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The Effect of Epicatechin-(4 β -8)-Catechin on Some Biomarkers of Fatigue in Packed Donkeys (*Equus Asinus*) during the Dry Season in Northern Nigeria

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ABSTRACT

Overworking and overloading of donkeys may cause fatigue. The experiment aims to determine the effects of epicatechin-(4 β -8)-catechin (EEC) on some fatigue biomarkers in packed donkeys during the dry season. 10 jacks, aged between four and five years were used for the study. Five were given EEC in feed seven days before packing (test), while another five were given feed only (control). Blood samples collected for biochemical analyses were analyzed for superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), total antioxidant capacity (TAC), creatine kinase (CK) and tumor necrosis factor-alpha (TNF- α). The SOD activity increased significantly ($P < 0.05$) in the test group during the cold-dry season after packing. The values of TAC during the packing period were significantly ($P < 0.05$) higher in test group during the hot-dry season. The TNF- α concentration was higher ($P > 0.05$) in the control group especially one-hour on route. The CK activity was significantly ($P < 0.05$) higher in the control donkeys during the hot-dry season. In conclusion, packing donkeys during the dry season resulted in fatigue, but EEC served as an anti-fatigue agent.

KEYWORDS

Donkeys; fatigue; oxidative status; inflammation; packing; welfare

Introduction

A high percentage of donkeys used for work are found mostly in developing countries (Fsahaye, Kumar, Kebede, & Abebe, 2018). These parts of the world depend on these animals for their livelihood, especially in the rural areas. Their use for work has replaced the use of expensive equipment in the rural areas, since they are comparatively inexpensive and “easy to maintain” (Singh, 2000). Despite the increase in the importation of cars with its advantages over animals and the drastic decrease in the use of animals for transportation, animal transport is still very relevant in northern Nigeria especially where there are only foot paths and also where motorable roads remain seasonal (Talla & Song, 2014). However, the welfare status of the donkey is still quite frequently suboptimal (Haddy, Rodrigues, Raw, Burden, & Proops, 2020). The result of a study conducted by Amante, Hunde, Endebu, Hirpa, and Mamo (2014) on the health and welfare of working equids in Ethiopia reported that management constraints like feed shortage, traditional health-care, lameness, wounds, overworking, overloading and housing problems are prevalent. Thus, further studies are required and necessary measures should be adopted to alleviate the welfare constraints that may hinder the efficient use of working donkeys.

The dry season is of paramount importance to pack donkeys in northern Nigeria. This is because during this period donkeys are subjected to packing (carrying load from one point to another) and used for transporting harvested farm produce. They are also used by low-income farmers for draught purposes such as plowing to make ridges in preparation for planting (Burden & Thiemann, 2015; Oudman, 2002). The cold-dry season also known as the harmattan season is a cold-dry, dusty season in sub-Saharan Africa, reported to be a stressful period (Okeahialam, 2016; Sufiyan, Mohammed, Bello, & Zaharadeen, 2020), while the hot-dry season is a transition period between the dry and rainy seasons. Vathana et al. (2002) reported that the transition period from dry to rainy season is more thermally stressful to animals in the tropics. Thus, the dry season is considered to be stressful to animals, which may be due to the fact that these animals are being subjected to high temperature-humidity index (THI) which is usually above the value of 80 as reported by Yousef (1985).

Biomarkers of fatigue include creatine kinase (CK) for muscle damage, tumor necrosis factor- α (TNF- α) for inflammation and activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and total antioxidant capacity (TAC) for oxidative stress (Maes, Rodriguez, & Morris, 2019). They are important in evaluating the physiological status of animals, especially when they are subjected or exposed to stressful environmental conditions (Fan, Dun, Gu, Guo, & Ikuyam, 2015). The fluctuations of these biomarkers give an indication of how the animal overcomes or is overwhelmed by stress. The recovery and ability to rapidly, accurately and non-invasively diagnose muscle fatigue is predicted, based on the ability to detect these biomarkers of stress in the blood (Finsterer & Drory, 2016). Fatigue may be due to mental disorder, central or peripheral nervous system dysfunction, skeletal muscle disease, involving mechanical fatigue or loss of force-generating capacity (Boyas & Guevel, 2011; Meeusen, Van Cutsem, & Roelands, 2020) and this may occur in donkeys subjected to strenuous work such as packing over a long distance. This strenuous work may result in localized damage to muscle tissue, which is observed in sarcolemma, basal lamina and contractile elements (Koch, Pereira, & Machado, 2014). Such damage is accompanied by the release of enzymes, including CK, which has been suggested as one of the best biomarkers for diagnosing muscle damage (Cheng, Jude, & Lanner, 2020; Koch et al., 2014). Physical exertion induces increased level of pro-inflammatory and anti-inflammatory cytokines. Thus, increased plasma levels of TNF- α and other cytokines are detected after strenuous work (Terink et al., 2018). The TNF- α is a pro-inflammatory cytokine that is predominantly produced by macrophages, and is able to induce inflammation (Finsterer, 2012). Spruit et al. (2005) reported an increase in serum levels of TNF- α in a patient subjected to maximal incremental exercise test. The antioxidant enzymes are found in significant amounts within the cytoplasm, and not in extracellular tissue (Bratovic, 2020). However, when detectable amounts are found in the blood, it suggests that break in the cell membrane of muscle cells has occurred (Gomes, Silva, & De Oliveira, 2012).

