological active properties in natural products **[27]**.*H. carnosa* flowers possess high levels of phenolic substances and associated antioxidant capacity. This means that their extracts can be used for pharmaceutical purposes or as food additives. In biochemical terms, however, there is no information in the literature regarding whether or not the plant is toxic. Therefore, in one study with experimental animals, rats were given aquatic plant extracts in three different concentrations. Blood specimens were collected over four days and biochemical parameters were investigated in serum. These were compared with control group values (**Table 3**). Blood sugar, triglyceride, liver function tests (ALT and AST), kidney function tests (urea, creatine, total protein), certain serum electrolytes (Na, K and Cl) and cardiac tests (LDH, CK) were investigated. Nothing was given to the control group apart from chow for three days. Statistical analysis of the results compared to the control group values revealed no significant difference among the groups ($p>0.05$). In conclusion, we think that since aquatic extracts of *H. carnosa* cause no biochemical change in experimental animals they may perhaps exhibit no such effects in humans, either. However, further tests are needed in order to be able to make use of *H. carnosa* aquatic extracts. Since this is the first study involving *H. carnosa,* with its high biological activity potential, it needs to be regarded as a beginning and further developed.

Table 3. Biochemical parameters measured in serum of rats (n = 28)

Parameters	**Group I Control**	**Group II** 200 mg/kg	**Group III** 400 mg/kg	**Group IV** 600 mg/kg	***p*-value**
Glucose (mg/dL)	156.66 ± 6.42	164.00 ± 11.26	158.00 ± 8.71	147.33 ± 3.21	0.122
Urea (mg/dL)	19.00 ± 1.73	19.66 ± 0.57	16.00 ± 0.00	18.33 ± 2.51	0.126
Creatine (mg/dL)	0.32 ± 0.04	0.37 ± 0.03	0.35 ± 0.05	0.36 ± 0.06	0.601
Sodium (mEq/days)	140.66 ± 4.04	142.33 ± 0.57	135.33 ± 0.57	137.66 ± 2.08	0.061
Potassium (mEq/days)	6.40 ± 0.45	6.73 ± 0.35	6.20 ± 0.86	6.90 ± 0.40	0.454
Chloride (mEq/days)	97.66 ± 1.15	99.33 ± 2.08	95.33 ± 1.52	95.33 ± 2.08	0.082
AST (U/L)	221.00 ± 36.71	190.66 ± 13.65	185.33 ± 25.69	288.33 ± 46.91	0.082
ALT (U/L)	80.33 ± 3.21	61.66 ± 6.35	65.00 ± 11.26	80.00 ± 13.11	0.095
LDH (U/L)	1317.33 ± 206.40	1649.66 ± 209.60	1135.00 ± 112.94	1775.33 ± 287.31	0.052
CK (U/L)	5212.00 ± 1037.64	5600.00 ± 396.79	4830.66 ± 1233.	6488.00 ± 354.06	0.121

Total protein (g/dL)	7.03 ± 0.35	7.30 ± 0.36	6.83 ± 0.32	7.13 ± 0.56	0.584
Triglycerides (mg/dL)	145.33 ± 49.32	153.33 ± 56.19	121.00 ± 8.71	110.00 ± 24.00	0.622

p, significant level a,b,c, the values of the results are significantlydifferent (p50.05) frome achother.
AST: Aspartate Aminotransferase, LDH: Lactate Dehydrogenase
CK: Creatine kinase, ALT: Alanine Aminotransferase

Conflict of Interest

The authors declare that they have no conflict of interests.

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