THE EFFECT OF DIETARY POLYPHENOLICS ON PEROXIDE INDUCED STRESS IN *LUMBRICUS TERRESTRIS*

THESIS

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by

Trey Steven Hutton, B.S.

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CHAPTER 1

THE EFFECT OF DIETARY POLYPHENOLICS ON PEROXIDE INDUCED STRESS IN *LUMBRICUS TERRESTRIS*

As part of their normal metabolic processes, cells continually produce free radicals (Aguilo et al., 2005). Generally, after free radicals are formed, they are neutralized by antioxidants so that the oxidative state of the cell is in physiological homeostasis (Powers, DeRuisseau, Quindry, & Hamilton, 2004). Under certain conditions, however, free radical production increases to the point that it exceeds the detoxification capacity of cellular antioxidants (Konig, Wagner, Elmadfa, & Berg, 2001). Broadly defined as oxidative stress, the disruption in this delicately balanced homeostasis may result in damage to lipids, proteins, and/or DNA, thereby inhibiting the normal function of these cellular components (Vollaard, Shearman, & Cooper, 2005). The oxidative stress that occurs during exercise is referred to as exercise-induced oxidative stress (EIOS). EIOS occurs during exercise of high intensity (Marzatico, Pansarasa, Bertorelli, Somenzini, & Della Valle, 1997; Sastre et al., 1992; Thompson et al., 2001) and/or long duration (Kanter, 1998; Mastaloudis, Leonard, & Traber, 2001; Miyazaki et al., 2001; Nieman et al., 2002), and may result in damage to the exercising muscles (Konig et al., 2001). Because muscle damage may directly affect exercise performance,

researchers have explored the use of antioxidant supplements by athletes in attenuating the negative effects of EIOS (Davies, Quintanilha, Brooks, & Packer,1982; Konig et al. 2001; Miyazaki et al., 2001; Morillas-Ruiz et al., 2005; Powers et al., 2004; Zhang et al., 2004). Researchers have postulated that dietary intake of antioxidants by athletes would raise antioxidant cellular levels so that the antioxidants would counteract the rise in free radical production during exercise (Bailey, Lander, & Darley-Usmar, 2005; Gomez-Cabrera, Borras, & Pallardo, 2005; McAnulty et al., 2005; Vassilakopoulos, Roussos, & Zakynthinos, 2005). The antioxidants most commonly investigated in athletes have been vitamins E and C (Alessio, Goldfarb, & Cao, 1997; Buchman et al., 1999; Goldfarb, Patrick, Bryer, & You, 2005; Itoh, Ohkuwa, & Yamazaki, 2000; Simon-Schnass & Pabst, 1988; van der Beek et al., 1990). Vitamin E, a lipid-soluble antioxidant, prevents lipid peroxidation and thus protects polyunsaturated fatty acids in cell membranes (Buchman et al., 1999). However, research on the effects of vitamin E supplementation on EIOS during exercise has produced discordant results (Buchman et al., 1999; Cannon et al., 1990; Helgheim, Hetland, Nilsson, Ingjer, & Stromme, 1979; Itoh et al., 2000; Nieman et al., 2004; Simon-Schnass & Pabst, 1988; Sumida, Tanaka, Kitao, & Nakadomo, 1989; Surmen-Gur, Ozturk, Gur, Punduk, & Tuncel, 1999). For example, Helgheim et al. (1979) reported that muscle damage following heavy exercise was not decreased in subjects who ingested 300mg/day of vitamin E for 6 weeks. In contrast, after a comparable bout of exercise, Sumida et al. (1989) found that supplementing athletes with 300mg/day of vitamin E for 4 weeks did reduce muscle damage.

Studies involving supplementing with vitamin C have produced equally conflicting results. *In vitro*, vitamin C has been shown to neutralize free radicals through the donation of a hydrogen ion (Connolly, Lauzon, Agnew, Dunn, & Reed, 2006). However, research has not been able to verify whether vitamin C supplementation has a significant effect on EIOS and/or muscle damage in the exercising muscle (Alessio et al., 1997; Goldfarb et al., 2005; Khassaf et al., 2003; van der Beek et al., 1990). The discordance in the literature may be due to the different dosages used in these studies. For instance, Khassaf et al. (2003) showed that a low dose of vitamin C (i.e., 0.5 g/day) for 8 weeks increased antioxidant enzyme response and attenuated EIOS following 45 minutes of cycling at 70% of VO_{2peak}, compared to a placebo. However, when a higher dosage of vitamin C was ingested regularly (i.e., 2 g/day) for 3 weeks, no significant reduction in lipid peroxidation was observed following a 10.5 km run (van der Beek et al., 1990).

The discordance in previous research regarding the effects of supplementation with the antioxidants, vitamins E and C, on EIOS in athletes may be attributed to the differences in experimental design, since the studies employed different vitamin dosages, different timing of supplementation, and different levels of training. However, it is also entirely possible that supplementation with these vitamins provided only limited protection against free radical damage (Peake, Suzuki, & Coobes, 2007; Urso & Clarkson, 2003). Moreover, it has been suggested that these antioxidants, when used alone or at high doses, may potentially exacerbate the oxidative stress response following exercise (Gleeson, Nieman, & Pedersen, 2004; Goldfarb, 1993), thereby producing an effect opposite of that intended. Thus, at present, given the discordant literature and the

potential for unintended harm, most researchers do not recommend supplementing with vitamin E or vitamin C to attenuate EIOS and consequent damage to muscle tissue.

Polyphenolics, compounds that attenuate oxidative stress and alleviate some of the detrimental effects associated with free radical production in muscles, have potential to serve as a novel source of antioxidant supplement for athletes (Pilaczynska-Szczesniak, Skarpanska-Steinborn, Deskur, Basta, & Horoszkiewicz-Hassan, 2005). Polyphenolics are products of the secondary metabolism of plants and exhibit a wide range of antioxidant properties (Urquiaga & Leighton, 2000). The hydrogen donating substituents attached to the aromatic rings of the polyphenolic compounds allow polyphenolics to effectively scavenge free radicals (Pari & Suresh, 2008). While a few studies have shown actual *in vivo* functionality of polyphenolics (Dunlap, Reynolds, & Duffy, 2006; Nakazato, Song, & Waga, 2007; O'Byrne, Devaraj, Grundy, & Jialal, 2002; Pilaczynska-Szczesniak et al., 2005), it is important to characterize the antioxidant capacities of specific polyphenolic containing foods *in vitro* before their *in vivo* function can be fully understood.

The first purpose of this study, therefore, was to characterize the antioxidant capacity of the effects of polyphenolic-rich extracts of commonly consumed fruits, herbs, and spices (i.e., basil, blackberry, blueberry, clove, cranberry, ginger, grape, grapefruit, mace, oregano, raspberry, rosemary, thyme, and turmeric) and to examine their effects on the quenching of free radicals and on reduction of lipid peroxidation, *in vitro*. The second purpose of this study was to test whether, and to what extent, the polyphenolic-rich extracts of these fruits, herbs, and spices decreased the generation of free radicals,

increased the antioxidant enzyme responses, and decreased cellular damage in an *in vivo* model, *Lumbricus terrestris* (i.e. earthworm), after exposure to a cellular pro-oxidant, hydrogen peroxide. The earthworm model was chosen as an *in vivo* model for assessing the effects of polyphenolics on muscle tissue because: (1) the body composition of earthworms is primarily muscle; (2) the immune function of the earthworm is sufficiently analogous to that of vertebrates; (3) earthworms are inexpensive and easy to maintain in the laboratory; and (4) earthworms can be quickly exposed to a test substance and rapidly harvested for analysis (Cooper, Kauschke, & Cossarizza, 2002; Goven, Fitzpatrick, & Venables, 1994). Based on previous research regarding polyphenolic supplementation (O'Byrne et al., 2008; Pilaczynska-Szczesniak et al., 2005; Wiswedel et al., 2004), we hypothesized that the most active polyphenolic-rich extracts of fruits, herbs, and spices *in vitro* would also be most active in the *in vivo* model, as determined by the reduced production of free radicals, the increased activities of antioxidant enzymes, and by the reduction of lipid peroxidation, all of which would protect the earthworm muscles from damage.

Methods

In Vitro Methods

Water Extraction One gram of the sample was suspended in 20ml of water and mixed thoroughly for 30 minutes. The sample was then vortexed for 10 minutes at 4,000 rpm and supernatant was removed for testing.

2, 2'-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS) Assay. Briefly, to 1 ml of 7 mM ABTS (in water, activated overnight with 140 mM potassium persulfate) was added 50 µl of extract and the mixture was incubated for 2.5 minutes at room temperature. The absorbance was measured at 734 nm and compared with control containing ethanol in place of the extract. The percentage inhibition in ABTS radical due to the extract was calculated by: [(AB – AS / AB) x 100].

Thiobarburic Acid Reactive Substances (TBARS) Assay· Briefly, an emulsion containing 250 µl linoleic acid (fish oil) and 250 µl tween in 25 ml deionized water was sonicated for 3 minutes. 0.8 ml of emulsion was added to 0.2 ml of extract to which 500 µl of 20% (w/v) trichloroacetic acid and 1 ml of 10 mM thiobarbutyric acid were added. Contents were vortexed and incubated for 45 minutes at 100°C. After incubation, tubes were centrifuged at 13,000 g for 10 minutes and the absorbance of the supernatant was measured at 532 nm. The concentration of malondialdehyde (MDA) was calculated from its molar extinction coefficient 156 μmol^{-1} cm^{-1} and expressed as $\mu mol\ g^{-1}$ FW. The percent inhibition was then calculated.

In Vivo Methods

Overview· Twenty eight mature and healthy earthworms were selected for this study. Earthworms were purchased from Wal-Mart and were selected based on presence of a clitellum, which denotes sexually maturation of the earthworm. The earthworms were randomly allocated to 4 groups comprised of a *C*, *P*, *T*, and *TP*. Earthworms in all groups were supported in an agar-based medium (Fisher Scientific) (15 ml), which

contained a oatmeal solution (Gerber Baby Oatmeal Single Grain). The Gerber oatmeal was purchased from Wal-Mart and added at an amount which optimized the earthworm's health. Gerber oatmeal was added at 1.25% to the agar medium, which nutritionally supported the earthworms during the testing process. The control medium contained no additional components. The *P* group was supported using the control medium with added hydrogen peroxide (H_2O_2) (0.13% per ml), to create a sub-lethal amount of oxidative stress (Goven et al., 1994). The *T* agar contained a polyphenolic-enhanced extract (10% W/W). The earthworms in the *TP* group were grown in medium including both H_2O_2 (0.13% per ml) and a polyphenolic extract (10% W/W). All the treatments (H_2O_2, Extract, Extract + H_2O_2) were added directly to the agar medium. Firstly, the earthworm were exposed to the extracts individually and tested, as described below. Secondly, an extract combination formulation were derived from the top 8 individually tested extracts to create four extract groups, which each contained a combination of four individual extracts. The extracts were divided into the following four groups: Group 1 included grapefruit, ginger, turmeric, and mace; Group 2 included grapefruit, ginger, blackberry, and oregano; Group 3 included raspberry, basil, turmeric, and mace; Group 4 included raspberry, basil, blackberry, and oregano.

Earthworm Culture and Maintenance

Testing. The mature earthworms were removed from soil 24 hours before the testing process begun. The worms were thoroughly washed to ensure their skin was free

from any soil or debris. The worms were then placed on an agar plate containing only the Gerber medium for 24 hours. The solution was designed to clear the earthworm's digestive system of any soil which reduced the soil interference during the chemical and enzymatic analysis. The earthworms were then divided into 4 groups of 7 and placed on plates containing one of the four treatments combinations.

Extraction. The earthworms were maintained in a dark-room on a Gerber based agar fed for 48 hours at room temperature before they were externally cleaned/washed. The worms were frozen at -20°C for 30 minutes and a muscle sample was then taken distal to the clitellum. A muscle sample, approximately one inch, was taken from two different earthworms every 48 hours for each of the 4 treatment groups. Each muscle sample was homogenized by a mortar and pestle and 5ml of 0.1 M Sodium Phosphate Buffer was added to create the supernatant. The mixture was then centrifuged at 4000 rpm for 10 minutes and the supernatant was collected for testing. Each sample was kept in an ice-bath to prevent loss of enzyme activity. This procedure was repeated every 48 hours for a 6-day time period. The supernatant was tested for lipid oxidation, antioxidant capacity, enzyme activity, polyphenolic bioavailability and skeletal muscle damage.

Chemical Analysis

Total Polyphenolics Assay The increase in polyphenolic content of the muscle was measured to determine the overall absorption and bioavailability. Total polyphenolics were measured following the protocol developed by Chandler and Dodds (1983) and modified by Vattem, Ghaedian, and Shetty (2005). Polyphenolics was

measured as gallic acid equivalents using the Folin Ciocalteu method. 0.25ml of the tissue homogenate was transferred to a test tube and 0.25 ml of 95% ethanol, 2.5 ml of distilled water (DW) and 0.125 ml of 50% (v/v) Folin– Ciocalteu phenol reagent (Sigma Chemical Co., St. Louis, MO) were added and vortexed. After an incubation period of 5 minutes, 0.25 ml of 5% Na_2CO_3 was added, again vortexed and the solution were kept in the dark for 1 hour. The absorbance of the samples was measured at 725 nm using a UV spectrophotometer (Thermo Biomate 3; Houston, TX).

ABTS Antioxidant Assay. The antioxidant activity of the samples were determined by the ABTS cation radical and antioxidant activity assay (Pari & Suresh, 2008). To 1 ml of ABTS, 50 µl of the tissue homogenate was added, vortexed and incubated at room temperature for 2.5 min. The absorbance was recorded at 734 nm. The radical scavenging activity of the sample was compared with a blank which contained DW in place of the sample. The percent inhibition was measured using the following formula: [(AB – AS / AB) x 100].

Malondialdehyde (MDA) Assay. Plasma levels of lipid peroxidation were measured by the formation of MDA which is a metabolite of lipid hydroperoxides (Vattem et al., 2005). MDA is a secondary oxidation product of lipids and serves as an acceptable marker for lipid oxidation and cell membrane injury. The MDA concentration in plasma was determined by its reaction with thiobarbituric acid (TBA). MDA was measured by modifying the method described by Vattem et al. (2005). In a test tube 0.8 ml of the tissue homogenate was mixed with 500 µl of 20% (w/v) trichloroacetic acid and 1 ml of 10 mM thiobarbutyric acid. The test tubes was incubated for 30 min at 100 °C

and then centrifuged at 13,000 rpm for 10 minutes. The absorbance of the supernatant was measured at 532 nm and the concentration of MDA was calculated from its molar extinction coefficient 156 μmol^{-1} cm^{-1} and expressed as μmol g^{-1} FW. The percent inhibition was then calculated.

Total Protein Assay. The protein content of the samples was measured by the Bradford method (1976). Bradford dye reagent (Bio-Rad protein assay kit II, Bio-Rad Laboratory, Hercules, CA) was prepared by diluting the commercial dye concentrate in a 1:4 ratio with distilled water. One milliliter of the dye was added to 20 µl of the tissue homogenate and blank (DW) in test tubes and incubated at room temperature for 5 minutes. The absorbance was measured at 595 nm against a 1ml reagent blank and 20 µl buffer.

Enzymatic Analysis

Superoxide Dismutase (SOD)-Riboflavin-NBT Assay SOD destroys the free radical superoxide by converting it to peroxide that can in turn be removed by CAT (Mates, 2000). SOD converts the highly reactive superoxide radical to the less reactive H_2O_2 (Mates, 2000). SOD activity was determined as described by Martinezet al. (2001) and Kumaran & Joel karunakaran (2006). The enzyme reaction mixture containing 50 mM KH_2PO_4 buffer (pH 8.0), 0.1 mM EDTA, 200 µM DTPA c and 57 µM NBT was prepared. The riboflavin [2 mM riboflavin (670 mg riboflavin and 10ml of buffer)] was added to the above solution to create the reaction mixture. In a clear test tube 200 µl of the tissue homogenate was mixed with 1.5 ml of the reaction mixture. It was then

incubated by exposing to fluorescent lamps in a dark room for 20 minutes. The reaction mixture with buffer was used as contrast. The absorbance was measured at 560 nm and the percent inhibition was determined by NBT oxidation [(Ac – AS / AC) x 100].

Catalase (CAT) Assay. Catalase protects cells from hydrogen peroxide generated within them. Even though CAT and SOD are not essential for some cells type under normal conditions, they play an important role in the acquisition of tolerance to oxidative stress in the adaptive response of cells. A method originally described by Beers and Sizer (1952) was used to assay the activity of CAT. A solution containing 1.9 ml of distilled water, 1 ml of 0.059 M H_2O_2 in 0.05 M potassium phosphate, pH 7.0 was prepared. The tissue homogenate was diluted to 1/10th of the original solution and 0.1 ml of the diluted tissue homogenate mixture was used. The disappearance of peroxide was followed spectrophotometrically by recording the decrease in absorbance at 240 nm from 0 to 3 minutes. The change in absorbance ΔA240/minutes from the initial (0 s) linear portion of the curve was calculated. One unit of catalase activity was defined as the amount that decomposes one micromole of H_2O.

Results

Single Extract in Vitro Data

Polyphenolic Content The polyphenolic content of the water extract was measured using the Folin–Ciocalteu assay. Our results indicate that Rosemary (RO) had the most polyphenolic content. This was followed polyphenolic content by Mace (MA),

Oregano (OR), Turmeric (TU), and Thyme (TH) extracts. Grape (GP), Blueberry (BU), Basil (BA), Cranberry (CR), and Blackberry (BK) extracts contained the next highest amount of polyphenolics. The extracts Ginger (GI), Grapefruit (GPF), Raspberry (RA), and Clove (CL) contained the least amounts polyphenolics (Figure 1).

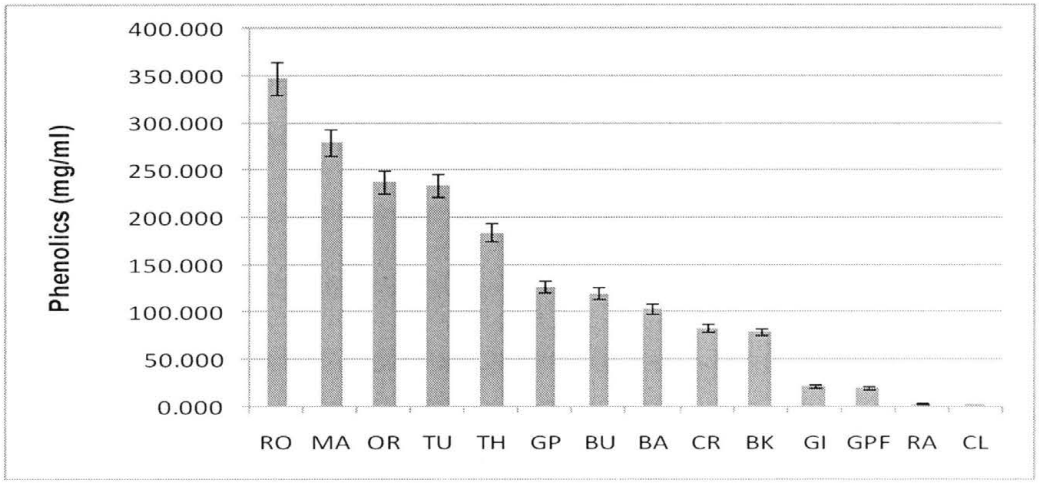

Figure 1. Phenolic bioavailability of single extract *in vitro.*

ABTS Radical Formation. We measured the effectiveness of water extracts on neutralizing the ABTS radical. A number of the extracts, which immediately decolorized the ABTS solution, were too powerful to be measured according to protocol. Thus, they were diluted 1/10th or 1/2 to allow for a more accurate calculation of their antioxidant activity. Our results indicate that CL had the most powerful ABTS neutralizing effect. Even at 1/10th dilution, CL neutralized 99% of the ABTS radical (Figure 2). This was followed by RO, OR, MA, TU, and RA, which were diluted to 1/10th and which reduced ABTS radicals by 60, 41.8, 41.5, 38, 17%, respectively (Figure 2). This was followed by CR, which at 1/2 dilution had 27% inhibition. Among the samples that did not have to be

diluted, TH, BU, GPF, GI, BA, BK, and GP, neutralized the ABTS radical formation by 98, 95, 92.4, 91.8, 90, 69, and 54%, respectively (Figure 2).