Epicatechin-(4 β -8)-catechin (EEC), also known as pycnogenol, is a well-known phytochemical compound and food component with antioxidant properties. The use of EEC has been reported in rats (Taner et al., 2014), dogs (Cerbo et al., 2017) and Chicken (Wu, Wang, & Qi, 2016). EEC has been reported to reduce fatigue by improving fitness and recovery in humans (Vinciguerra et al., 2013; Williamson & Manach, 2005). The active ingredients can be extracted from other sources, such as peanut skin, berries, apples and grape seeds (Gu et al., 2004). Information on its effects on physical fatigue in work animals is lacking. The aim of the study was to determine the effect of EEC on some biomarkers of fatigue in working donkeys during the dry season. The experimental hypothesis is that administration of EEC prior to work will reduce the level of fatigue.

Materials and methods

Study area

The study was carried out during the cold- (between November and December, 2016) and hot-dry (between March and June, 2016) seasons at the donkey farm of the Equine and Camel Research Programme of National Animal Production Research Institute, Shika-Zaria, located in the Northern Guinea Savannah zone of Nigeria (11° 12' N; 7° 33' E). The ambient temperature (AT), relative humidity (RH) and THI were $17.3 \pm 1.1^{\circ}\text{C}$, $20.9 \pm 2.5\%$ and 88.8 ± 1.9 , respectively during the cold-dry season and $32.8 \pm 0.9^{\circ}\text{C}$, $49.2 \pm 3.3\%$ and 100.2 ± 1.7 , respectively during the hot-dry season.

Ethical clearance

Ethical clearance for the use of donkeys in this study was sought and approval was obtained from Ahmadu Bello University Committee on Animal Use and Care (ABUCAUC), Ahmadu Bello University, Zaria (Approval No: ABUAUC/2020/68).

Measurement of thermal environmental conditions

The AT was recorded simultaneously at the time when the blood samples were collected at the experimental site, using a wet- and dry-bulb thermometer (Brannan, England), and RH was obtained using Osmond's hygrometric table (Narindra Scientific industries, Haryana, India). THI was calculated using the formula of Bianca (1962) as seen below.

$$\text{THI} = (0.35t_d + 0.65 t_{wb}) \times 1.8 + 32$$

Where, t_d = dry-bulb temperature ($^{\circ}\text{C}$)

t_{wb} = wet-bulb temperature ($^{\circ}\text{C}$)

Experimental animals and management

Ten, apparently, healthy male donkeys between the ages of 4–5 years with average body condition score of seven over nine with average weight of 141 ± 2.2 kg, were used as the experimental animals. The donkeys were pre-conditioned for two weeks, during which they were screened and treated against ectoparasites and endoparasites. They were packed with load of sand weighing 50% of their bodyweight for two hours. They were kept under semi-intensive management system. At night, they were kept under a roofed pen without walls. Two donkeys were kept in each pen with standard dimension of 9 m^2 . During the day, the donkeys were subjected to packing. They were allowed to graze during the day after packing on natural pasture, comprising mainly bermuda grass (*Cynodon dactylon*), gamba grass (*Andropogon gayanus*), Egyptian crowfoot grass (*Dactyloctenium aegyptium*), and supplemented with maize and sorghum bran. In addition, 1 kg of whole sorghum grain was fed to each donkey per experimental day. The donkeys were given access to water *ad libitum* before and after packing. Salt licks were also provided.

Experimental design

Briefly, 10 jacks, randomly assigned to two groups (test and control) of five donkeys each jacks per, were used for the field experiment. Baseline data of all the biomarkers used in this study were recorded prior to the beginning of the trial and the test group was supplemented with EEC for seven days while the control group was given feed only. Thereafter, each group was subjected to packing for three days, one day apart. They were loaded with sand, weighing 50% of their body weight and trekked for two hours (10:00 h to 12:00 h) on each experimental day.

Administration of epicatechin-(4 β -8)-catechin

Epicatechin-(4 β -8)-catechin (EEC) (pynogenol®; a registered trademark of Horphag Research Ltd., Geneva, Switzerland) was commercially sourced from Viva Naturals Inc., New York, USA and administered to the test group 7 days before packing (Grimm et al., 2006) at a dose of 10 mg/kg mixed in feed (Yang et al., 2008).

Packing procedure

All donkeys with average body weight of 141 ± 2.2 kg were saddled according to the methods of Oudman (2002). They were loaded with sand at a loading rate of 50% of their body weight (Pearson, 2003) every morning on each experimental day and were subjected to trekking a distance of 10 km. The donkeys were weighed using a Lider B-650® digital electronic scale of 1000 kg capacity.