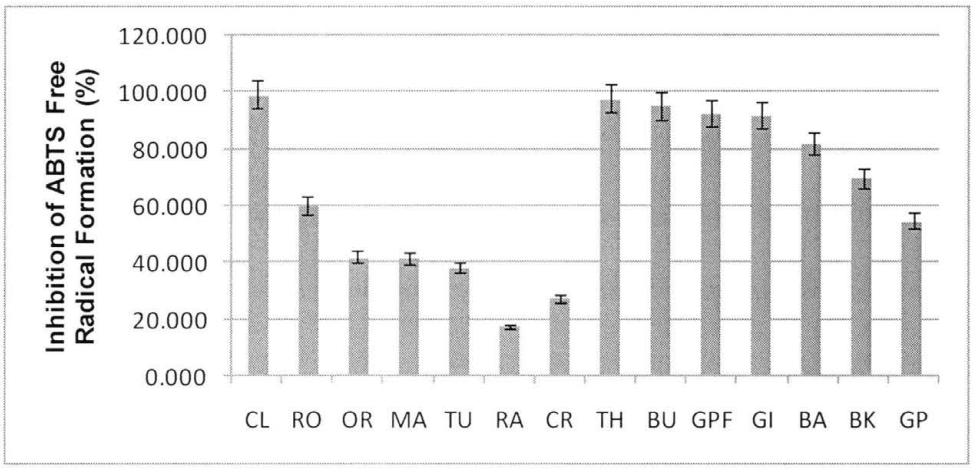

Figure 2. Effect of single extract on reducing ABTS free radicals *in*

TBARS Formation. The potential of the water extracts in reducing the formation of TBARS due to the oxidation of linolenic acid was assessed. Among the extracts, RO, TU, TH, BU, and MA reduced the formation of TBARS by 91, 86, 75, 67, and 62%, respectively (Figure 3). This was followed by BK, RA, BA, CR, and OR, which inhibited TBARS formation by 60, 58, 48, 43, and 37%, respectively (Figure 3). CL, GI, GP, and GPF all decreased TBARS formation by less than 20% (Figure 3).

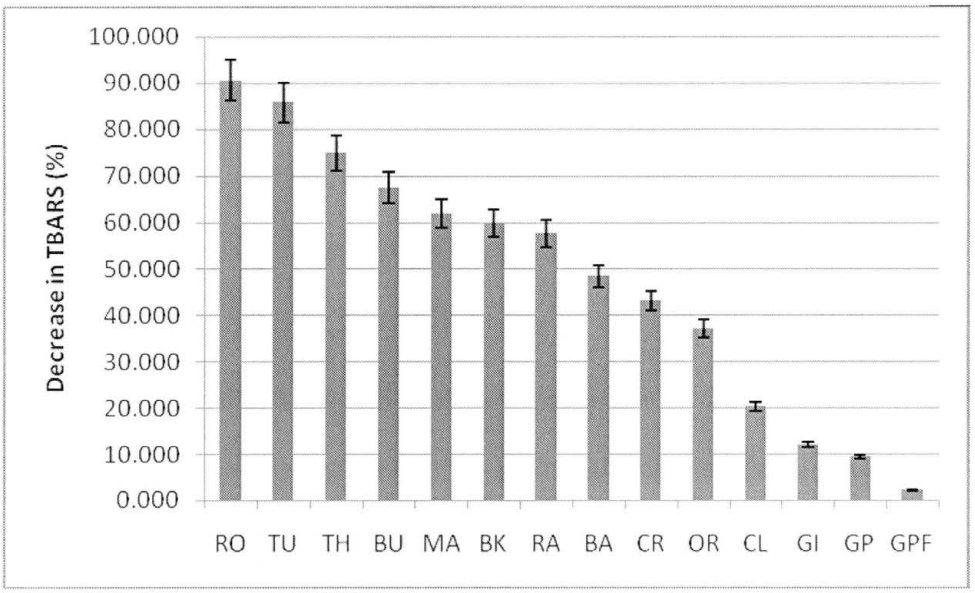

Figure 3. Effect of single extract on decreasing TBARS formation *in*

Single Extract in Vivo Data

Polyphenolic Content. After the ingesting the single extracts the amount of polyphenolics absorbed into the earthworms muscle tissue was assayed using the Folin–Ciocalteu assay. In the Control (*C*) and Peroxide (*P*) samples, the value of polyphenolics did not change over the course of the 6-day treatment. Our results indicate that compared to the *C*, the Treatment-Peroxide (*TP*) earthworms ingesting the TU extract had the highest total amount of bioavailable polyphenolics. This was followed in polyphenolic bioavailability by the *TP* earthworms ingesting the BU, MA, GI, and GP extracts. BK, RA, OR, TH, and GPF extracts contained the next highest amounts of bioavailable polyphenolics. The extracts BA, CR, RO, and CL were the least effective in increasing polyphenolic bioavailability (Figure 4).

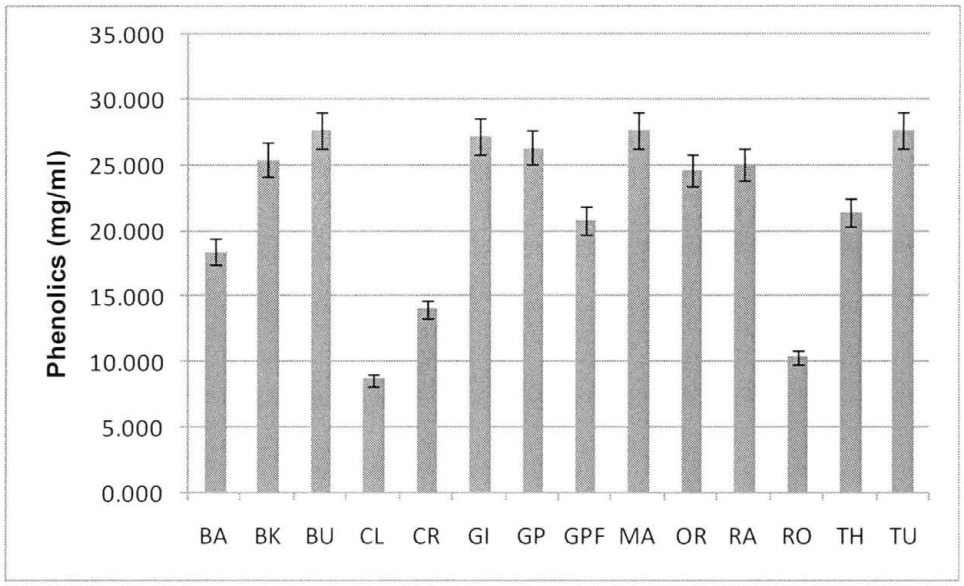

Figure 4. Phenolic bioavailability of single Treatment-Peroxide extract *in*

In the group fed Treatment (*T*) only, the earthworms which ingested the GP extract had the highest total amount of bioavailable polyphenolics. This was followed in polyphenolic bioavailability by the earthworms ingesting the BK, RA, MA, and BU extracts. TU, GI, GPF, TH, and OR extracts contained the next highest amount of bioavailable polyphenolics. The extracts RO, BA, CR, and CL had the lowest amounts of bioavailable polyphenolics (Figure 5).

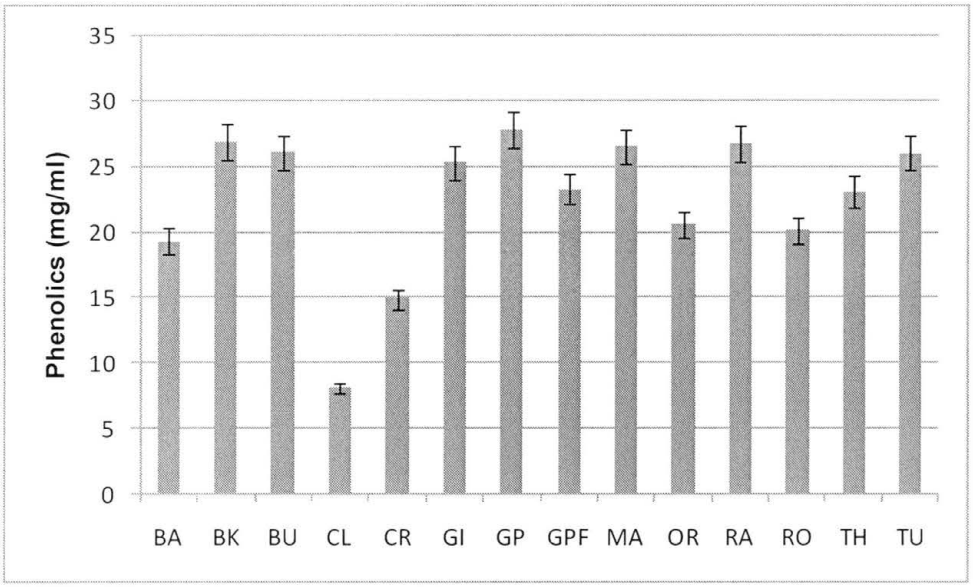

Figure 5. Phenolic bioavailability of single Treatment extract *in vivo.*

ABTS Radical Formation. We measured the effectiveness of single extracts on neutralizing the ABTS radicals. In the *C* sample, which did not have any polyphenolic treatment, and the *P* alone treated tissue sample, the inhibition of ABTS radical did not change over the course of the 6-day treatment. Our results indicate that compared to *C*, the *TP* earthworms ingesting the CL extract had the most powerful ABTS neutralizing effect. The CL extract inhibited 42% of the ABTS radicals (Figure 6). This was followed by the *TP* earthworms ingesting the RO, CR, OR, and BA extracts which reduced ABTS formation by 38, 37, 32, and 28%, respectively (Figure 6). TH, MA, BK, BU, and RA reduced ABTS radicals by 26, 25, 24, 22, and 20%, respectively (Figure 6). TU, GP, GI, and GPF all decreased ABTS by less than 20% (Figure 6).

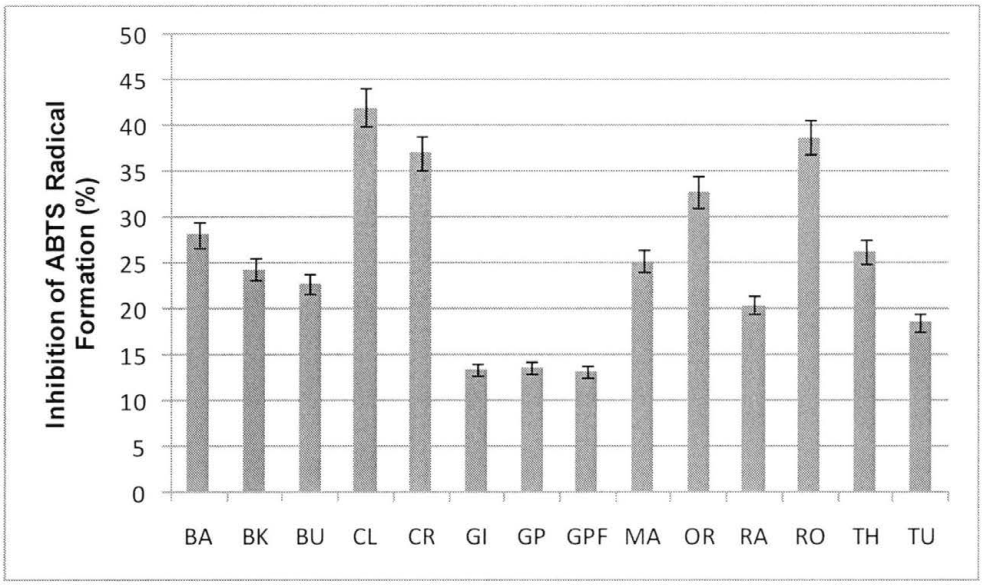

Figure 6. Effect of extract on reducing ABTS free radical in Treatment-Peroxide group *in vivo.*

In the *T* only group, the earthworms which ingested the CL extract had the most powerful ABTS neutralizing effect. The CL extract inhibited 40% of the ABTS radicals (Figure 7). This was followed in ABTS inhibition by the *T* earthworms ingesting the CR, OR, TH, and BA extracts which reduced ABTS formation by 36, 31, 26, and 25%, respectively (Figure 7). BK, BU, RA, MA, and RO reduced ABTS radicals by 24, 23, 22, 21, and 17%, respectively (Figure 7). TU, GPF, GP, and GI all decreased ABTS by less than 17% (Figure 7).

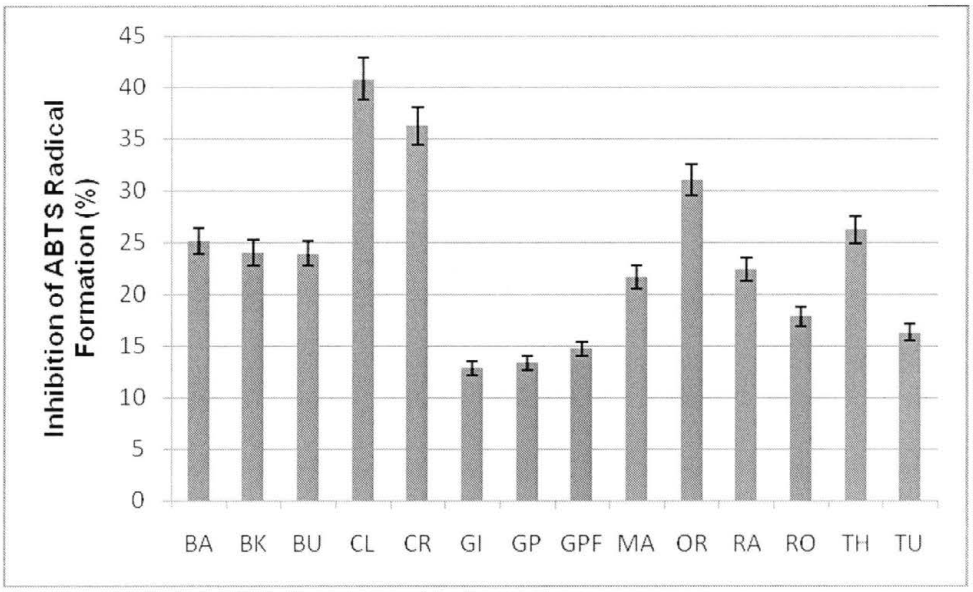

Figure 7. Effect of extract on reducing ABTS free radical in Treatment

Malonaldehyde (MDA) Content. The MDA content of the earthworm muscle sample was measured to study the extent of the membrane degradation as a result of H_2O_2 induced oxidative stress over 6-days of treatment. In the *P* earthworm sample, the amount of MDA formed was the highest. The earthworms stressed with H_2O_2 and contained a polyphenolic treatment (*TP* group) showed a different trend. Our results indicate that compared to the control, the *TP* earthworms ingesting the GP extracts had the lowest total amount of MDA. This was followed by the *TP* earthworms ingesting the GPF, GI, RA, and CL extracts. BK, CR, BU, RO, and TH extracts contained the next lowest amount of MDA. The extracts BA, OR, TU, and MA had the highest amounts of MDA (Figure 8).

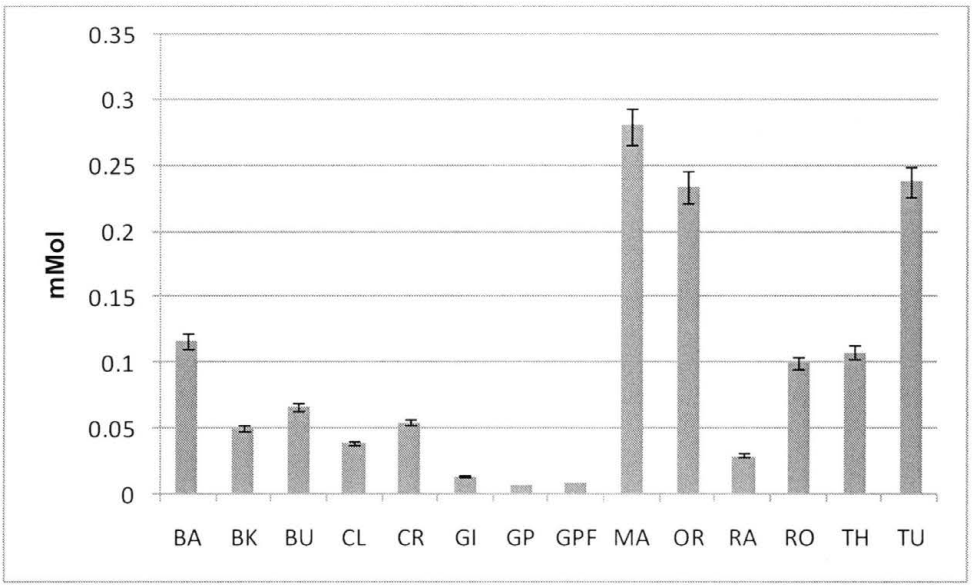

Figure 8. Effect of extracts on reducing formation of MDA in Treatment-Peroxide group *in vivo.*

In the group fed *T* only, the earthworms which ingested the GP extracts had the lowest total amount of MDA. This was followed by the *T* earthworms ingesting the GPF, GI, RA, and BK extracts. CL, CR, BU, BA, and TH extracts contained the next lowest amount of MDA. The extracts RO, MA, OR, and TU had the highest amounts of MDA (Figure 9).

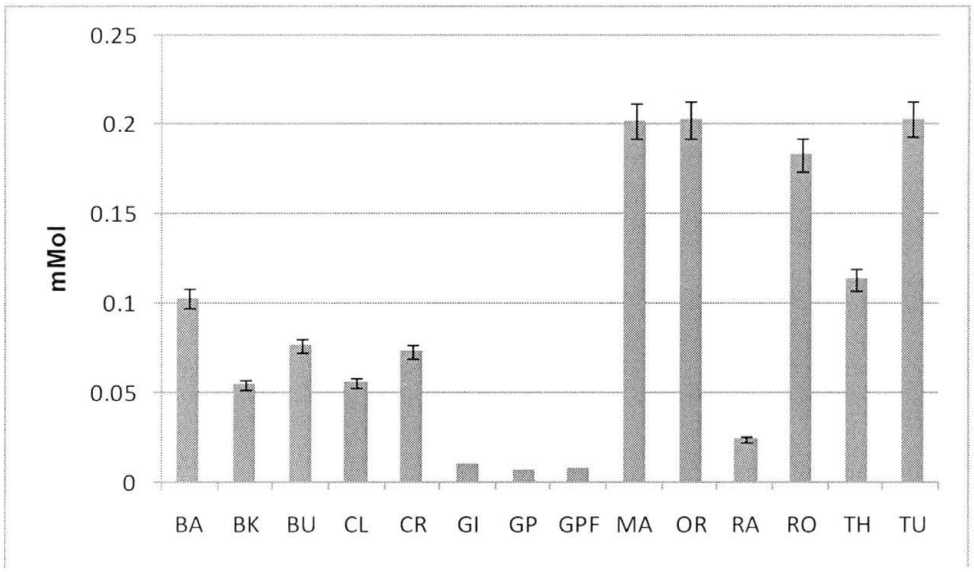

Figure 9. Effect of extracts on reducing formation of MDA in Treatment

Superoxide Dismutase (SOD) Activity. We measured the activity of the extracts to increase the amount of SOD expressed within the muscle. In the *C* and *P* treated tissue samples the SOD activity did not change over the course of the 6 days treatment, although, the *TP* and *T* groups showed a variety of results. The *TP* earthworms ingesting the TU extract were the most effective in increasing enzyme expression. This was followed by the *TP* earthworms ingesting the MA, BK, OR, and RO. TH, BU, BA, CL, and CR extracts contained the next highest amounts of SOD. While the RA, GI, GPF, and GP extracts had the lowest amounts of SOD expression (Figure 10).

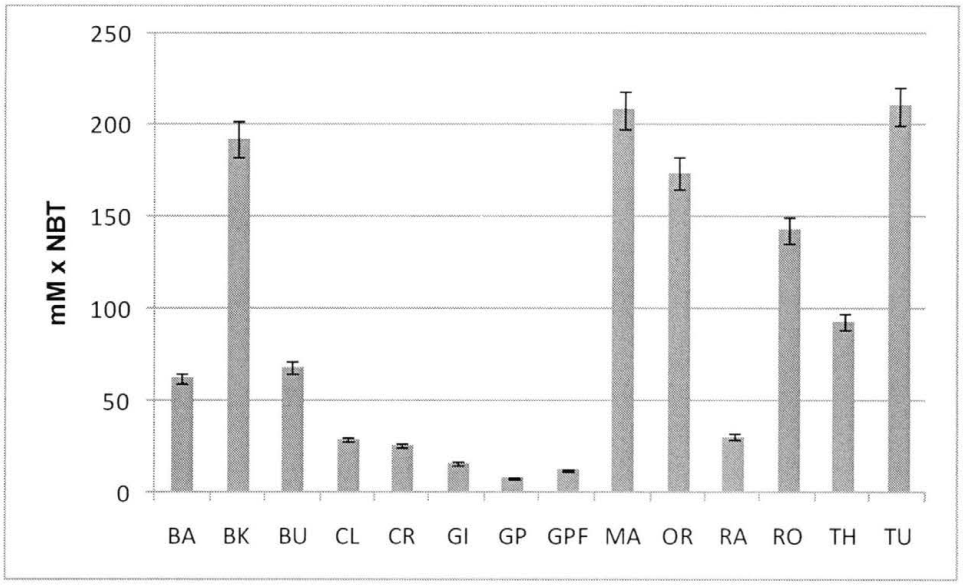

Figure 10. Effect of extracts on increasing the expression of SOD in Treatment-Peroxide group *in vivo*.

In the group fed *T* only, the earthworms which ingested the TU extract had the highest increase in SOD expression. This was followed in SOD expression by the earthworms ingesting the MA, OR, BK, and RO extracts. TH, BU, BA, CR, and CL extracts contained the next highest amounts of SOD. The RA, GI, GPF, and GP extracts had the lowest amounts of SOD (Figure 11).

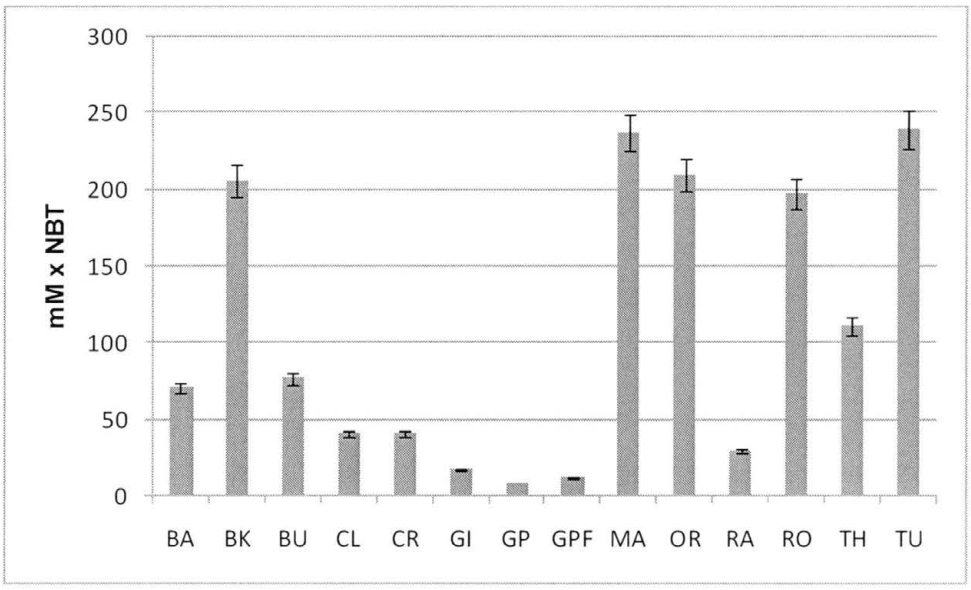

Figure 11. Effect of extracts on increasing the expression of SOD in Treatment group *in vivo*.

Catalyse (CAT) Activity. We measured the activity of the extracts to increase the amount of CAT expressed within the muscle. In the *C* and the *P* sample, the levels of CAT did not change over the course of the 6 days of treatment. The *TP* earthworms ingesting the GPF extract was the most effective in increasing CAT enzyme expression. This was followed by the *TP* earthworms ingesting the GI, RA, CR and BU extracts. CL, TH, TU, OR, and RO extracts contained the next highest amounts of CAT. While the BK, MA, BA, and GP extracts had the lowest amounts of CAT (Figure 12).

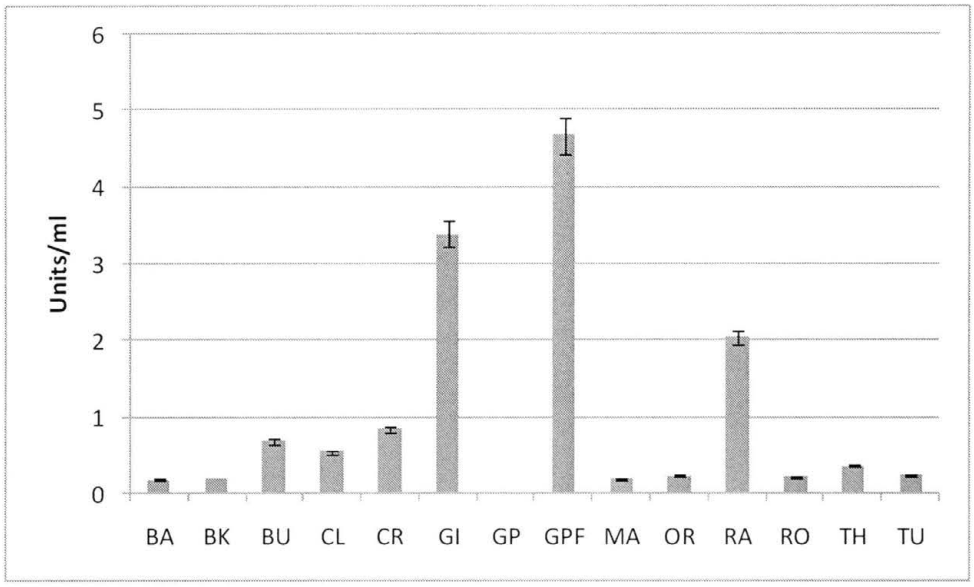

Figure 12. Effect of extracts on increasing the expression of CAT in Treatment-Peroxide group *in vivo*.

Of the earthworms which were fed the *T* extracts, the muscles which showed the highest expression of CAT was found in those which ingested the GI extract. This was followed by the earthworms ingesting the GPF, RA, CR, and BU extracts which were also effective in increasing expression of the enzyme. CL, TH, TU, OR, and BA extracts contained the next highest amounts of CAT. While BK, MA, RO, and GP extracts had the lowest amounts of CAT within the *T* earthworms (Figure 13).

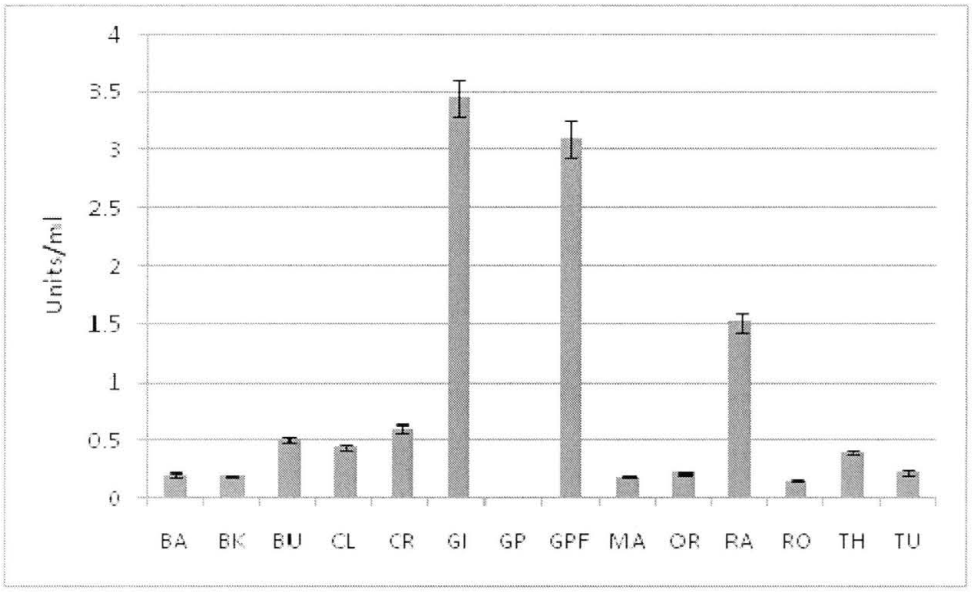

Figure 13. Effect of extracts on increasing the expression of CAT in Treatment group *in vivo.*

Summary of Results

Extracts were ranked by activity of SOD and CAT, from greatest increase in expression to least. The most active extracts in each category are shown in Table 1. Formulations of the most active extracts were created, in terms of enzyme activity, to determine if activity was increased when phenolic extracts were combined. Specifically, we combined the top four extracts which increased CAT expression (Table 1), with the top four extracts which increased SOD expression (Table 1). The four formulations were as follows: Group 1: GPF+GI+TU+MA, Group 2: GPF+GI+BK+OR, Group 3: RA+BA+TU+MA, and Group 4: RA+ BA+ BK+OR (Table 2).

Table 1

Extract Enzyme Rankings

Rank[a]	Superoxide Dismutase (SOD)	Catalase (CAT)
1	Turmeric	Grapefruit
2	Mace	Ginger
3	Blackberry	Raspberry
4	Oregano	Cranberry
5	Rosemary	Blueberry
6	Thyme	Clove
7	Blueberry	Thyme
8	Basil	Turmeric
9	Clove	Oregano
10	Cranberry	Rosemary
11	Raspberry	Blackberry
12	Ginger	Mace
13	Grapefruit	Basil
14	Grape	Grape

[a]Extracts ranking of CAT and SOD expression listed from greatest to least.

Table 2

Extract Group Combinations

Group 1	Group 2	Group 3	Group 4
Grapefruit	Grapefruit	Raspberry	Raspberry
Ginger	Ginger	Basil	Basil
Turmeric	Blackberry	Turmeric	Blackberry
Mace	Oregano	Mace	Oregano

To form the extract groups, 0.25g of each extract was used to create the mixture and extracted in 20ml of water as described before. *C*, *P*, *T*, *TP* groups were prepared by using the combined extracts in place of the pure extract. Also, determine if the combination extracts were more effective then the single extracts alone, the peroxide challenge was increased to 20%.

Group Extract Data

Polyphenolic Content After the earthworms were exposed to the Group 1 (GP1), Group 2 (GP2), Group 3 (GP3), and Group 4 (GP4) extracts the amount of polyphenolics absorbed into the muscle tissue was determined using the Folin–Ciocalteu assay. In the *C* and *P* samples the value of polyphenolics did not change over the course of treatment. Our results indicate that compared to the *C*, the *TP* earthworms ingesting the GP1

absorbed the greatest amount of bioavailable polyphenolics. This was followed in polyphenolic bioavailability by the earthworms ingesting the GP4 (Group 4), GP3 (Group 3), and GP2 (Group 2) extracts (Figure 14). The *T* earthworms ingesting the GP4 extracts had the highest total amount of bioavailable polyphenolics. This was followed in polyphenolic bioavailability by the *T* earthworms ingesting the GP1, GP3, and GP2 extracts (Figure 15).

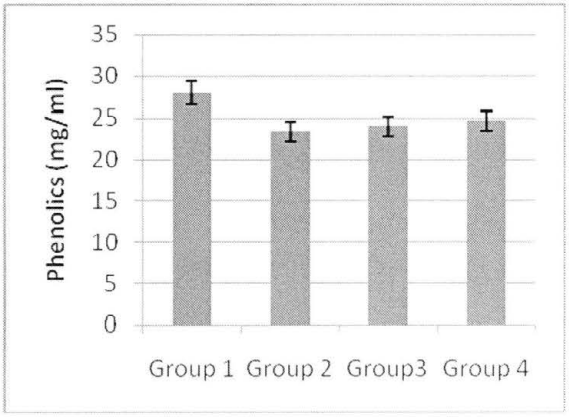

Figure 14. Phenolic bioavailability of Treatment-Peroxide grouped extracts *in vivo.*

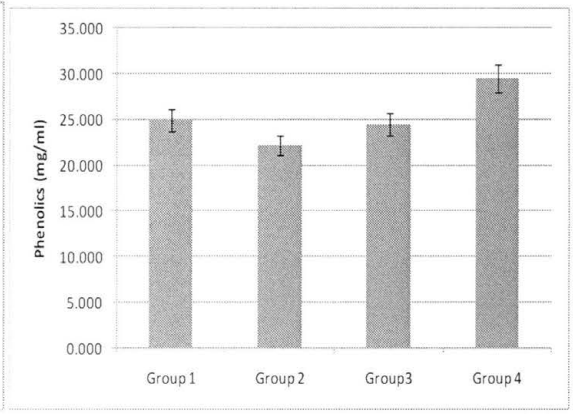

Figure 15. Phenolic bioavailability of Treatment grouped extracts *in vivo.*

ABTS Radical Formation. We measured the effectiveness of extracts on neutralizing the ABTS radicals. In the *C* sample, which did not have any polyphenolic treatment and in the *P* alone treated tissue sample, the inhibition of ABTS radical did not change over the course of treatment. Compared to *C*, the muscles of the *TP* earthworms ingesting the GP4 extracts had the most powerful ABTS neutralizing effect. The GP4 extract inhibited 15.7% of the ABTS radicals. This was followed in ABTS inhibition by the *TP* earthworms ingesting the GP1, GP2, and GP3 extracts which reduced ABTS

formation by 13.1, 12.9, and 12.5%, respectively (Figure 16). The *T* earthworms ingesting the GP4 extracts had the most powerful ABTS neutralizing effect. The GP4 extracts inhibited 16.3% of the ABTS radicals. This was followed in ABTS inhibition by the *T* earthworms ingesting the GP2, GP3, and GP1 extracts which reduced ABTS formation by 15.8, 12.6, and 10.9%, respectively (Figure 17).

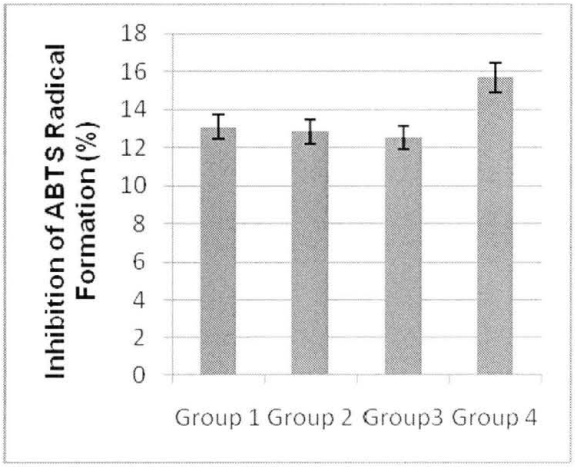

Figure 16. Effects of grouped extracts on reducing ABTS free radical in Treatment-Peroxide group *in vivo.*

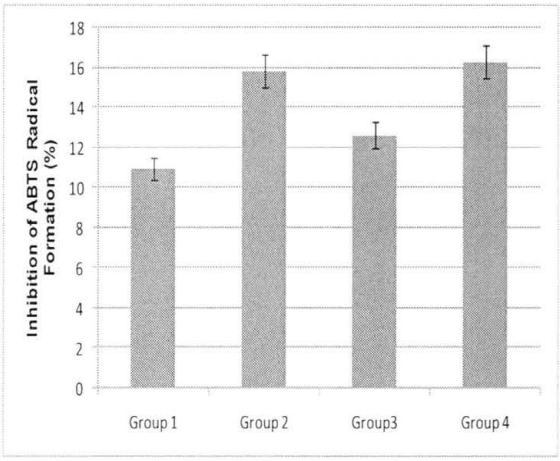

Figure 17. Effects of grouped extracts on reducing ABTS free radical in Treatment group *in vivo.*

Malonaldehyde (MDA) Content. The MDA content of the earthworm muscle sample was measured to study the extent of the membrane degradation as a result of H_2O_2 induced oxidative stress. In the *P* earthworm sample, which was stressed with H_2O_2 but did not contain any polyphenolic treatment, the amount of MDA formed was the highest. The earthworms stressed with H_2O_2 and contained a polypolyphenolic treatment (*TP* group) showed a different trend. Our results indicate that compared to the control, the *TP* earthworms ingesting the GP2 extracts had the lowest total amount of MDA. This was followed by the *TP* earthworms ingesting the GP3, GP1, and GP4 extracts (Figure 18).

The *T* earthworms ingesting the GP2 extracts had the lowest total amount of MDA. This was followed by the *T* earthworms ingesting the GP3, GP4, and GP1 extracts (Figure 19).