Blood collection

Blood sample for baseline values was collected one week before the blood collection during the blood sampling episodes. Blood sample was collected from each donkey before packing (6:00 h), one-hour on route (11:00 h), 10 minutes after packing (12:10 h) and three and seven days after packing. The head of the donkeys were stabilized gently and slanted to the left before collecting the blood from the jugular vein. The collection was performed by jugular venipuncture using a 10-mL syringe, connected to an 18-gauge needle. The sample was placed in a potassium ethylenediaminetetraacetate coated tube to obtain plasma which was stored at -20°C before biochemical analyses (Clark, Youngman, Palmer, Parish, & Collins, 2003).

Determination of superoxide dismutase activity

The activity of superoxide Dismutase (SOD) was measured using the Northwest Life Science Specialties SOD kit (NWLSS™ NWK-SOD02) based on the method of monitoring the auto-oxidation rate of hematoxylin originally described by Martin, Dailey, and Sugarmann (1987) and modified to enhance reliability. Briefly, 230 μL of assay buffer was added to wells of the microplate. Thereafter, 10 μL of assay buffer (for blank) and 10 μL of plasma sample were added. The wells were shaken properly, mixed, and incubated for 2 minutes. A multichannel pipette was used to add 10 μL of hematoxylin reagent to begin the reaction. The content of each well was mixed quickly using instrument's shaker function, and immediately, the absorbance at 560 nm was recorded. The activity was calculated as: $\text{SOD U/mL} = 1.25 \times \% \text{ inhibition}$.

Determination of catalase activity

The catalase (CAT) kit was purchased from Abcam PLC, 330 Cambridge Science Park, UK. Briefly, 12 μL of fresh 1 mM H_2O_2 was added into each well of plasma samples and positive control solution and sample high control (HC) to start the reaction. Then the samples were incubated at 25°C for 30 minutes, and 10 μL of stop solution added into each sample well to terminate the reaction. For each well, 50 μL of developer mix, containing: 46 μL of assay buffer, 2 μL of OxiRed probe, and 2 μL of horseradish peroxidase solution was prepared. Fifty microliter of the developer mix was added to each test sample, control, and standard. The samples were mixed well and incubated at 25°C for 30 minutes and then optical density of 570 nm in a plate reader was measured (Aebi, 1984).

Calculation: Signal change by CAT in the sample was $\Delta A = \text{AHC} - A \text{ sample}$. AHC was the reading of sample HC; sample A was the reading of sample in 30 minutes. H_2O_2 standard curve was plotted, and the ΔA was applied to the H_2O_2 standard curve to get B nmol of H_2O_2 decomposed by CAT in 30 minutes reaction. The activity of CAT was calculated as follows:

$$\text{CAT activity} = \frac{B}{30 \times V} \times \text{Sample dilution factor} = \text{nmol/min/ml} = \mu\text{U/ml}$$

Where B was the decomposed H_2O_2 amount from H_2O_2 standard curve (in nmol); V was the pretreated sample volume added into the well (in mL); 30 was the reaction time in 30 minutes; one unit of CAT was the amount of CAT that decomposed 1.0 μmol of H_2O_2 per minute at pH 4.5 at 25°C.

Determination of glutathione peroxidase activity

The activity of glutathione Peroxidase (GPx) was measured using the Northwest Life Science Specialist, Vancouver, Canada (NWLSS™) GPx assay kits protocol NWK-GPX01. Briefly, for standard procedure for microplate assay, all reagents were brought to room temperature (25°C). Diluted plasma sample (50 μL) was added to the wells and then 50 μL of working nicotinamide adenine dinucleotide phosphate (NADPH) was added to each well. Working H_2O_2 (50 μL) was added to each well. After waiting for 1 minute, microplate was placed in plate reader and read at 340-nm wavelength.

To calculate GPx activity using the NADPH absorption coefficient: the GPx activity, expressed as mU/mL, was calculated using the GPx activity definition.

$$\text{Glutathione peroxidase} = \frac{2 (\text{mRate}_s - \text{mRate}_b) \cdot V_{\text{Rxm}}}{2.74 \cdot V_s} \cdot \text{df}$$

Where $\text{mRate}_s = -1,000 \Delta\text{A}_{340}/\text{min}$ of sample; $\text{mRate}_b = -1,000 \Delta\text{A}_{340}/\text{min}$ of blank; 2.74 = NADPH 340 nm millimolar absorption coefficient at 1 cm path length; V_{Rxm} = volume of reaction mixture; V_s = volume of sample; 2 = correction for 2 moles reduced glutathione oxidized to 1 mole glutathione disulfide (oxidized glutathione) per mole NADPH oxidized; df = sample dilution factor.

Determination of total antioxidant capacity

Total antioxidant status was determined using the Trolox Equivalent Antioxidant Capacity method (TEAC). 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) solution (14 mmol/l ABTS, 4.9 mmol/ $\text{K}_2\text{S}_2\text{O}_8$) was diluted with deionized water until the absorbance was 0.7–0.02 at 734 nm. To 1980 ml of ABTS working solution 20 mL of plasma was added and the absorbance was recorded at the wavelength of 734 nm against deionized water at time 0 and after 10 min. Absorbance was measured spectrophotometrically (UV-1700 PharmaSpec of Shimadzu, Japan) at a wavelength of 734 nm (Miller, Rice-Evans, Davies, Gopinathan & Milner, 1993). Plasma antioxidant capacity was determined from the analytical curve for trolox as a standard. Total antioxidant capacity (TAC) was expressed in mmolTrolox/L.

Determination of tumor necrosis factor- α concentration

The Donkey tumor necrosis factor- α (TNF- α) enzyme-linked immunosorbent assay test kit was purchased from GeniRose Scientific Inc., Paoli, Pennsylvania, USA. This assay employed the quantitative sandwich enzyme immunoassay technique and used biotin-streptavidin chemistry to improve the performance of the assays. An antibody specific for equine TNF- α was pre-coated onto a microplate. 100 μL of standards and plasma samples were pipetted into the wells and any TNF- α present bound by the immobilized antibody. After washing away any unbound substances, 100 μL of detection antibody specific for equine TNF- α was added to the wells. Following wash to remove any unbound antibody reagent, a detection reagent was added. After intensive wash 100 μL of substrate solution was added to the wells and color developed in proportion to the amount of TNF- α

bound in the initial step. The color development was stopped and the intensity of the color was measured (Brennan & McInnes, 2000). TNF- α was expressed in ng/mL.

Determination of creatine kinase activity

The activity of creatine kinase (CK) was assayed spectrophotometrically using CK reagent kit (Pointee scientific, Canton MI 48188, USA). Briefly, working reagent was prepared according to manual's instruction. Exactly 0.1 mL of working reagent was pipetted into appropriate tubes and pre-warmed at 37°C for 4 minutes. The spectrophotometer (UV-1700 PharmaSpec of Shimadzu, Japan) was primed to zero using distilled water at 360 nm. 0.05 mL of the sample was added to the respective tubes and mixed gently. After 2 minutes, absorbance was read and recorded. Readings were repeated every minute for 2 minutes. Average absorbance difference per minute (Δ Abs/min) was calculated. The Δ Abs/min was multiplied by the factor 3376 for results in U/L (Swason & Wilkinson, 1972).

Statistical analysis

Statistical differences between groups and seasons were analyzed using two sample *t*-test and paired *t*-test respectively. Repeated measures analysis of variance (ANOVA) was used to analyze between sampling times. Correlation coefficients between environmental parameters, biomarkers of fatigue and oxidative stress parameters were determined. Data were expressed as mean $\pm$ standard error of the mean (Mean $\pm$ SEM). GraphPad Prism 5.3 for Windows (GraphPad Software, San Diego, California, USA) was used for the analyses. Values of $P < 0.05$ were considered significant.

Results

Table 1 shows the activity of SOD in donkeys administered with EEC and subjected to packing during the dry season. The activity of SOD increased during the cold-dry season when compared with the hot-dry season. The activity of SOD increased in the test group during the cold-dry season especially after packing with the value of 755.7 ± 74.1 U/L which was significantly ($P < 0.05$) higher than the value of 443.2 ± 59.9 U/L obtained in control group. During the hot-dry season, the activities of SOD in the test and control groups were not significantly ($P > 0.05$) different during the packing period.

Table 2 shows the activity of CAT in donkeys administered with EEC and subjected to packing during the dry season. CAT activity increased significantly ($P < 0.05$) during the cold-dry season when compared with the hot-dry season. Seven days after packing, during the cold-dry season, CAT activity increased significantly ($P < 0.05$) in control group (18.6 ± 1.1 μ mol/min/mL). Although during the hot-dry season, the activity of CAT (8.2 ± 0.3 μ mol/min/mL) increased relatively, the differences in the value was not significant.

Table 1. Activities of superoxide dismutase in donkeys administered with epicatechin-(4 β -8)-catechin and subjected to packing during the dry season.

Packing episodes	Cold-dry season		Hot-dry season	
	Control (n = 5)	Test (n = 5)	Control (n = 5)	Test (n = 5)
Baseline (U/L)	700 ± 0.2^a	700 ± 0.2^a	382.9 ± 12.5^b	382.9 ± 12.5^b
Before (U/L)	390.8 ± 49.2^a	$520.4 \pm 76.6^{a,1}$	$337.5 \pm 76.6^{b,1}$	$456.5 \pm 29.9^{a,1}$
One-hour on route (U/L)	$537.8 \pm 87.2^{a,1}$	$677.7 \pm 81.7^{a,1}$	$460.9 \pm 10.2^{a,2}$	$379.2 \pm 24.7^{a,2}$
10 minutes after (U/L)	443.2 ± 59.9^a	755.7 ± 74.1^b	407 ± 29.6^a	401.3 ± 16.4^a
3 days after (U/L)	$674.4 \pm 21.5^{a,2}$	592.4 ± 118.5^a	$373.7 \pm 27.2^{a,3}$	$291.9 \pm 35.1^{a,3}$
7 days after (U/L)	$298.1 \pm 40.4^{a,2}$	$326.5 \pm 111.8^{a,2}$	$347.2 \pm 39.3^{a,3}$	$329 \pm 21.9^{a,3}$

Values with superscript ^{a,b} on the same row are significantly different ($P < 0.05$).

Values with superscript ^{1,2,3} on the same column are significantly different ($P < 0.05$).