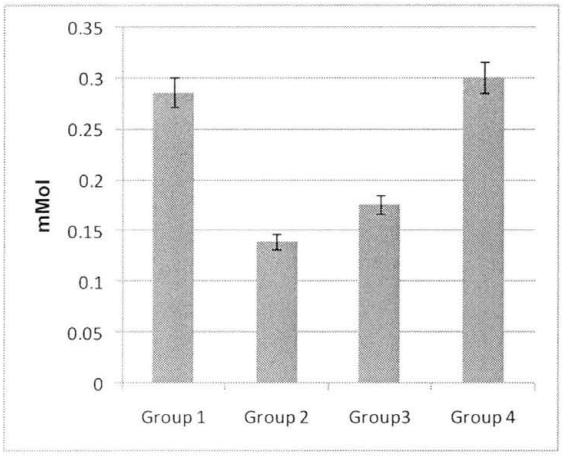

Figure 18. Effect of grouped extracts on reducing formation of MDA in Treatment – Peroxide group *in vivo.*

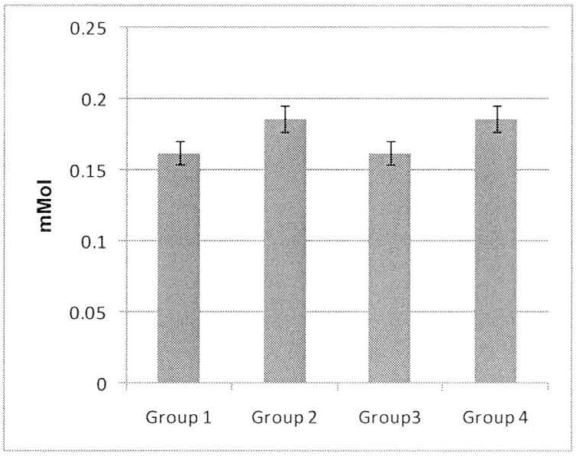

Figure 19. Effect of grouped extracts on reducing formation of MDA in Treatment group *in vivo.*

Superoxide Dismutase (SOD) Activity. The activity of the extracts to increase the amount of SOD available within the muscle was measured. In the *C* and *P* treated tissue samples, the value of polyphenolics did not change over the course of treatment. The *TP* earthworms ingesting the GP4 extract were the most powerful, increasing activity by 207 units/mg of protein. This was followed by the *TP* earthworms ingesting the GP1, GP3, and GP2 which were also effective and increased the activity of the enzyme by 182, 180, and 100 units/mg of protein, respectively (Figure 20). Of the earthworms which were fed the *T* extracts, the muscles which showed the highest expression of SOD was found in those ingesting the GP4 extract by 207 units/mg of protein. This was followed in SOD activity by the *T* earthworms ingesting the GP1, GP3, and GP2 extracts which were also

effective and increased the activity of the enzyme by 199, 189, and 99 units/mg of protein, respectively (Figure 21).

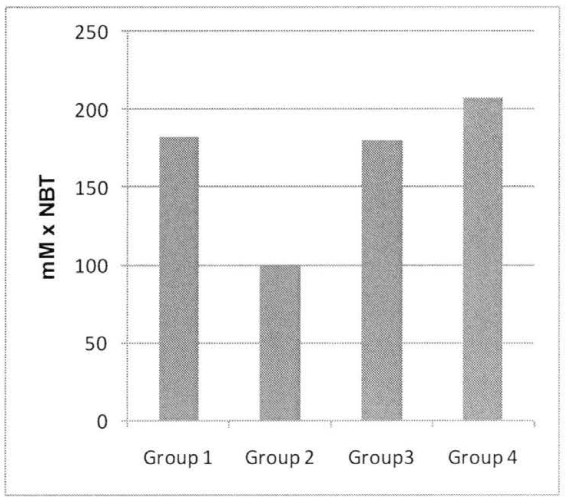

Figure 20. Effect of grouped extracts on increasing the expression of SOD in Treatment-Peroxide group *in vivo.*

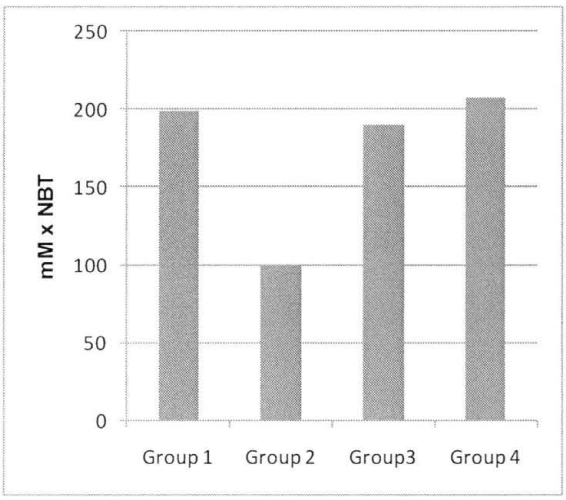

Figure 21. Effect of grouped extracts on increasing the expression of SOD in Treatment group *in vivo.*

Catalyse (CAT) Activity. The activity of the extracts to increase the amount of CAT available within the muscle was measured. In the *C* and *P* samples the enzyme activity, levels did not change over the course of treatment. The *TP* earthworms ingesting the GP2 extract were the most powerful, increasing activity by 0.409 units/mg of protein. This was followed by the *TP* earthworms ingesting the GP1, GP3, and GP4 which were also effective and increased the activity of the enzyme by 0.184, 0.178, 0.170 and units/mg of protein, respectively (Figure 22). Within the *T* earthworms, ingesting the GP2 extract had the highest increase CAT increasing activity by 0.364 units/mg of protein. This was followed in CAT activity by the *T* earthworms ingesting the GP1, GP4, and

GP3 extracts which were also effective and increased the activity of the enzyme by 0.213, 0.211, and 0.168 units/mg of protein, respectively (Figure 23).

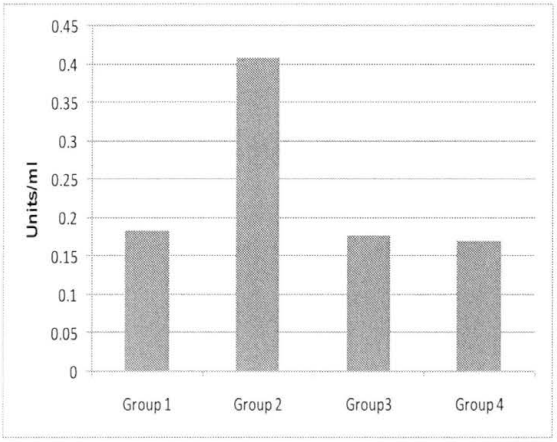

Figure 22. Effect of grouped extracts on increasing the expression of CAT in Treatment-Peroxide group *in vivo.*

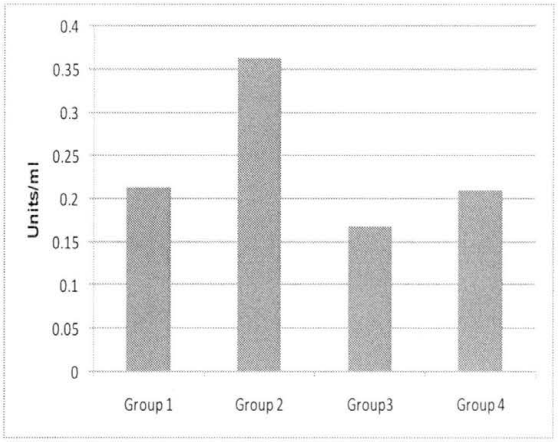

Figure 23. Effect of grouped extracts on increasing the expression of CAT in Treatment group *in vivo.*

Summary of Results. The groups were ranked on their ability to both increase the expression of SOD and CAT, and decrease the amount of MDA. The best extracts in each category are shown in Table 3.

Table 3
Extracts Group Rankings

Rank	SOD	CAT	MDA
1	Group 4	Group 2	Group 2
2	Group 1	Group 1	Group 3
3	Group 3	Group 3	Group 1
4	Group 2	Group 4	Group 4

According to the results GP2 was the best at increase CAT and MDA, although, GP 4 was the best at increasing SOD expression. Overall, GP2 had the highest rank, followed by GP4, GP1, and GP3, respectively.

Discussion

Effectiveness of Extracts in vitro vs. in vivo

Many polyphenolic compounds are known to possess antioxidant activity *in vitro* and *in vivo* (Fernandez-Panchon, Villano, & Troncoso, 2008; Jensen et al., 2008). In this study, we examined the free radical neutralizing ability of the extracts *in vitro* and *in vivo*. *In vitro*, even at a 1/10th dilution, CL was shown to have the highest free radical scavenging ability, neutralizing 99% of the ABTS radicals. This was followed RO, OR, and MA which scavenged greater than 40% of the ABTS radicals. Similar results were found *in vivo*. CL was also the most effective extract *in vivo*, and was followed by RO, CR, and OR. In short, the extracts with the highest antioxidant activity *in vitro* also showed activity *in vivo*.

Examining the reduction of lipid peroxidation *in vitro* versus *in vivo* produced very discordant results. *In vitro*, the lowest amount of lipid peroxidation was exhibited by RO. This was followed by TU, TH, and BU. *In vivo*, these extracts ranked 9th, 13th, 10th, and 8th, respectively. However, *in vivo*, results indicated GP, GPF, GI, and RA extracts decreased lipid peroxidation the greatest, as noted by MDA. In contrast, when these extracts were compared *in vitro* they ranked 13th, 14th, 12th, and 7th. The results suggest

that protection from lipid oxidation *in vivo* is a consequence of several antioxidant mechanisms in addition to the direct participation of the extract in inhibiting free radicals.

Polyphenolic Content of Extract in vitro vs. Bioavailability of Extract in vivo

We also examined the polyphenolic content of the water extracts *in vitro* and its relationship to the bioavailability of these polyphenolics in the muscle (i.e, the *in vivo* model). *In vitro*, the extract which had the highest polyphenolic content was RO, followed by MA, OR, and TU. The bioavailability of these extracts *in vivo* was quite different. *In vivo*, the extracts which had the highest bioavailability were TU, BU, MA, and GI. When these extracts were compared *in vitro*, they ranked 4^{th}, 7^{th}, 2^{nd}, and 12^{th}, respectively. Thus, high polyphenolic content does not necessarily correspond to increased bioavailability. This could be due to synergistic interactions between the individual polyphenolics present in some extracts, which may have increased their bioavailability (Vattem et al., 2005).

Bioavailable Polyphenolics vs Antioxidant Protection in Muscle

According to published literature, a high serum/muscle antioxidant concentration is believed to offer protection against stress, but the results of our investigation did not support this protective role of antioxidant concentration with functionality (Karakaya, 2004). In our current study, *in vivo,* the extracts which exhibited the highest polyphenolic bioavailability (e.g. absorption into muscle) were, TU, BU, MA, and GI, were not the most effective in reducing MDA. In fact, when the amount of MDA formed in the muscle was measured, TU, BU, MA, and GI ranked 13^{th}, 8^{th}, 14^{th}, and 3^{rd}, respectively. Also,

when TU, BU, MA, and GI were ranked for their ability to quench free radicals, they were 11th, 9th, 7th, and 13th, respectively. The greatest antioxidant functionality *in vivo* was offered by extracts whose bioavailability was not high, when compared with other extracts (Figure 7). These observations suggest that high bioavailability of polyphenolic/antioxidants does not equate to an increase in antioxidant protection within the cell. This is possibly due to the non-free radical mediated antioxidant function carried out by activating signaling pathways (Rechner et al., 2002; Williams, Spencer, & Rice-Evans, 2004). Low antioxidant activity could also be due to structural changes in polyphenolics caused by *in vivo* metabolism. However, in some extracts, these structural changes may not affect the ability to activate some signaling pathways, which result in increased antioxidant defense response, such as increased SOD and CAT (Rechner et al., 2002; Williams et al., 2004).

Furthermore, high amounts of polyphenolics within the muscle do not have an effect on the expression of antioxidant enzymes SOD and CAT. The highest ranked bioavailable polyphenolic was TU. This was followed by BU, MA, and GI. When these extracts were assessed for their ability to increase CAT, they ranked 8th, 5th, 12th, and 1st, respectively. Furthermore, when assessed for their ability to increase expression of SOD, the extracts ranked 1st, 7th, 2nd, and 12th, respectively. These results further reiterate the earlier finding that a reduction in oxidative stress is not only dependent on antioxidant activity but also dependent on the ability of extracts to induce an antioxidant response by increasing the expression of antioxidant enzymes, such as SOD and CAT (Vattem et al., 2005).

Non-free Radical Mediated Antioxidant Response

The grouped extracts were created based on their measured ability to increase the amount of the CAT and SOD groups expressed. The current study investigated whether these extracts have an enhanced effect on decreasing oxidative muscle damage at a higher hydrogen peroxide challenge (20%). This amount was found to be the highest sub-lethal concentration tolerated by *Lumbricus terrestris*. Even after a hydrogen peroxide (H_2O_2) challenge, the extracts offered antioxidant protection to the muscle, as evidenced by the reduction in MDA levels (Figure 18). These results suggest that the combined extracts were even more effective in conferring antioxidant protection than single extracts. This protection was not only due to increased free radical quenching ability but was also due to increased expression of SOD and CAT (Figures 21 and 23). Thus, the polyphenolics in the combined extracts appeared to be acting synergisticially (Chu, Sun, Wu, & Liu, 2002; Sun, Chu, Wu, & Liu, 2002). As described previously, synergy can be defined as the ability of two or more bioactive components such as antioxidants in a polyphenolic background to mutually enhance their functionality (Vattem et al., 2005). This synergistic intervention significantly improves the polyphenolic function of the mixture, thereby reducing the overall dosage required to observe the desired positive effect (Vattem et al., 2005). This was true with all our groups, where considering the groups outperformed the individual extracts and they did so at a much higher oxidative challenge.

Conclusion

Oxidative stress, especially in muscle tissue, can contribute to loss of performance in athletes (Davies et al., 1982; Konig et al., 2000; Miyazaki et al., 2001; Morillas-Ruiz et

al., 2005; Powers et al., 2004; Zhang et al., 2004). Several articles examining the effect of antioxidant supplementation on decreasing physical and/or oxidative muscle damage have shown to be inconclusive (Buchman et al., 1999, Cannon et al., 1990; Helgheim et al., 1979; Itoh et al., 2000; Nieman at al., 2004;Simon-Schnass & Pabst., 1988; Sumida et al., 1989; Surmen-Gur et al., 1999). The first purpose of this *in vitro* investigation was to determine whether there is a basis for examining polyphenolic extracts using the *in vivo* model. The effects of several antioxidant-rich dietary herbs, spices, and fruits on reducing oxidative damage to muscle tissue were examined. Results indicated that *in vitro* antioxidant function did not always translate similar results *in vivo*. We also demonstrated that the extracts improved antioxidant defenses by increasing the expression of CAT and SOD, which is a more significant mechanism by which oxidative stress related muscle damage, can be reduced. The results also suggest that bioavailability of phenols from different extracts is quite varied. While some phenols are more bioavailable than others, it did not translate into increased effectiveness. This could be due to metabolic alterations in the structure of the phenols (Dunlap et al., 2006; Nakazato et al., 2007; O'Byrne et al., 2002;Pilaczynska-Szczesniak et al., 2005). Through a series of experiments, we were able to establish that there are synergistic interactions between polyphenolics and that they play a critical role in increasing the overall bioactivity. Thus, the synergistic behavior between extracts allows them to have an increased functionality in group formulations than compared to the single extracts alone.

Since oxidative stress plays an important role in muscle damage, several antioxidant supplements in various forms have been sold and marketed to athletes. Most

are combinations of natural antioxidants (e.g., vitamins E and C) or contain natural products extracts (e.g., green tea, blueberry, and quercetin). A survey of these products indicated that they have been developed only based on their *in vitro* activity. However, the current work indicates that this rationale, using compounds that demonstrate *in vitro* functionality, without prior *in vivo* testing, may prove this to be ineffective. A complete understanding of *in vivo* and *in vitro* functionality of these antioxidants or natural products via free radical or non-free radical antioxidant activity is imperative to develop effective supplementation to manage EIOS.

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CHAPTER 2

THE EFFECTS OF ANTIOXIDANTS ON EXERCISE INDUCED OXIDATIVE MUSCLE DAMAGE

Unaccustomed exercise, in a very basic sense, is a threat to homeostasis (Powers, DeRuisseau, Quindry, & Hamilton, 2004). Depending on the mode, intensity, and duration, unaccustomed exercise is purported to produce physical and/or metabolic changes that may lead to skeletal muscle damage (Armstrong, Warren, & Warren, 1991). A common physical change is the overstretching of the myofibrils, potentially damaging sarcomeres, myocyte membranes, sarcoplasmic reticulum, transverse tubules, or any combination of these (Niess & Simon, 2007; Peake et al., 2005). Damage to these structures may then trigger a series of events, including the uncontrolled influx of calcium into the sarcoplasm, proteolysis, and the ultimate invasion of neutrophils and macrophages (Niess & Simon, 2007; Peake et al., 2005), into the working muscle, causing pain, discomfort, and soreness (Close, Kayani, Vasilaki, & McArdle, 2005).

Muscle damage can also occur due to events which are metabolic in nature. Metabolic damage is primarily attributed to an increased production of oxygen radicals which exceeds the detoxification capacity of antioxidant enzymes (Aguilo et al., 2005). Metabolic muscle damage attributed to free radicals is referred to as exercise-induced

oxidative stress (EIOS) (Vollaard, Shearman, & Cooper, 2005). Specifically, EIOS refers to an increase in free radical production within the working muscle during strenuous exercise (Konig, Wagner, Elmadfa, & Berg, 2001; Vollaard et al., 2005). While the formation of free radicals is a normal consequence of a variety of essential biochemical reactions (Konig et al., 2001; Machlin & Bendich., 1987), a physiological balance generally exists between the formation of free radicals and the neutralization of free radicals by antioxidants in order to maintain physiological homeostasis. If this balance is disrupted, such as during strenuous physical activity, so that free radical production exceeds neutralization, muscle damage may occur, having a negative impact on muscle performance (Konig et al., 2001; Powers et al., 2004).

When homeostasis is not threatened (e.g., at rest), free radicals, such as hydrogen peroxide (H_2O_2), superoxide (O_2^-) hypochlorous acid (HOCl), and nitric oxide (NO^-), play an important role in the body's immune response, redox regulation of gene transcription, cell signaling, and enzymology (Niess & Simon, 2007; Vollaard et al., 2005). Basically, appropriate amounts free radicals participate in normal bodily functioning. However, when homeostasis is threatened, such as during unaccustomed bouts of physical activity or an activity of great intensity and/or prolonged duration, large amounts of free radicals are produced in amounts which exceed the body's antioxidant capacity.

In order to decrease the effects of EIOS researchers have examined the effects of supplementation with a variety of antioxidants (e.g. vitamin C, vitamin E, and polyphenolic compounds). However, due to differences in methodology, including the

type of antioxidant investigated, the type of physical activity employed, the training status of the subjects, and the biological markers used to measure EIOS, results of these studies have been discordant and warrant clarification. Thus, the purpose of this paper is to clarify the published literature by organizing previous studies according to methodological approach. While this paper will provide a brief review of the mechanisms involved in EIOS and the rationale for antioxidant supplementation with exercise, the primary focus of this paper will be to determine if antioxidant supplementation can be effective when used at the proper time and amount after a strenuous bout of exercise.

Exercise-Induced Oxidative Stress (EIOS)

EIOS occurs during high intensity and/or prolonged exercise when free radical production exceeds the body's ability to maintain a physiological homeostasis. Oftentimes, this imbalance is associated with muscular fatigue, increased inflammation, and/or muscle damage, and may ultimately result in a reduction in performance (Davies, Quintanilha, Brookes, & Packer, 1982; Miyazaki et al., 2001; Morillas-Ruiz et al., 2005; Zhang et al., 2004). In light of these potential consequences, a thorough understanding of EIOS is warranted. Thus, this section will review how free radical induced muscle damage is formed, measured, and why free radical accumulation during exercise may lead to EIOS.