Table 2. Activities of catalase in donkeys administered with epicatechin-(4 β -8)-catechin and subjected to packing during the dry season.

Packing episodes	Cold-dry season		Hot-dry season	
	Control (n = 5)	Test (n = 5)	Control (n = 5)	Test (n = 5)
Baseline ($\mu\text{mol}/\text{min}/\text{mL}$)	5.9 ± 1.3^a	5.9 ± 1.3^a	4.9 ± 0.6^b	4.9 ± 0.6^b
Before ($\mu\text{mol}/\text{min}/\text{mL}$)	13.3 ± 2.0^1	8.9 ± 1.6	10.9 ± 1.3^1	9.6 ± 2.2
One-hour on route ($\mu\text{mol}/\text{min}/\text{mL}$)	15.5 ± 1.6^1	8.1 ± 1.1	12.2 ± 0.4^1	10.8 ± 0.7
10 minutes after ($\mu\text{mol}/\text{min}/\text{mL}$)	12.7 ± 1.9	7.7 ± 0.9	11.8 ± 0.6^1	12.2 ± 0.5
3 days after ($\mu\text{mol}/\text{min}/\text{mL}$)	7.4 ± 0.8^2	7.7 ± 0.1	11.7 ± 0.8^2	11 ± 1.9
7 days after ($\mu\text{mol}/\text{min}/\text{mL}$)	$18.6 \pm 1.1^{a,2}$	16.2 ± 2.8^a	8.2 ± 0.3^b	7.2 ± 1.5^b

Values with superscript ^{a, b} on the same row are significantly different ($P < 0.05$).

Values with superscript ^{1,2} on the same column are significantly different ($P < 0.05$).

Table 3 shows the activity of GPx in donkeys administered with EEC and subjected to packing during the dry season. There was no significant ($P > 0.05$) difference between all the values obtained during the season. However, higher ($P > 0.05$) activities with values of 25.4 ± 2.4 U/L was recorded 1 hour en route and 39.9 ± 2.1 U/L was obtained 7 days after packing during the cold-dry season in the test group while during the hot-dry season, higher ($P > 0.05$) activities with values of 19.7 ± 1.4 U/L was recorded one-hour on route and 15.8 ± 1.7 U/L was obtained 3 days after packing in test group.

Table 4 shows the TAC of donkeys administered with EEC and subjected to packing during the dry season. The values of TAC obtained in all the packing episodes during the dry season were significantly ($P < 0.05$) higher in the test group. Higher ($P < 0.05$) values were obtained in test group during the hot-dry season, with a value of 3.4 ± 0.3 mmolTrolox/L when compared with the value of 2 ± 0.1 mmolTrolox/L obtained one-hour on route. Furthermore, during the cold-dry season, a significantly ($P < 0.05$) higher value of 1.9 ± 0.3 mmolTrolox/L was obtained in the test group when compared with the value of 1.6 ± 0.2 mmolTrolox/L obtained in control group.

Table 3. Activities of glutathione peroxidase in donkeys administered with epicatechin-(4 β -8)-catechin and subjected to packing during the dry season.

Packing episodes	Cold-dry season		Hot-dry season	
	Control (n = 5)	Test (n = 5)	Control (n = 5)	Test (n = 5)
Baseline (U/L)	21.1 ± 3.3	21.1 ± 3.3	17.6 ± 1.9	17.6 ± 1.9
Before (U/L)	13.2 ± 0.4	22.4 ± 2.8^1	18.4 ± 1.9^1	22.2 ± 1.4
One-hour on route (U/L)	20.2 ± 2.8	25.4 ± 2.4^2	12.4 ± 1.2^2	19.7 ± 1.4^1
10 minutes after (U/L)	16.7 ± 1.9	27.3 ± 2.9	23.9 ± 1.0	23.2 ± 2.8^2
3 days after (U/L)	13.5 ± 2.9	14.5 ± 2.4^1	10.3 ± 1.9^2	15.8 ± 1.7^3
7 days after (U/L)	20.9 ± 5.2	39.9 ± 2.1	17.2 ± 1.9^2	21.8 ± 1.8^3

Values with superscript ^{1,2,3} on each column are significantly different ($P < 0.05$).

Table 4. Total antioxidant capacity of donkeys administered with epicatechin-(4 β -8)-catechin and subjected to packing during the dry season.

Packing episodes	Cold-dry season		Hot-dry season	
	Control (n = 5)	Test (n = 5)	Control (n = 5)	Test (n = 5)
Baseline (mmolTrolox/L)	0.8 ± 0.2^a	0.8 ± 0.2^a	2.3 ± 0.4^b	2.3 ± 0.4^b
Before (mmolTrolox/L)	0.6 ± 0.1^a	1.2 ± 0.2^a	2.4 ± 0.7^b	4.1 ± 1.2^b
One-hour on route (mmolTrolox/L)	1.5 ± 0.2	1.5 ± 0.3	2.0 ± 0.1	3.4 ± 0.3^a
10 minutes after (mmolTrolox/L)	1.6 ± 0.2	1.9 ± 0.3^b	3.3 ± 0.2^c	3.1 ± 0.3^c
3 days after (mmolTrolox/L)	1.2 ± 0.5^a	1.4 ± 0.3^a	3.2 ± 0.2	3.7 ± 0.7
7 days after (mmolTrolox/L)	1.0 ± 0.2^a	0.5 ± 0.1^a	3.1 ± 0.8^b	2.9 ± 0.3^b

Values with superscript ^{a,b,c} on the same row are significantly different ($P < 0.05$).

Table 5 shows the concentration of TNF- α of donkeys administered with EEC and subjected to packing during the dry season. The concentration of TNF- α was significantly ($P < 0.05$) higher during the cold-dry season when compared with the hot-dry season. In the control group, during the cold-dry season, TNF- α concentration was higher ($P < 0.05$) especially during packing (35.6 ± 3.3 ng/mL) when compared to the value of 29.6 ± 2.4 ng/mL obtained in test group. Furthermore, during the hot-dry season, the control group recorded a higher concentration of TNF- α with value of 15.3 ± 1.4 ng/mL, when compared with that of 7.4 ± 0.6 ng/mL recorded in test group. However, the increases were higher but not significant ($P > 0.05$).

Table 6 shows the activity of CK in donkeys administered with EEC and subjected to packing during the dry season. On the overall, significantly ($P < 0.05$) lower CK activities were recorded during the cold-dry season when compared to the hot-dry season. During the hot-dry season, CK activity was higher ($P < 0.05$) in control group one-hour on route (95.9 ± 12.4 U/L), 10 minutes after packing (186.5 ± 12.9 U/L), 3 days after packing (60.3 ± 8.8 U/L) and 7 days after packing (205.8 ± 8 U/L) when compared with test group with values of 71.1 ± 5.3 U/L recorded one-hour on route, 140.2 ± 19.8 U/L obtained 10 minutes after packing, 49.2 ± 20.3 U/L obtained 3 days after packing and 71.9 ± 15.7 U/L obtained 7 days after packing. In all the packing period during the cold-dry season, the values of CK recorded in control group were not significantly ($P > 0.05$) different from those obtained in test group.

Tables 7–10 show the relationship between thermal environmental parameters, biomarkers of fatigue and oxidative-stress parameters. The relationship between AT and TAC in the control donkeys (**Table 7**) was negative and significant ($r = -0.3609$, $P < 0.01$) during the cold-dry season. During the hot-dry season (**Table 8****Table 9**), in the control donkeys, the relationship between AT and GPx was positive and significant ($r = 0.4796$, $P < 0.01$) while the relationship between RH and GPx was negative and significant ($r = -0.4777$, $P < 0.01$).

Table 5. Concentration of tumor necrosis factor-alpha of donkeys administered with epicatechin-(4 β -8)-catechin and subjected to packing during the dry season.

Packing episodes	Cold-dry season		Hot-dry season	
	Control (n = 5)	Test (n = 5)	Control (n = 5)	Test (n = 5)
Baseline (ng/mL)	37.9 ± 1.9^a	37.9 ± 1.9^a	6.9 ± 0.5^b	6.9 ± 0.5^b
Before (ng/mL)	$32.3 \pm 3.6^{a,1}$	27.3 ± 1.1^a	9.7 ± 2.6^b	$9.5 \pm 1.1^{b,1}$
One-hour on route (ng/mL)	35.6 ± 3.3^a	$29.6 \pm 2.4^{a,1}$	15.3 ± 1.4^b	$7.4 \pm 0.6^{b,1}$
10 minutes after (ng/mL)	$29.6 \pm 3.6^{a,2}$	$31.6 \pm 2.9^{a,2}$	5.6 ± 0.6^b	8.9 ± 1.7^b
3 days after (ng/mL)	$26.9 \pm 5.6^{a,3}$	$25.5 \pm 2.4^{a,2}$	6.0 ± 0.5^b	$5.1 \pm 0.8^{b,2}$
7 days after (ng/mL)	$25.8 \pm 2.6^{a,3}$	$24.8 \pm 1.9^{a,2}$	7.7 ± 0.1^b	6.2 ± 0.8^b

Values with superscript ^{a,b} on the same row are significantly different ($P < 0.05$).

Values with superscript ^{1,2,3} on the same column are significantly different ($P < 0.05$).

Table 6. Activities of creatine kinase in donkeys administered with epicatechin-(4 β -8)-catechin and subjected to packing during the dry season.

Packing episodes	Cold-dry season		Hot-dry season	
	Control (n = 5)	Test (n = 5)	Control (n = 5)	Test (n = 5)
Baseline (U/L)	8.6 ± 1.1^a	8.6 ± 1.1^a	41.5 ± 3.2^b	41.5 ± 3.2^b
Before (U/L)	9.3 ± 0.6^a	$8.6 \pm 1.4^{a,1}$	49.9 ± 2.8^b	$47.6 \pm 6.2^{b,1}$
One-hour on route (U/L)	$10.6 \pm 1.1^{a,1}$	12.9 ± 1.7^a	95.9 ± 12.4^b	71.1 ± 5.3^c
10 minutes after (U/L)	$11.9 \pm 1.6^{a,2}$	9.7 ± 0.9^a	186.5 ± 12.9^b	$140.2 \pm 19.8^{c,2}$
3 days after (U/L)	8.4 ± 1.1^a	$6.1 \pm 0.9^{a,2}$	60.3 ± 8.8^b	$49.2 \pm 20.3^{c,3}$
7 days after (U/L)	6.6 ± 0.6^a	$6.3 \pm 0.2^{a,2}$	205.8 ± 22.1^b	$71.9 \pm 15.7^{c,3}$

Values with superscript ^{a,b,c} on the same row are significantly different ($P < 0.05$).