Free radicals, produced in cells by electron transfer reactions are molecules that contain one or more unpaired electrons and are capable of existing independently (Aruoma, 1994). Because they contain at least one unpaired electron, free radicals are

highly reactive (Close, Ashton, McArdle, & Maclaren, 2005). Specifically, the unpaired electron attempts to stabilize itself by pairing with an electron from another free radical, a non-radical, or from an antioxidant (Nayak, Karmen, Frishman, & Vakili, 2001). If an electron is obtained from another free radical, then the free radical becomes a nonradical (Close et al., 2005). However, when an electron is obtained from a nonradical, then a new free radical may be generated from this reaction (Close et al., 2005). This new free radical may then take an electron from another nonradical, and so on, so that a chain reaction occurs. This propagation of free radicals by the continual joining of a free radical and nonradical is known as a free radical chain (Close et al., 2005). This free radical chain will continue until a termination reaction occurs, such as when the free radical reacts with either another free radical or an antioxidant, forming a nonradical (Nayak et al., 2001).

Formation of Free Radicals during Exercise

EIOS occurs as a result of high intensity/prolonged physical exercise. The three most common sources of free radical generation during exercise are mitochondrial respiration, the reaction catalyzed by xanthine oxidase (XO), and a variety of immune responses within the body.

Physical exercise increases mitochondrial oxygen consumption by muscle tissue resulting in elevated free radical generation (Davies et al., 1982). The free radicals are created in the electron transport chain (ETC) when a molecule of oxygen is reduced by one electron to form an unprotonated form of superoxide ($O_2^{\bullet -}$) (Sachdev & Davis, 2008).

This form of superoxide is particularly harmful because it can be easily converted to hydrogen peroxide (H_2O_2) which may then be cleaved to form the highly reactive hydroxyl radical ($^{\bullet}OH$) (Deaton & Marlin, 2005). The hydroxyl radical is a very powerful oxidant and can produce damage to several different biological systems (Deaton & Marlin, 2005). Research has shown that 2-5% of oxygen consumed during exercise is reduced to unprotonated superoxide which is due to mitochondrial leakage (Halliwell, 2001). During respiration the protons that leak between Complex I and Complex III of the ETC account for most of the superoxide that is generated during heavy exercise (Bokov, Chaudhuri, & Richardson, 2004). Thus, when muscle metabolic rate is elevated, like during long duration/high intensity exercise, there is an increase mitochondrial oxygen consumption, which will generate a large amounts free radicals (Tonkonogi, Walsh, Svensson, & Sahlin, 2000). The leakage of protons across the mitochondrial membrane may be significant source of free radicals, although, the molecular mechanisms are still poorly understood and need to be studied further (Brookes, 2005; McArdle et al., 2004; Tonkonogi et al., 2000).

XO is an enzyme that has been associated with the production of free radicals. When exercise of high intensity occurs the muscle fibers may undergo relative hypoxia/ischemia, as the oxygen supply cannot match the energy demand (Packer, 1997). The ischemic conditions trigger conversion of xanthine dehydrogenase (XD) to XO (Hellsten, Hansson, Johnson, Frandsen, & Sjodin, 1996). The function of XD is to reduce NAD+ to NADH. However, once XD is converted to XO, it is no longer available to perform this conversion (McCord, 1983). Furthermore, XO preferentially reduces oxygen

directly to superoxide and hydrogen peroxide (Vina et al., 2000). When exercise levels decrease and oxygen is reintroduced, a burst of superoxide and hydrogen peroxide can occur, thus resulting in an increase in damage to the surrounding tissue (Vina et al., 2000). Vina et al. (2000) demonstrated the potential importance of XO in rats and in humans. These researchers showed that, in both species, exercise to exhaustion did result in increased blood XO activity. Furthermore, once they experimentally inhibited activity of XO, exercise-induced oxidative damage decreased in both humans and rats. Thus, results suggest that XO is responsible for the free radical production and tissue damage during exhaustive exercise.

Another theory of free radical production which has recently been introduced involves the production of superoxide as a result of neutrophils and other phagocytic cells overpowering the body's antioxidant defenses as an immune response to the tissue damage (Peake & Suzuki, 2004). Neutrophils produce free radicals which act as defense mechanisms against pathogens, infections, damaged tissue following a injury (Weiss, 1989). Although, when exercise causes muscle damage the excessive production of these toxic molecules it may lead to oxidative stress (Peake & Suzuki, 2004). Intense exercise has been shown to cause an increase in neutrophil activities and impair antioxidant enzymes (Tauler et al., 2003). Powers et al. (2004) running downhill on a treadmill for three 15 minute periods of 75% of participates max heart rate. There was an increase in capacity of neutrophils to generate superoxide after exercise for up to 12 days. Quindry, Stone, King, & Broeder (2003) tested the idea that circulating neutrophils will impose oxidative stress when exercise exceeds the LT. The authors found the highest amount of

oxidative stress immediately after the maximal treadmill exercise test which directly coincides with the largest neutrophil activity. Thus the authors reasoned that most of the exercise induced oxidative damage was produced as a result of the inflammatory response. Similarly, McArdle et al. (1999) examined the extensor digitorum longus muscle of rats after inducing an extensive bout of injury-inducing protocol. The authors found elevated levels of oxidized muscle damage 3 days after the exercise protocol was completed. This delayed muscle damage was likely attributed to an aggressive inflammatory response (McArdle et al., 1999). A hypothesis to account for this oxidative damage that occurs from neutrophils has been purposed by Tauler et al. (2003) who suggest that neutrophils posses the ability to release oxidants after an intense bout of physical activity. Typically neutrophils have available antioxidant defenses, attributed to antioxidant enzyme secretion, which are lowered after an intense bout of physical exercise. The lowered antioxidant enzyme secretion may explain for the oxidative damage created by neutrophils although still further research is needed to verify Tauler et al. (2003) hypothesis.

Currently the overall systems, conditions, and reactions that produce free radicals and cause oxidative damage are yet to be fully clarified and validated. Although there appears to be substantial evidence for the generation of superoxide and H_2O_2 by the mitochondria, XO, and phagocytes during endurance exercise or exercise of high intensity. Still more research is needed to definitively prove which source plays the most substantial role during exercise-induced free radical formation. The focus of the next

sections will be to determine which oxidative damage markers can be verified by the direct measurement of damage to lipids, protein and DNA (Djordjevic, 2004).

Lipid Peroxidation

A common marker of EIOS is lipid peroxidation (Konig et al., 2001; Radak et al., 2001; Vollaard et al., 2005). Lipid peroxidation refers to the oxidative degradation of lipids (Hyslop et al., 1988). As mentioned in the previous section, when free radicals are formed, they may stabilize by removing electrons from other compounds, including lipids (Halliwell & Chirco, 1993). In particular, polyunsaturated fatty acids (PUFAs) within cell membranes and intracellular organelles contain methylene-CH_2 groups between many carbon-carbon double bonds (Djordjevic, 2004; Halliwell & Chirco, 1993). Because hydrogens can be easily removed from these methylene-CH_2 groups, PUFAs are particularly susceptible to peroxidation (Halliwell & Chirco, 1993). The removal of the single electron from the hydrogen leaves an unpaired electron on the carbon atom to which it was originally attached. The carbon atom becomes a lipid radical (Halliwell & Chirco, 1993). This, too, is an unstable species and will react with another fatty acid resulting in the continuation of a free radical chain reaction mechanism (Djordjevic, 2004). This mechanism will continue until either two radicals combine, producing a non-radical species or until a free radical reacts with an antioxidant. For a more detailed discussion on lipid peroxidation, see Halliwell & Chirico (1993).

Typically, by-products of lipid peroxidation are the main markers used to measure free radical damage (Vollaard et al., 2005). The primary measures of lipid damage are

malondialdehyde-thiobarbituric acid (MDA-TBARS) and isoprostanes. TBARS, as measured by MDA, has been extensively studied since the 1980's (Kanter, Lesmes, Kaminsky, La Ham-Saeger, & Nequin, 1988; Lovlin, Cottle, Pyke, Kavanagh, & Belcastro, 1987; Viinikka, Vuori, & Ylikorkala, 1984). Several of the previous studies have found significant increases in TBARS levels, some by as much as 220% (Marzatico, Pansarasa, Bertorelli, Somenzini, & Della Valle, 1997). While somewhat discordant, numerous research studies have reported, MDA, a by-product of the peroxidation of PUFAs (Vollaard et al., 2005), has been shown to be elevated post-exercise (Quindry, Stone, King, & Broeder, 2003; Radak et al., 2001; Vollaard et al., 2005; Waring et al., 2003). The inconsistencies in findings, however, are most likely due to differences in methodology, including intensity and duration of the exercise tests. For example, Quindry et al. (2003) showed that intensity, but not duration, was a key factor in increasing oxidative damage. The authors reported elevated levels of MDA in 9 trained men who completed one maximal treadmill test and three sub-maximal treadmill exercise sessions performed at different intensities: 1) 10% above lactate threshold (LT) for 45 minutes, 2) 10% below LT for 45 minutes, and 3) sub-maximal session at 10% below LT until the caloric expenditure equaled the first exercise session. Significant blood oxidative stress was observed immediately after maximal, but not sub-maximal, exercise intensities. The researchers concluded that exercise intensity, not total energy expenditure or duration, was associated with oxidative stress. This study suggested that EIOS, as measured by MDA, may be most consistently induced with exercise that is performed at a high intensity above the LT. This research confirmed previous studies that reported elevated

MDA post-high intensity exercise. For instance, Szczesniak et al. (1998) observed elevated levels of MDA in 13 males following a graded maximal treadmill exercise test.

Similarly, elevated levels MDA has also been seen in moderate intensity exercise of long duration. Borsheim, Knardahl, and Hostmark (1999) discovered elevated levels of MDA in 8 moderately trained males who were cycling for 90 minutes at 58% of their VO_2 max. In a similar study, Laaksonen et al. (1999) observed that levels of plasma MDA increased by 50% following cycling at 60% of their VO_2 max for 40 minutes in 14 untrained males. Based on previous results, oxidative stress may be created and accurately measured by MDA in long-term exercise preformed at moderate intensity and/or short-term exercise performed at high intensity.

While MDA is the most well studied marker for lipid peroxidation, levels of isoprostanes are considered a more suitable marker for lipid peroxidation (Vollaard et al., 2005). Because isoprostanes have both a well documented mechanism of production and a potential for oxidative capacity, they are well suited for measurement *in vivo*. Isoprostanes form through the peroxidation of arachidonic acid which can be easily detected in the blood, urine, and saliva (Vollared et al., 2005). Waring et al. (2003) observed that cycling at 80 W for 20 minutes induced EIOS, as determined by increased levels of plasma isoprostanes post-exercise, in 20 untrained males and females. Similarly, Mastaloudis, Morrow, Hopkins, Devaraj and Traber (2004) tested 11 trained males and females during a 50 km race of long duration. The average run time was 423 ± 11 minutes at a pace of 13.7 ± 0.4 minutes/mile and an intensity of 71 ± 2% VO_2 max. Mastaloudis et al (2004) reported that isoprostanes levels peaked directly post-exercise

which was substantial enough to cause EIOS. As reflected from these studies, isoprostanes are a more sensitive and reliable measure of peroxidation, *in vivo*, than MDA due to the better measured source of production. In summary, most methods to asses oxidative stress have suffered from a lack of reliability, whereas, the research articles that have measured oxidative damage through isoprostanes have shown to produce significant reliability (Morrow & Robarts, 2002). Lipid peroxidation is damaging because of the subsequent chain reactions, the byproducts of which will target proteins and DNA.

Protein Oxidation

The primary mechanism for protein oxidation is the abstraction of hydrogen from amino acid residues which form free radicals (Niess & Simon, 2007). The modifications caused by the change in the amino acids will result in impaired physiological function (Levine et al., 1983) and a significant loss in the biological activity of proteins (Radak et al., 2001). Furthermore, mitochondrial enzymes like ATP synthetase can be oxidized by free radicals which will interfere with muscle contractions and reduce contractility (Vollaard et al., 2005). The typical measured markers for oxidative protein damage are carbonyl derivatives. There has been a significant link established between accumulation of carbonyl derivatives and impaired physiological processes and disorders (Radak et al., 2001; Stadtman, 1992). Using protein carbonyls as a definite marker for protein oxidation, however, is still unproven because most of the studies have been limited to animals (Griffiths, 2000). Reznick, Witt, Matsumoto, and Packer (1992) subjected rats to

a single bout of exhaustive exercise and then observed an increase rate of protein oxidation as shown by the accumulation of carbonyl derivates in the rat's skeletal muscle. These results were confirmed by Witt, Reznick, Viguie, Starke-Reed, and Packer (1992), who elicited a three month period of endurance exercise which consisted of treadmill running lasting two hours, three days per week. Witt et al. (1992) also observed increased oxidative damage to proteins. Radak, Pucsuk, Boros, Josafi, and Taylor (2000), reported an increase in serum and urinary carbonyl proteins in a 4-day super marathon racing of 8 male trained athletes. The carbonyl levels increased after the first day of running 93 km and then hit a plateau on the following 3 days of competition. This study showed oxidative stress which was caused by exhaustive aerobic exercise increases the nitration and carbonylation of serum proteins. Recently, Morillas-Ruiz, Villegas Garcia, Lopez, Vidal-Guevara, and Zafrilla (2006), reported oxidative stress in 30 trained males who cycled for 90 minutes at 70% of VO_2 max. There was 12% increase in protein carbonyls which is sufficient enough to note that protein oxidation did occur. The exact mechanism for exercise-induced protein oxidation is still yet to be conclusively determined, but there does seem to be enough evidence to state that protein oxidation will adversely affect exercise performance due to either effecting either the contractile elements or inhibiting enzymes (Niess & Simon, 2007). For a more detailed look at protein oxidation see Dean, Fu, Stocker, and Davies (1997).

DNA Damage

Oxidative damage which has been linked to modifications to mitochondrial DNA can lead to an accelerated rate of cellular mutations (Johns, 1995). Mutations to the nuclear DNA are considered a potential pathophysiological factor in the development of cancer (Niess & Simon, 2007). These modifications can then result in deficient mitochondrial respiratory function and disturbances in the cellular energy supply (Johns, 1995). Although 8-ox-odG has been shown to only represent approximately 5% of the total DNA oxidative damage, urinary levels of 8-dihydro-2'-deoxyguanosine (8-ox-odG) are typically measured in human studies to identify DNA damage (Radak et al., 2000). DNA degradation studies, using 8-ox-odG as a marker have shown inconsistent results. Poulsen, Loft, and Vistisen (1996), found a 33% increased rate of oxidative DNA damage in 20 men who participated in vigorous exercise which amounted to be ~10 hours per day for 30 days. Poulsen et al. (1996) found an increased excretion of oxidatively modified 8-ox-odG. Likewise, Tsai et al. (2001) found an increase in urinary 8-ox-odG after 14 male runners participated in a marathon. However, Sumida, Doi, Sakurai, Yoshioka, and Okamura (1997) studied the effects of various types of exercise on urinary excretion of 8-ox-odG. The authors implemented three types of exercise; an incremental exhaustive treadmill exercise test, an incremental exhaustive cycling exercise test, and a 20 km run, and assessed urinary excretion of 8-ox-odG. There were no significant findings in either trained or untrained subjects. Due to the problem in accurately measuring DNA damage levels, it appears 8-ox-odG cannot be used as a definite measure.

Despite the small variations in the findings primarily due to the differences in type/intensity of exercise protocol and oxidative damage markers employed, research suggest that an exhaustive or prolonged bout of exercise may cause oxidative damage to lipids, proteins, and possibly DNA. As oxidative damage takes place, due to free radical generation, the body's increased oxidative activity will need to be compensated for by antioxidants, which has the ability to quench the free radicals.

Adaptations to EIOS

It should be noted that there is a distinction between regular, moderate physical exercise and maximal bout of high intensity exercise. There is an adaptive response to regular, moderate physical exercise that over time increases both endurance capacity and antioxidant defense (Radak, Chung, & Goto, 2008). This adaptation is primarily due to increase in mitochondrial biogenesis and total number of muscle mitochondria (Davies et al. 1982). These adaptive mechanisms seem to decrease oxidative stress and increase the antioxidant defenses, reduce resting production of oxidants, reduce the free radical leak that occurs with oxidative phosphorylation (Ji, Gomez-Cabrera, & Vina, 2006; Leeuwenburgh & Heinecke, 2001). Moderate exercise has been shown to increase the body's protection and decrease the several detrimental effects which occur with oxidative stress (Navarro, Gomez, Lopez-Cepero, & Boveris, 2004). Navarro et al. (2004) showed that moderate exercise in rats can significantly decrease the age-associated oxidative stress. The authors were able to show increases in life span, prevention of the decay of mitochondrial function, and improvement in behavior. Although moderate intensity

exercise plays a key role in preventing oxidative damage, high-intensity exercise has been shown disrupt the fragile balance of oxidants and antioxidants within the body (Radak et al., 2008). This imbalance has lead to the significance of supplementing dietary antioxidants during physical exercise.

Antioxidants

The uncertain physiological role that free radicals play on the human body has led to new areas of research and supplementation. In an effort to decrease some of the oxidative damage that occurs with supplementation, antioxidants has been recently been examined. The body contains elaborate antioxidant defense systems, including antioxidant enzymes that aid in prevention of various diseases and attenuate the aging process (Arguilo et al., 2005). Given that free radicals have been proven to be a factor in oxidative muscle damage (Davies et al., 1982), research has suggested that antioxidant supplementation may minimize or prevent the damaging effect of free radicals (Banerjee, Mandal, Chanda, & Chakraborti, 2003; Connolly, Lauzon, Agnew, Dunn, & Reed, 2006; Goldfarb, Patrick, Bryer, & You, 2005; Pfeiffer et al., 1999). Dietary antioxidants can control oxidation and the excessive formation of free radicals by eliminating oxygen radicals, inactivating enzymes that cause oxidation, and activating systems that control oxidation (Conolly et al., 2006). Dietary supplementation with vitamins that act directly as antioxidants themselves or as cofactors for cellular and/or extracellular antioxidant enzymes may reduce free radical generation by strengthening antioxidant defense systems within the body (Pfeiffer et al., 1999). Thus, the next section will review the

effects that specific antioxidants have on reducing the oxidative stress that is specific to exercise.

Antioxidants and Exercise

Given the potential involvement of free radicals in detrimental cellular processes, research has focused on the potential health benefit of antioxidant consumption (Navarro et al., 2004). An antioxidant is defined as a substance which will inhibit or prolong the oxidation of a substrate when it is in lower concentrations compared to that substrate (Adams, Wermuth, & McBride, 1999; Halliwell & Gutteridge, 1989). In other words, antioxidants are substances which slow or prevent the oxidation of other molecules through the termination of the free radical chain by removing free radical intermediates, inhibiting other oxidation reactions, or by being oxidized themselves (Adams et al., 1999). Antioxidants occur naturally within the body and are used in several cell protecting roles throughout many biological processes (Packer, 1997). It has also been established that antioxidants act in prevention of cardiovascular and cerebrovascular diseases, some types of cancers, and age-related disorders (Packer, 1997). These consistent findings which suggest antioxidants protective activities do occur at rest are highly suggestive of a protective effect which could help to decrease the habitual stress of strenuous exercise. Although antioxidants may play an important role in reducing certain types of diseases within the body, the studies are still discordant to their potential for use during exercise. The next section will focus on specific antioxidants and their potential

mechanism of action within the body and its relationship to the oxidative stress that is caused by high intensity/prolonged physical exercise.