Values with superscript ^{1,2,3} on the same column are significantly different ($P < 0.05$).

Table 7. Correlation coefficient between environmental parameters, biomarkers of fatigue and oxidative stress parameters in control donkeys during the cold-dry season.

	Ambient temperature	Relative humidity	Temperature humidity index
Tumor necrosis factor-alpha	0.2549	0.2967	0.0998
Creatine kinase	0.3038	-0.1820	-0.1514
Total antioxidant capacity	-0.3609*	-0.2800	-0.0279
Glutathione peroxidase	0.0864	-0.0281	-0.0229
Catalase	0.2316	-0.2623	-0.1944
Superoxide dismutase	0.2598	0.0459	-0.0759

*P < 0.01.

Table 8. Correlation coefficient between environmental parameters, biomarkers of fatigue and oxidative stress parameters in test donkeys during the cold-dry season.

	Ambient temperature	Relative humidity	Temperature humidity index
Tumor necrosis factor-alpha	0.2804	-0.0845	-0.3032
Creatine kinase	-0.0302	-0.0189	-0.2327
Total antioxidant capacity	-0.4848	-0.1051	-0.4017
Glutathione peroxidase	-0.1249	0.4062	-0.2393
Catalase	0.3349	-0.0630	0.0242
Superoxide dismutase	0.1636	-0.1998	-0.1420

Table 9. Correlation coefficient between environmental parameters, biomarkers of fatigue and oxidative stress parameters in control donkeys during the hot-dry season.

	Ambient temperature	Relative humidity	Temperature humidity index
Tumor necrosis factor-alpha	0.2144	-0.1946	-0.1681
Creatine kinase	0.1749	-0.0494	0.0524
Total antioxidant capacity	0.0674	-0.0889	-0.0608
Glutathione peroxidase	0.4796*	-0.4777*	-0.4501
Catalase	0.2535	-0.2729	-0.2710
Superoxide dismutase	0.0791	-0.0713	-0.0611

*P < 0.01.

Table 10. Correlation coefficient between environmental parameters, biomarkers of fatigue and oxidative stress parameters in test donkeys during the hot-dry season.

	Ambient temperature	Relative humidity	Temperature humidity index
Tumor necrosis factor-alpha	0.2502	-0.2873	-0.3019
Creatine kinase	-0.2309	0.2610	0.2739
Total antioxidant capacity	0.1662	-0.2300	-0.2797
Glutathione peroxidase	-0.0558	0.0095	-0.0412
Catalase	0.3643	-0.2932	-0.2209
Superoxide dismutase	-0.2070	0.1499	0.0794

Discussion

The increased activity of SOD observed in the test group denotes that EEC played a role in reducing oxidative stress. The result agrees with the findings of Lee et al. (2017) that EEC has the ability to improve the activity of enzymatic antioxidants by stimulating their synthesis of antioxidants (Fan et al., 2015). The increased activity of SOD observed during the cold-dry season shows that the donkeys were exposed to higher level of oxidative stress during the cold-dry season than during the hot-dry season. Therefore, the cold-dry season may have been more stressful to the donkeys than the hot-dry season.

The decrease in CAT activity observed in the test group especially during the cold-dry season did not agree with the findings of Rohdewald (2002) that EEC enhances catalase activity dose-

dependently. Therefore, it may be that the dose of EEC used in this study during the cold-dry season was not optimum enough to improve CAT activity or that the CAT as an enzyme was used up, since its activity recovered 7 days after packing. The increase in CAT activity in control group, especially during the cold-dry season, denotes that the donkeys were exposed to oxidative stress.

The insignificant increase in GPx in test group agrees with the report of Lee et al. (2017) that EEC enhances the activities of antioxidant enzymes. It also gives credence to the fact that increased GPx activity improves the stability of RBC thereby reducing hemolysis (Remigante, Morabito, & Marino, 2019). Gpx does this by converting hydrogen peroxide, a potent reactive oxygen species (ROS) into harmless water and oxygen (Ighodaro & Akinloye, 2018). The increased activity during the cold-dry season denotes that donkeys were more exposed to oxidative stress during the cold-dry season. Intake of selenium has also been reported to improve the activity of GPx (Nogales, Ojeda, Fenutria, Murillo, & Carreras, 2013), however in this study selenium was a deficient mineral in the feed.

The SOD is the major cytoplasmic antioxidant enzyme that catalyzes dismutation of superoxide radical to molecular oxygen and hydrogen peroxide which is in turn converted by GPx or CAT into water and oxygen, thus providing a combined enzymatic action against oxygen toxicity (Ighodaro & Akinloye, 2018). If there is an imbalance in these enzymes, this results in the accumulation of hydrogen peroxide which may participate in Fenton reaction and form formation of noxious hydroxyl radical (Rodriguez-Sureda, Vilches, Sanchez, Audi, & Dominguez, 2015). ErbenBayeta, Benjamin, and Lau (2000) reported that the activity of CAT and SOD increased with administration of EEC and this report supported the above findings. Lee, Moon, Bae, Park, and Jang (2013) also reported that EEC enhances antioxidant enzyme activity. The EEC has been shown to double the level of antioxidant enzymes in endothelial cells (Simpson, Kure, & Stough, 2019).

The TAC is the summation of the action of antioxidants within the body system against ROS (Rubio, Hernandez-Ruiz, Martinez-Subiela, Tvarijonavicueta, & Ceron, 2016). The higher value of TAC recorded in the test group agreed with the findings of Cao, Russel, Lischner & Prior (Cao, Russel, Lischner, & Prior, 1998), who reported an increase in TAC following consumption of rich fruits and vegetables. In the present study, the result obtained indicates that the body's antioxidant status may counteract oxidative damage due to increased generation of ROS more in the test group than in the control group (Suresh, Annam, Pratibha, & Prasad, 2009). EEC has been reported to be not only a free radical scavenger, but is also capable of prolonging the activity and life-time of ascorbic acid which in turn regenerates tocopherol, thereby improving antioxidative status (Cossins, Lee, & Packer, 1998).

The increase in TNF- α level in control donkeys during the dry season shows clearly that inflammation occurred in the control group. The result agrees with the findings of Salamat, Azarbayjani, Yusof & Dehghan (2016) that endurance exercise has a positive effect on TNF- α , a pro-inflammatory cytokine. The lower values obtained in the test group during the hot- and cold-dry seasons indicate that EEC may have played a role in ameliorating inflammation since it has been known to play a major role in prevention of inflammation (Malekahmadi et al., 2020) and TNF- α has been known to stimulate leucocytes and vascular endothelial cells to release other cytokines, express cell surface adhesion molecules and increase arachidonic acid turnover (Taner et al., 2014). In addition, the higher levels of TNF- α concentration obtained in control group points to the fact that there was an on-going inflammation, but in the tested group the administration of EEC, a good anti-inflammatory agent (Fan et al., 2015), may play a positive role by preventing inflammatory processes through inhibition of nuclear factor-kappa B and activator protein-1 pathway which controls transcription of cytokine production (Grimm et al., 2006). The result agrees with the finding of Tumen, Akkol, Tastan, Suntar & Kurtca (2018), who reported that EEC is a very important anti-inflammatory agent. It is also pertinent to note that packing donkeys may lead to inflammation of the endothelium of vascular tissue supplying the contractile muscle tissue; however, it is unknown if it leads to muscle damage.

In this study, muscle damage was evaluated by measuring the activity of CK. The significantly lower activity of CK in the test group when compared with the control group during the seasons

denotes that all donkeys were exposed to muscle damage, but was observed more in the control group. The activity of CK is the best marker for diagnosing overtraining (Cheng et al., 2020; Koch et al., 2014) and it is also a good biomarker for detecting muscle damage (AbuMoh'd, Shamrokh, Bataineh, & Al-Horani, 2020; Brancaccio, Lippi, & Maffuli, 2010). In this study, muscle damage was observed to be significantly higher in the control group than in the test group during the packing episodes, especially during the hot-dry season. It may be postulated that exhaustive exercise leads to inflammation which is followed by muscle damage. The damage may occur for some days after the donkeys have been subjected to packing; however, EEC reduced muscle damage in the test group. The mechanism by which EEC exerted its action was not elucidated in the study. However, EEC has been reported to increase nitric oxide production, which leads to increased release of acetylcholine by nerves and vasorelaxation (Yagi et al., 2017). Thus, EEC improves blood flow in arteries of working muscles and provides more oxygen to working muscles, which may have reduced muscle damage (Aldret & Bellar, 2019; Gulati, 2014).

The positive relationship between AT and TAC in the control donkeys during the cold-dry season denotes that TAC increases with increase in AT. This agrees with the findings of Lopez-Vidana, Figueroa, Cortes, Rogano & Ocana (2016). The negative relationship between AT and GPx in the control donkeys during the hot-dry season denotes that the activities of GPx increased with decrease in AT (Miao et al., 2020).

The concept of welfare cannot be achieved under stress (Viessier & Boissy, 2007). The changes in the oxidative stress parameters and biomarkers of fatigue in this study generally reveals that the donkeys were exposed to stress which is an unspecific response to noxious agents in which hypothalamo-pituitary axis plays a central role (Selye, 1936). The donkeys were in a state of continuum between poor and good while trying to adapt (Broom, 1991). Packing the donkeys with load while trekking over long distances and exposure to thermal stress may have combined to trigger stress responses (Mason, 1971). In conclusion, the donkeys were exposed to oxidative stress, inflammation and muscle damage after packing and loading during the dry season, ameliorated by EEC administration. The EEC is a potential anti-fatigue agent, which may be administered to working donkeys to improve their welfare status.

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