Vitamins

In the 1970s some of the first studies to examine vitamin C and E supplementation and exercise appeared. Although the mechanism of action was not known at this time, the studies examined the effect of supplementation on performance. Lawrence, Bower, Riehl, & Smith (1975) found a greater work capacity in subject who ingested 900IU/day of vitamin E, compared to a placebo. Although most early studies involving vitamin E have inconclusive results (Sharman, Down, & Norgan, 1976), Sumida et al. (1989) was one of the first to show vitamin E supplementation of 300mg/day reduced the increase in MDA in response to exercise. Additional research has studied the effect of ingesting the antioxidants vitamin E and vitamin C on counteracting exercise-induced muscle damage. Although through the years the studies involving these vitamins have been discordant. The next section will seek to confirm the use of vitamins during strenuous/prolonged exercise.

Vitamin C is serves an important role as an antioxidant within the body (Connolly et al., 2006). Vitamin C is capable of reducing free radical within the body and has been shown to interact with free radicals before they can initiate damage to cells (Connolly et al., 2006). Vitamin C can reduce oxidants via the donation of a hydrogen ion to prevent or delay the oxidation of a molecule (Goldfarb et al., 2005). The evidence for vitamin C supplementation is still mixed, this was shown by Alessio, Goldfarb, and Cao (1997) who

had 9 male subjects supplement with vitamin C (1 g/day) for 1 day or 2 weeks. The subjects then performed a 30min bout of exercise at 80% of their VO_{2max}. The highest oxidative stress occurred in the placebo group compared to the 1 day or 2 week supplementation group. Although, there was a difference in oxidative stress between the two groups it was minimal and not statistically significant. Vitamin C given in a higher amount of 2g/day also demonstrated minimal protection against oxidative stress created by a 10.5km run on 9 male athletes who were supplemented for a 3 week time period (van der Beek et al., 1990). Recently, Goldfarb et al. (2005) demonstrated that at 0.5/day and 1 g/day for 2 weeks vitamin C can attenuate oxidative stress as indicated by a decrease in protein carbonyl damage for 12 male subjects. Even though most of the studies supplemented subjects for long periods of time, Ashton et al. (1999) had 10 subjects ingest 1g of vitamin C two hours before and exercise to VO_{2max}. This was enough of a time period to attenuate oxidative stress as noted by decreased levels lipid peroxidation within the working muscles. Since there have been several different doses of vitamin C used it is unclear as to what amount would be appropriate in reducing EIOS. Vitamin C is often studied along with vitamin E because vitamin C can directly scavenge free radicals and helps to recycle vitamin E so it can perform its appropriate action (Packer, Slater, & Willson, 1979).

Vitamin E is an important lipid soluble, chain breaking free radical scavenger (Buchman et al., 1999). Vitamin E reacts rapidly with free radicals and it is widely accepted that the antioxidant properties of this compound are responsible for it biological activity (Gaeini, Rahanma, & Hamedinia, 2006; Packer et al., 1979). Since vitamin E is

lipid soluble it is sought to be the first line of defense against lipid peroxidation, protecting PUFAs in cell membranes, and quenching free radicals in the early stages of attack (Gaeini et al., 2006). Vitamin E possesses one of the most potent antioxidant activity levels and helps it protect against highly reactive radicals (Packer et al., 1979). The location of vitamin E in the mitochondrial inner membrane enhances its ability to quench free radicals (Banerjee, Mandal, Chanda, & Chakraborti, 2003). There is much evidence to support the use of vitamin E to scavenge free radicals. As early as 1982 Davies et al. (1982) found that a deficiency in vitamin E increased muscle and liver free radical production, lipid peroxidation, and mitochondrial dysfunction in exhaustively exercised rats. Similar results were found by Jackson, Jones, and Edwards (1983), who found restricted vitamin E in the diets of rats and found increase in the skeletal muscle damage. These two separate authors have shown that with low levels of vitamin E in ones diet there is increased amounts of oxidative stress formed during exercise. Simon-Schnass and Pabst (1988) showed that vitamin E can be beneficial in exercise performance and cell protection in high altitude mountain climbers. The authors had one group of 6 high altitude mountain climbers supplement their diet with 2 x 200 mg of vitamin E per day and a second of 6 were given placebos during a 10 weeks expedition. The authors concluded based on the synthesis of lactic acid and on the exhalation of pentane that vitamin E significantly decreased the oxidative stress within the body. Furthermore, Itoh et al. (2000) performed a randomized and placebo-controlled study on fourteen male runners who took 1200 IU/day of vitamin E 4 weeks prior to and during 6 days of successive running. The runners were training 48.3 +/- 5.7 km/day in their 6 days

of successive running. The authors found that vitamin E offers protective effects against free radicals which can inhibit free-radical-induced muscle damage caused by a sudden large increase in the running distance. Although, not all studies have been able to demonstrate improvements in exercise performance when supplementing with vitamin E. Lawrence et al. (1975) tested 48 well trained athletes and give each one a vitamin E supplement for 6 months containing 900IU of vitamin E. A swimming endurance test was performed before the start of the supplementation period, and after 1, 2, 5, and 6 months. Lawrence et al showed no increase in a swimming endurance during the 6 month period. More recently, Nieman at al. (2004) tested the effects of supplementing with 800 IU of vitamin E per day on 36 trained males and females in a randomized double-blind, placebo controlled study. The subjects supplemented for 2 months prior to a triathlon (3.9k swim, 180k cycle, 42k run). The findings showed that vitamin E had no effect on exercise induced oxidative stress. These findings were also confirmed by Cannon et al. (1990) and Surmen-Gur, Ozturk, Gur, Punduk, and Tuncel (1999), who both showed no increase in performance when supplemented with 800IU/day and 400mg/day of vitamin E, respectively. Thus, based on the information from the previous studies it cannot be determined if vitamin E supplementation is needed during exercise. For more information on vitamin E and use during exercise see the review article by Takanami, Iwane, Kawai, and Shimomitsu (2000).

Polyphenols

Bioactive polyphenolics, found in plant based foods, have diverse biochemical activities, which include antioxidant properties (Alessio et al., 2002). Polyphenols are a type of polyphenolic that contain polyphenolic structures, a characteristic that makes them potent antioxidants (Hagerman, Riedl, & Rice, 1999). Polyphenols are the most ample source of antioxidants in the diet (Tapiero, Tew, Ba, & Mathe, 2002). Recent interest in polyphenols has increased due to their potential for antioxidant capacity (Bravo, 1998), health benefits (Miller, Appel, Levander, & Levine, 1997), and treatment of diseases (Tapiero et al., 2002).

Consumption of fruits, vegetables, and spices have been shown to provide protection from diseases by providing polypolyphenolic compounds that supplement or enhance activities of known dietary or endogenous antioxidants (Alessio et al. 2002). For instance, van Acker, Schouten, Haenen, van der Vijgh, and Bast (2000) showed that a type of polyphenolic compound can mimic the antioxidant activity of vitamin E in the cell membrane. The authors were able to show high levels of antioxidant activity and the potential health benefits for the use of polyphenolic compounds. Alessio et al. (2002) recently tested a polyphenolic compound found in green tea on reducing the oxidative stress attributed to exercise in rats. The authors found a small influence on total plasma antioxidant and a decrease in the exercise-induced kidney damage in the rats that were supplemented with green tea. Pilaczynska-Szczesniak, Skarpanska-Steinborn, Deskur, Basta, and Horoszkiewicz-Hassan (2005) tested the polyphenolic compounds found in Chokeberry juice to determine if they could reduce the effects of oxidative stress on a

rowing ergometer exercise. The authors had 19 trained male members of the Polish rowing team participate in the study. Each subject was supplemented 3 times a day for 4 weeks. The results indicated that an increased intake of polyphenolic compounds limits the exercise-induced oxidative damage to red blood cells, by enhancing the endogenous antioxidant defense system.

Given that there has not been much research done on polyphenolic compounds and EIOS we must also look at general oxidative stress to see if polyphenolic compounds have an impact there as well. O'Byrne, Devaraj, Grundy, and Jialal (2008) tested the polyphenolic compounds found in Concord grape juice, to see if they have a greater antioxidant efficacy in vivo than vitamin E. In the subjects who consumed the Concord grape juice the authors found an increased serum antioxidant capacity and protection of LDL against oxidation to an extent similar to that obtained with 400 IU vitamin E per day but decreased plasma protein oxidation significantly more than did vitamin E. The authors concluded that the Concord grape juice polyphenolic compounds are potent antioxidants that may protect against oxidative stress and reduce the risk of free radical damage and chronic diseases.

Also, given that there is little research conducted on humans we must also look to see if polyphenolic compounds can reduce the effects of EIOS in animals. Dunlap, Reynolds, and Duffy (2006) conducted a study to analyze the effects that blueberry polyphenolic compounds would have on oxidative stress to 35 Alaskan huskies who exercised at 70% of their VO2 max for 2 consecutive days. The dogs were fed a diet containing blueberries 2 months prior to the exercise session and were then tested for

were antioxidant serum levels and muscle damage. Although the bout of exercise that was subjected on the dogs did not cause muscle damage there a significantly elevated antioxidant status in the sled dogs post exercise. Thus suggesting that because of the elevated antioxidant status the animals could be better protected against EIOS. Due to the lack of evidence substantially supporting the use of polyphenolic compounds to prevent EIOS it is worth further examining their effect on animals before a human study can be conducted.

Conclusion

Research suggests that exercise of long duration and/or of high intensity may disrupt the fragile homeostasis between oxidants and antioxidants that exists within the body during resting conditions. Although the exact mechanism for free radical production is yet to be fully understood, it is known that free radicals are produced in substantial quantities within the body during exercise. This phenomenon is commonly referred to as Exercise Induced Oxidative Stress (EIOS). Throughout the past several decades, research has indicated that EIOS may be deleterious to an athlete's performance and recovery following performance. Thus, supplementation with various antioxidants has increased among athletes in order to attenuate the effects of this oxidative stress. Currently, there are a small number of studies which prove there is definite need for antioxidant use. However, there is a lack of substantial evidence which supports the efficacy of supplementing an athlete's diet with vitamins C and E. Nevertheless, because of its potential benefit as indicated by preliminary research, the effects of polyphenolic

compounds on reducing the effects of EIOS should continued to be examined. In summary, more scientific evidence is needed to support the recommendation for antioxidant supplements by athletes during training in order to enhance performance or reduce oxidative damage.

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APPENDIX

SINGLE EXTRACT FIGURES

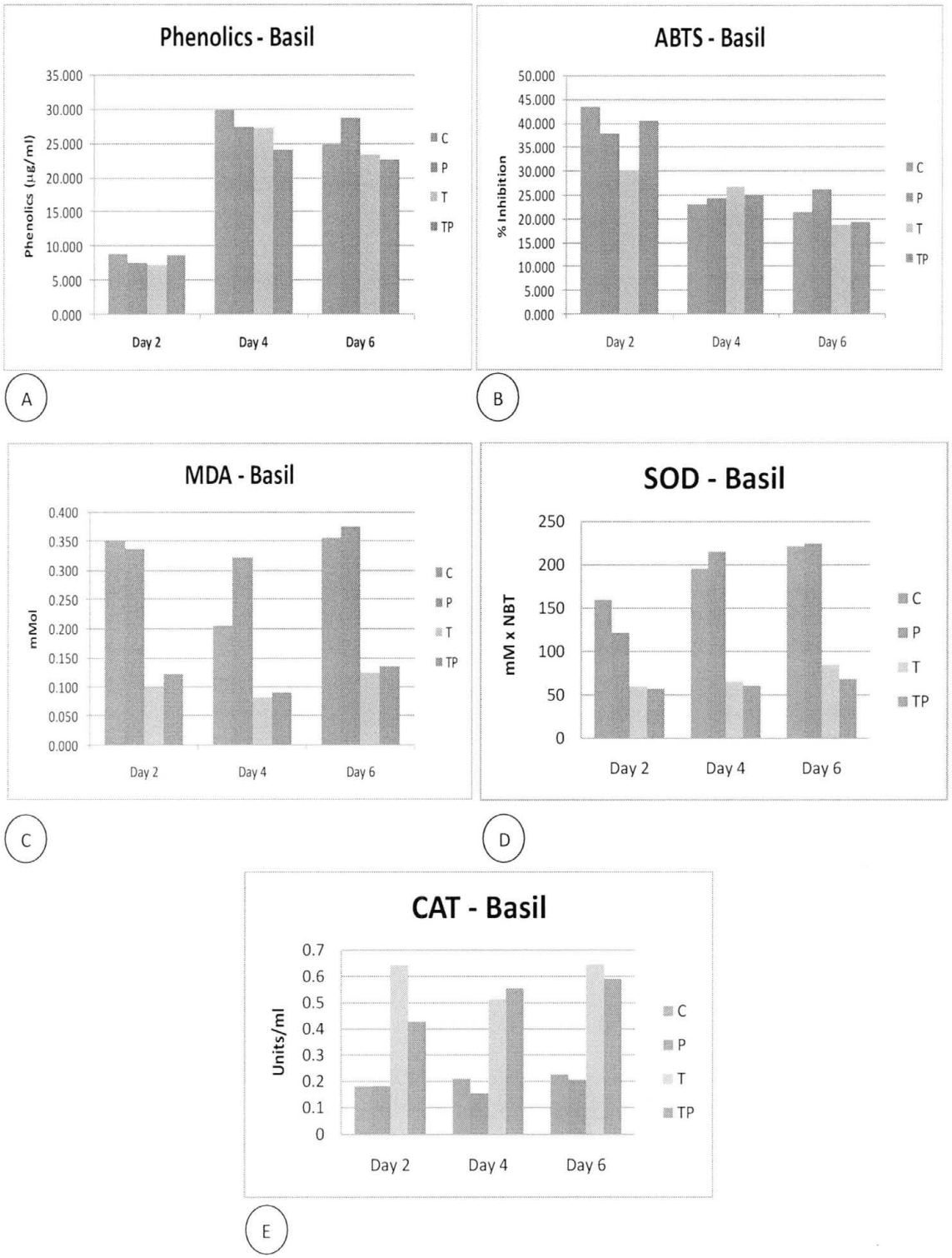

Figure 24. Effect of basil on oxidative stress in *Lumbricus terrestris*. A) Phenolic content, B) ABTS radical inhibition AOX activity, C) MDA formation, D) SOD activity by NBT formation, E) CAT activity.

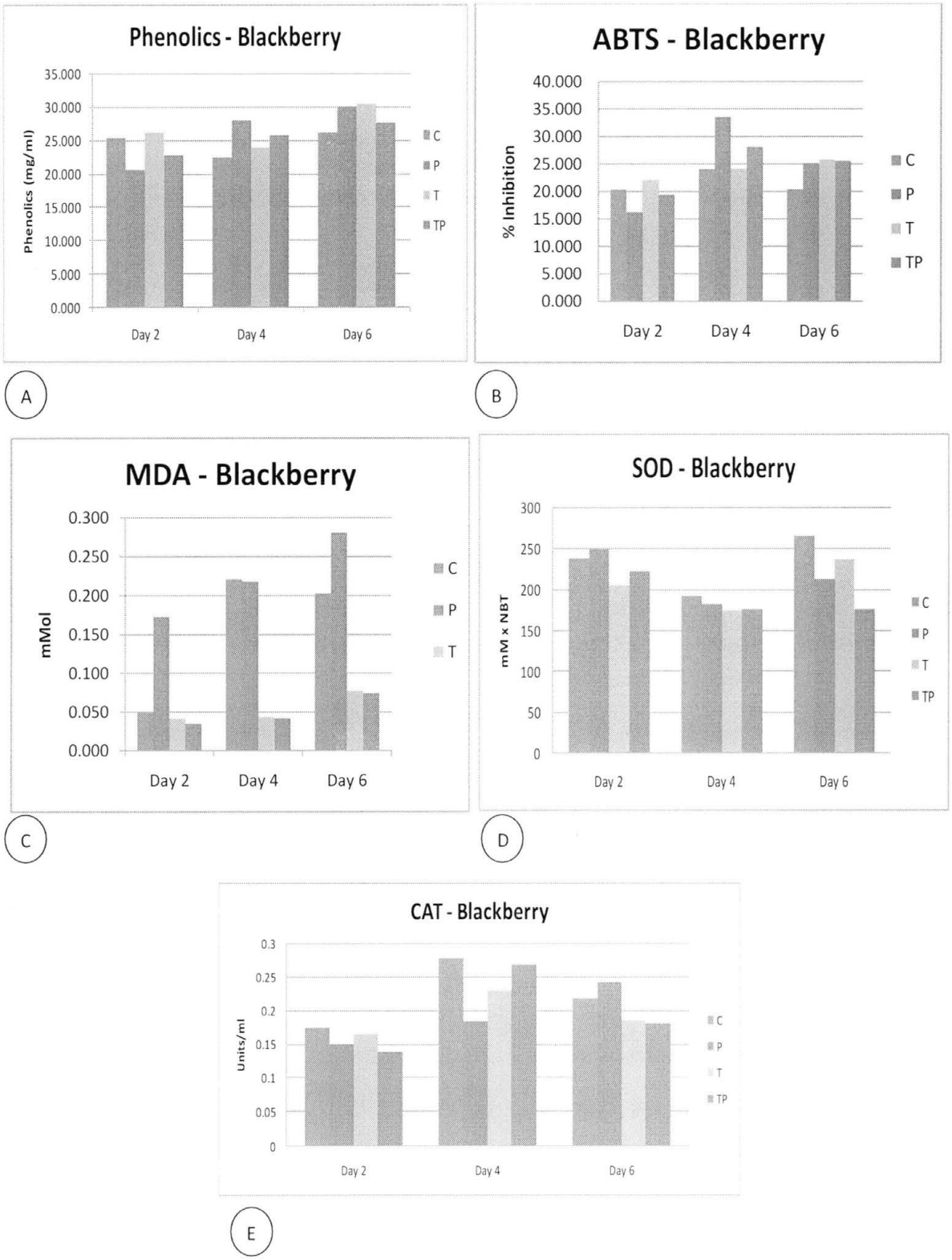

Figure 25. Effect of blackberry on oxidative stress in *Lumbricus terrestris*. A) Phenolic content, B) ABTS radical inhibition AOX activity, C) MDA formation, D) SOD activity by NBT formation, E) CAT activity.

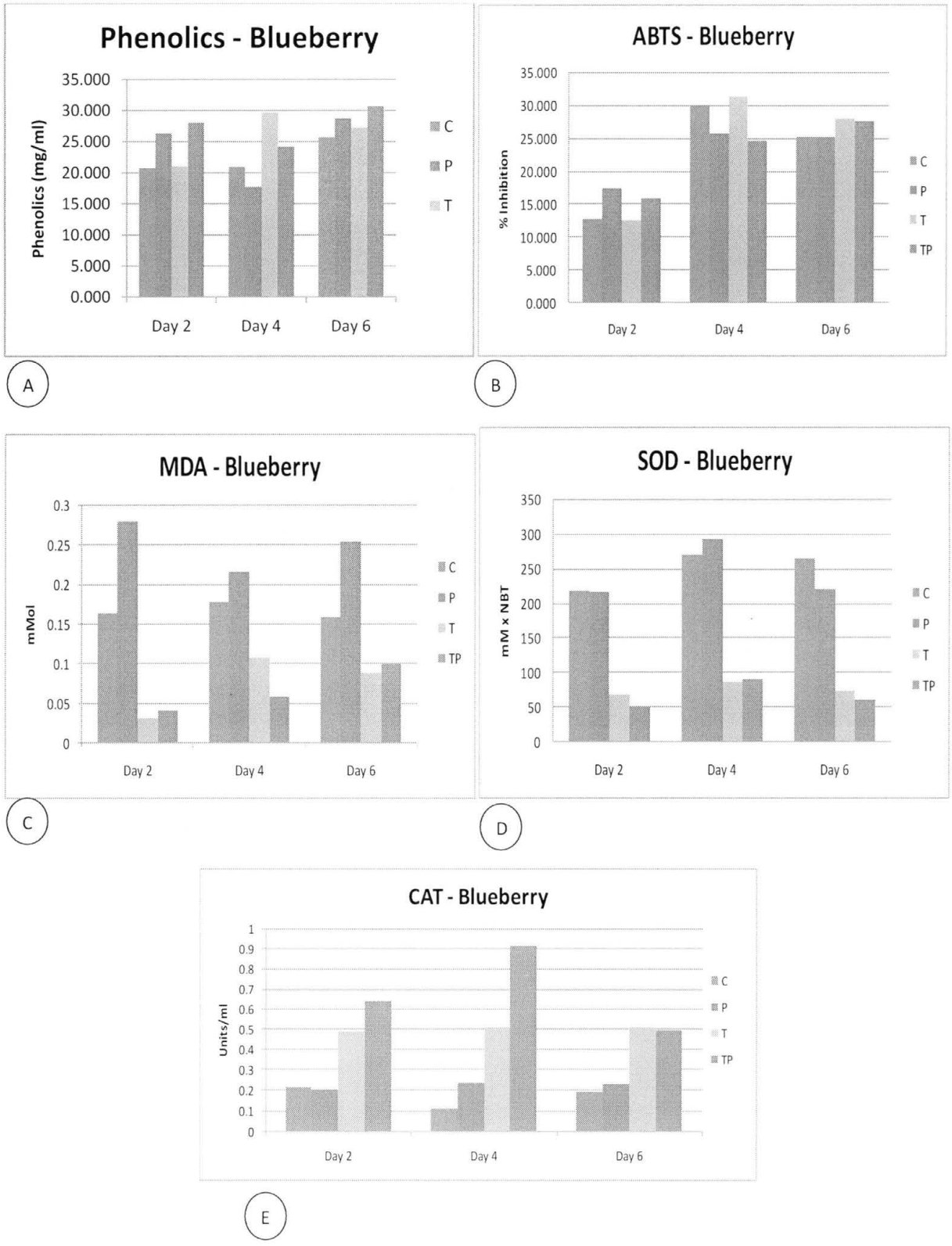

Figure 26. Effect of blueberry on oxidative stress in *Lumbricus terrestris*. A) Phenolic content, B) ABTS radical inhibition AOX activity, C) MDA formation, D) SOD activity by NBT formation, E) CAT activity.

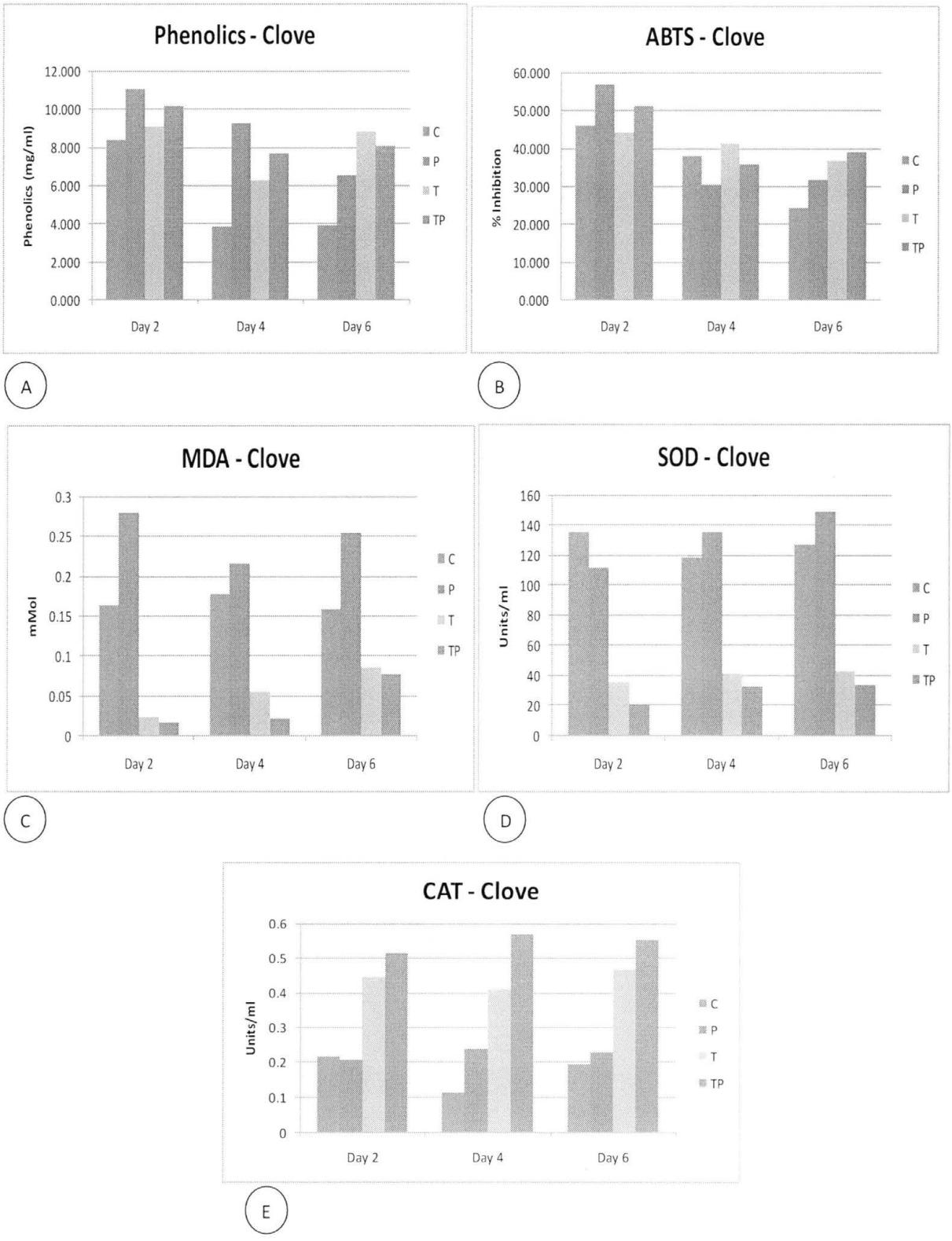

Figure 27. Effect of clove on oxidative stress in *Lumbricus terrestris*. A) Phenolic content, B) ABTS radical inhibition AOX activity, C) MDA formation, D) SOD activity by NBT formation, E) CAT activity.

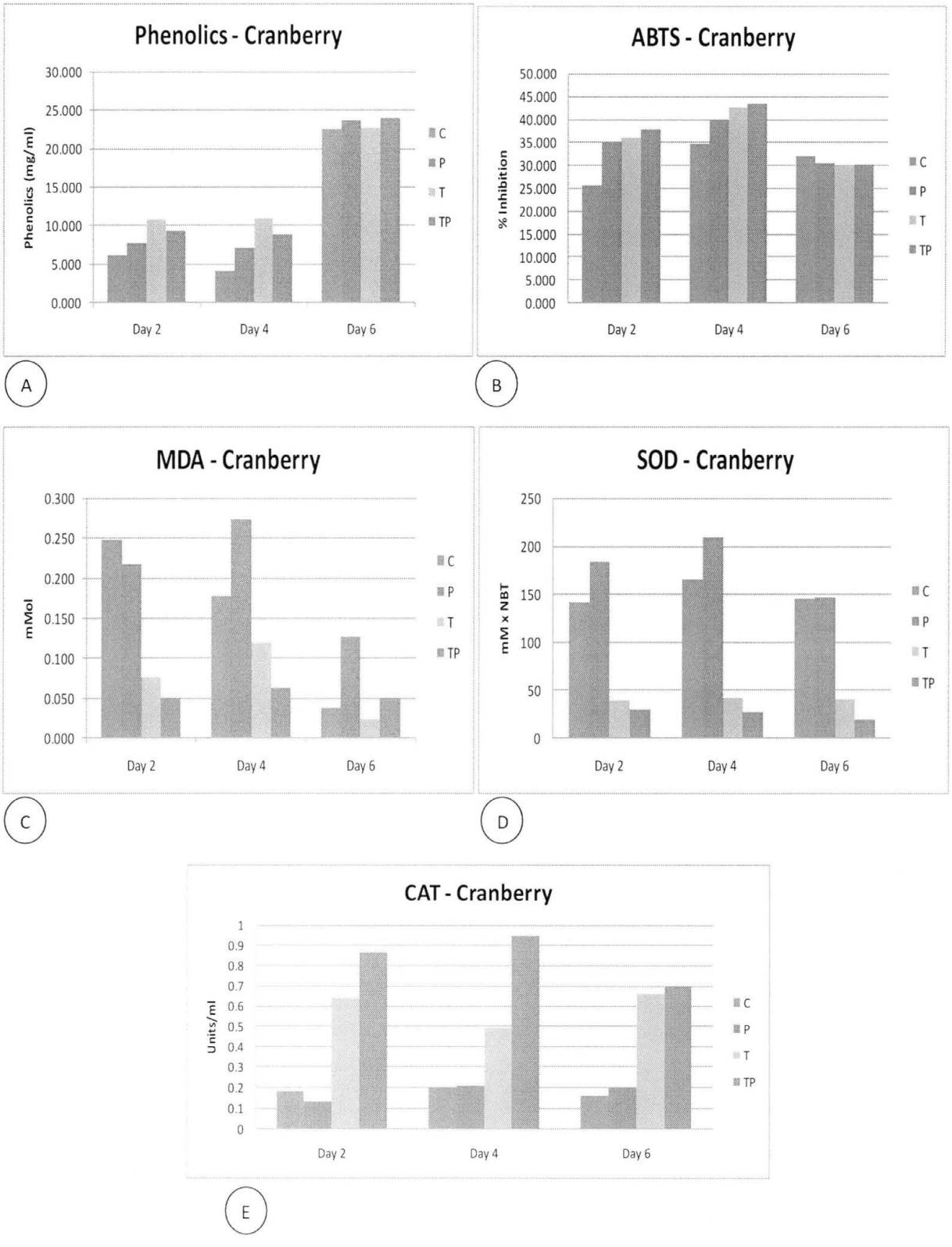

Figure 28. Effect of cranberry on oxidative stress in *Lumbricus terrestris*. A) Phenolic content, B) ABTS radical inhibition AOX activity, C) MDA formation, D) SOD activity by NBT formation, E) CAT activity.

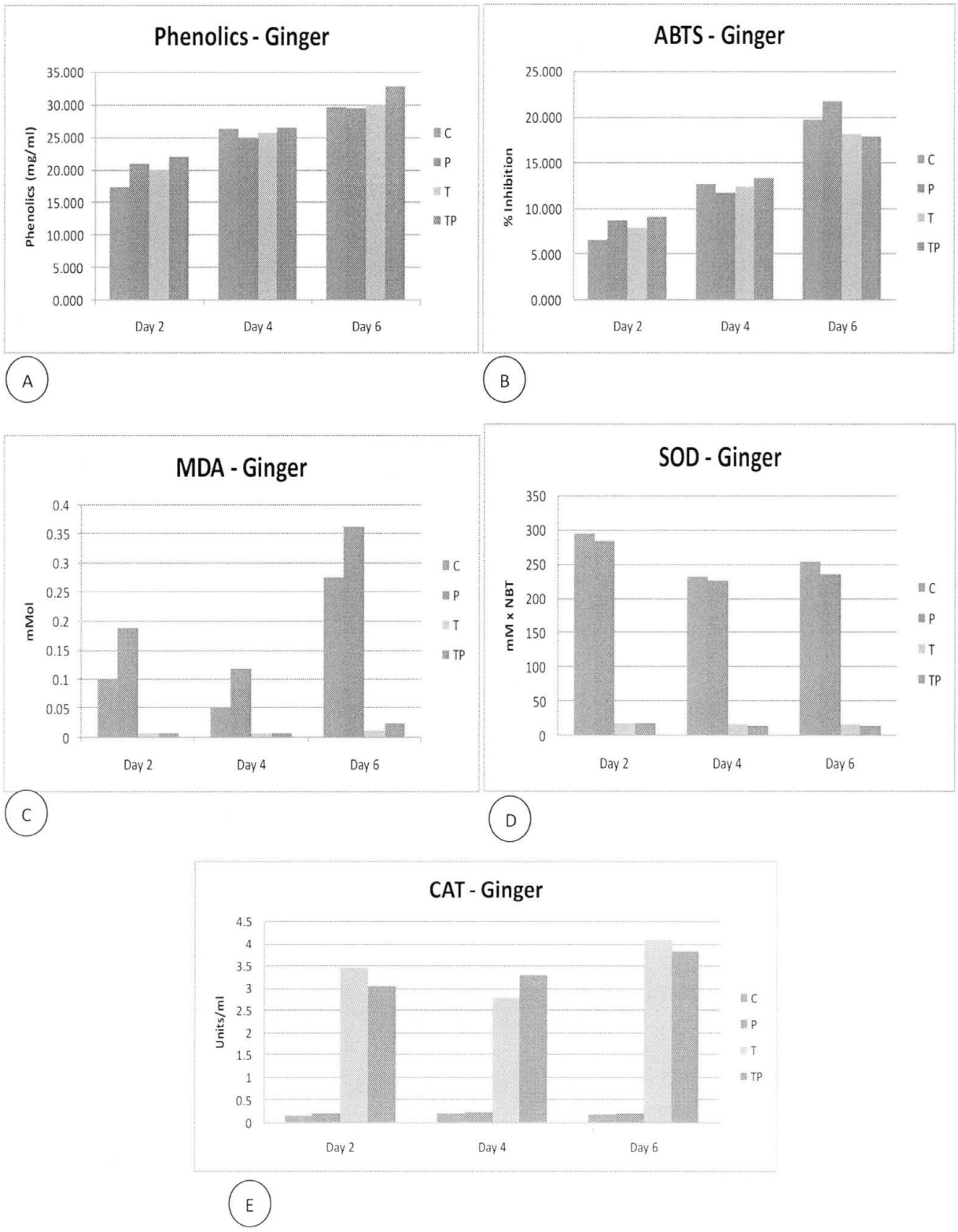

Figure 29. Effect of ginger on oxidative stress in *Lumbricus terrestris*. A) Phenolic content, B) ABTS radical inhibition AOX activity, C) MDA formation, D) SOD activity by NBT formation, E) CAT activity.

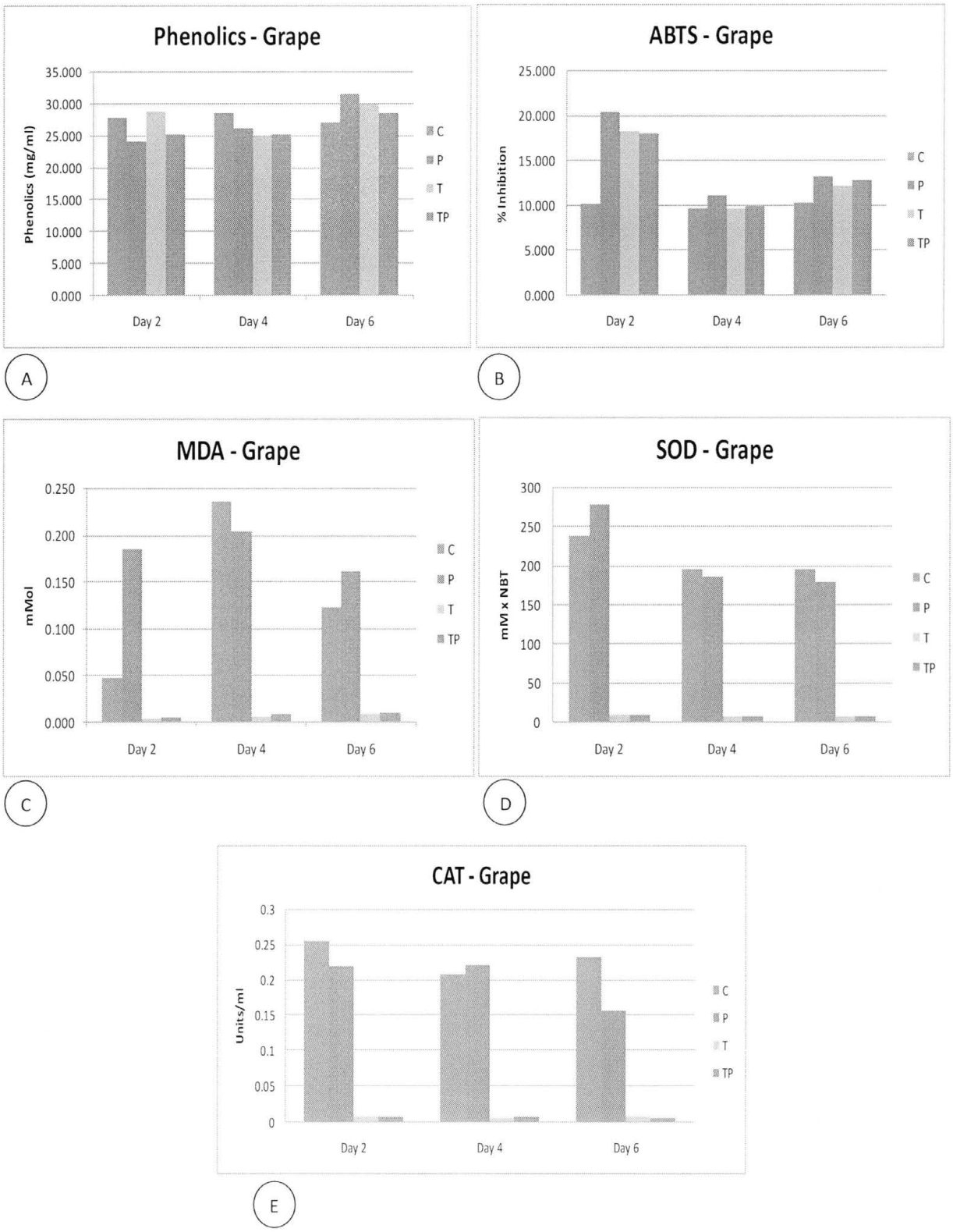

Figure 30. Effect of grape on oxidative stress in *Lumbricus terrestris*. A) Phenolic content, B) ABTS radical inhibition AOX activity, C) MDA formation, D) SOD activity by NBT formation, E) CAT activity.

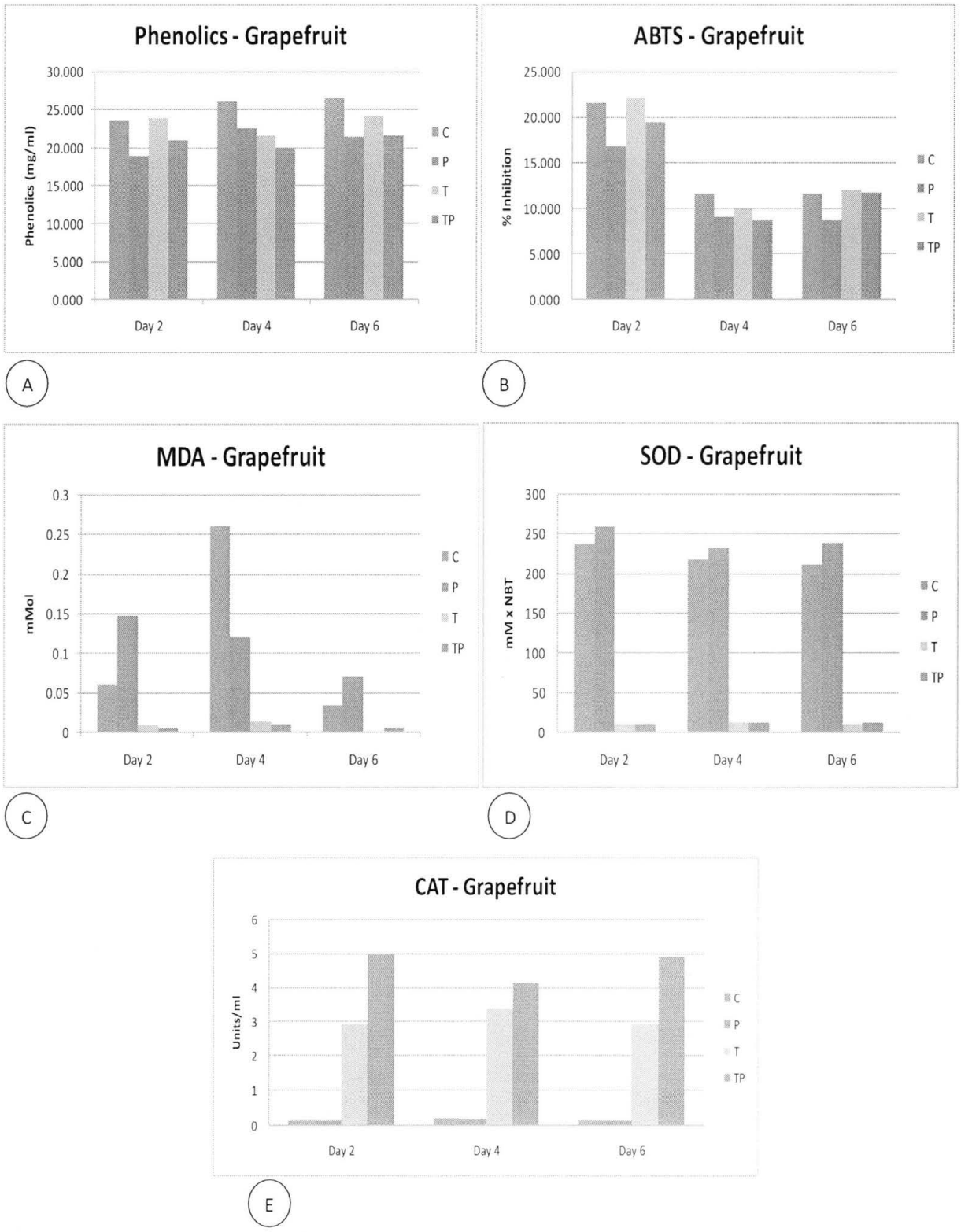

Figure 31. Effect of grapefruit on oxidative stress in *Lumbricus terrestris*. A) Phenolic content, B) ABTS radical inhibition AOX activity, C) MDA formation, D) SOD activity by NBT formation, E) CAT activity.

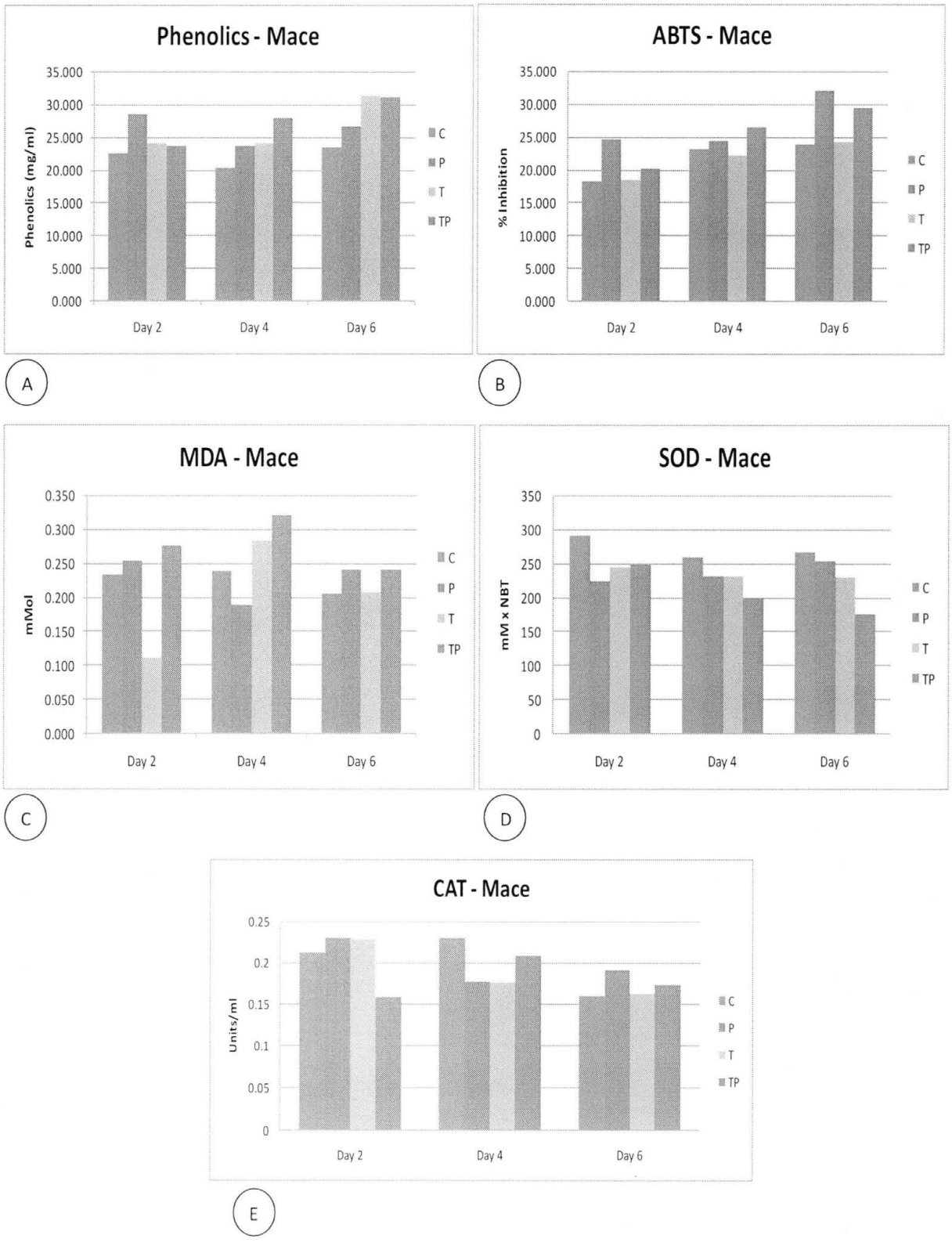

Figure 32. Effect of mace on oxidative stress in *Lumbricus terrestris*. A) Phenolic content, B) ABTS radical inhibition AOX activity, C) MDA formation, D) SOD activity by NBT formation, E) CAT activity.

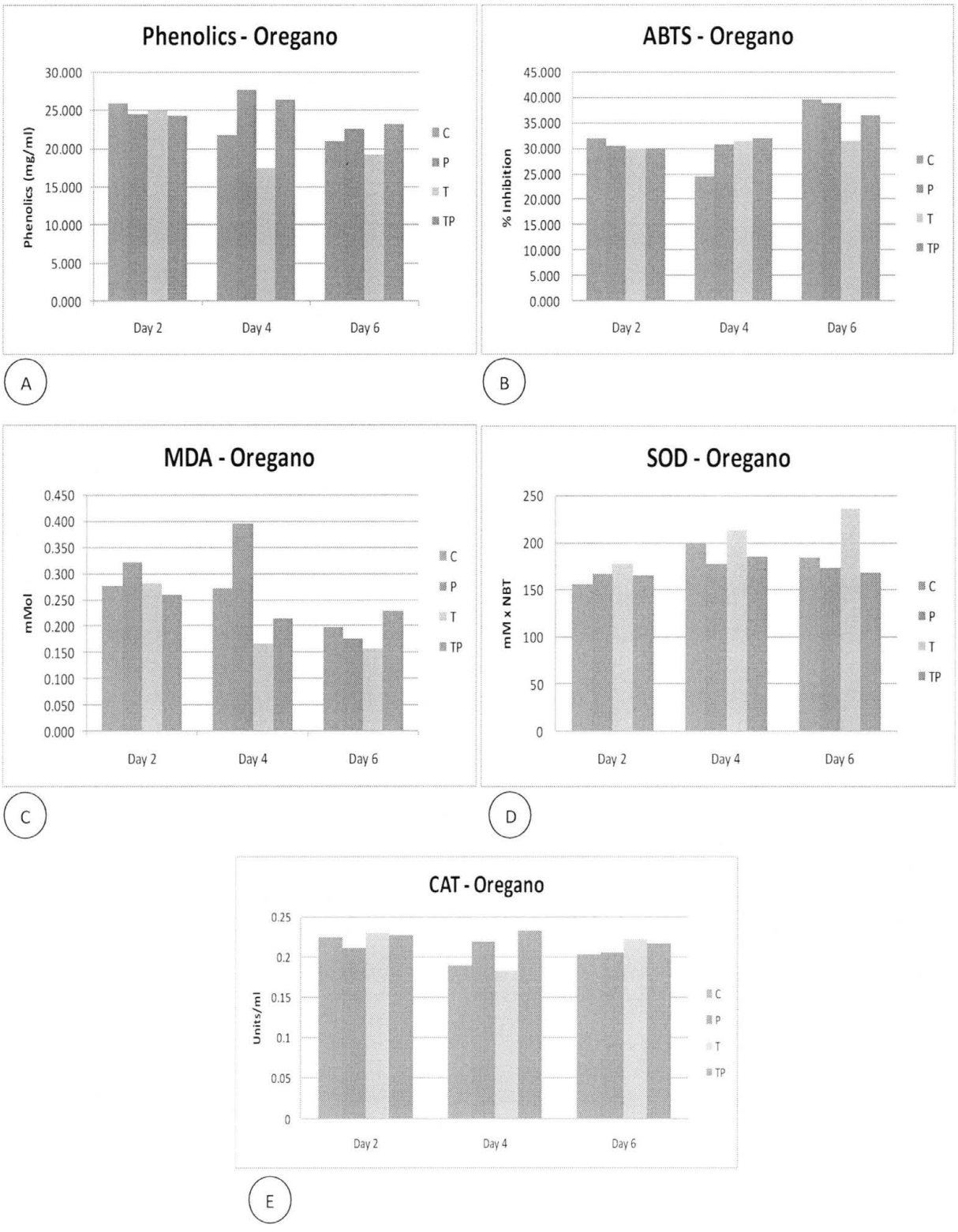

Figure 33. Effect of oregano on oxidative stress in *Lumbricus terrestris*. A) Phenolic content, B) ABTS radical inhibition AOX activity, C) MDA formation, D) SOD activity by NBT formation, E) CAT activity.

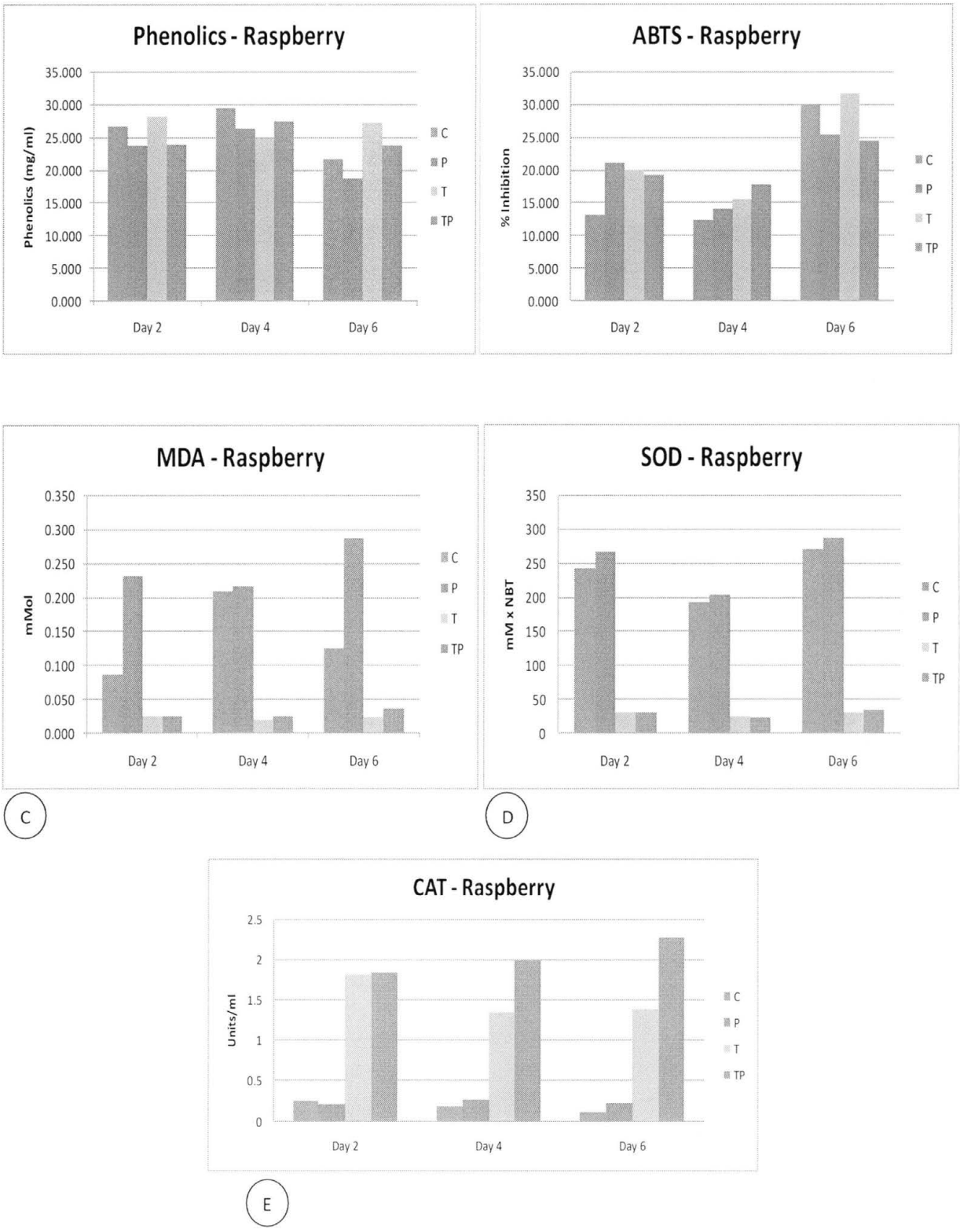

Figure 34. Effect of raspberry on oxidative stress in *Lumbricus terrestris*. A) Phenolic content, B) ABTS radical inhibition AOX activity, C) MDA formation, D) SOD activity by NBT formation, E) CAT activity.

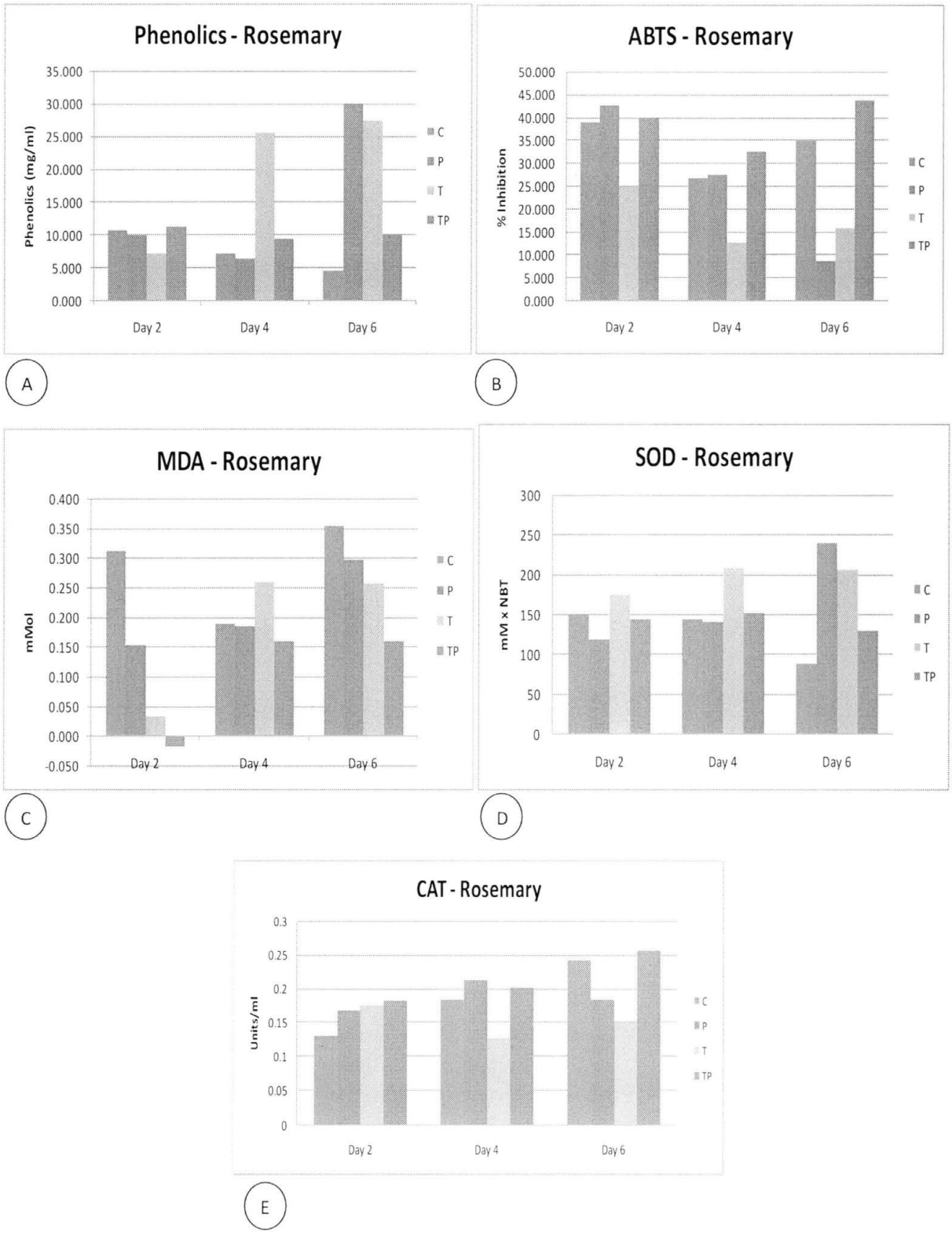

Figure 35. Effect of rosemary on oxidative stress in *Lumbricus terrestris*. A) Phenolic content, B) ABTS radical inhibition AOX activity, C) MDA formation, D) SOD activity by NBT formation, E) CAT activity.

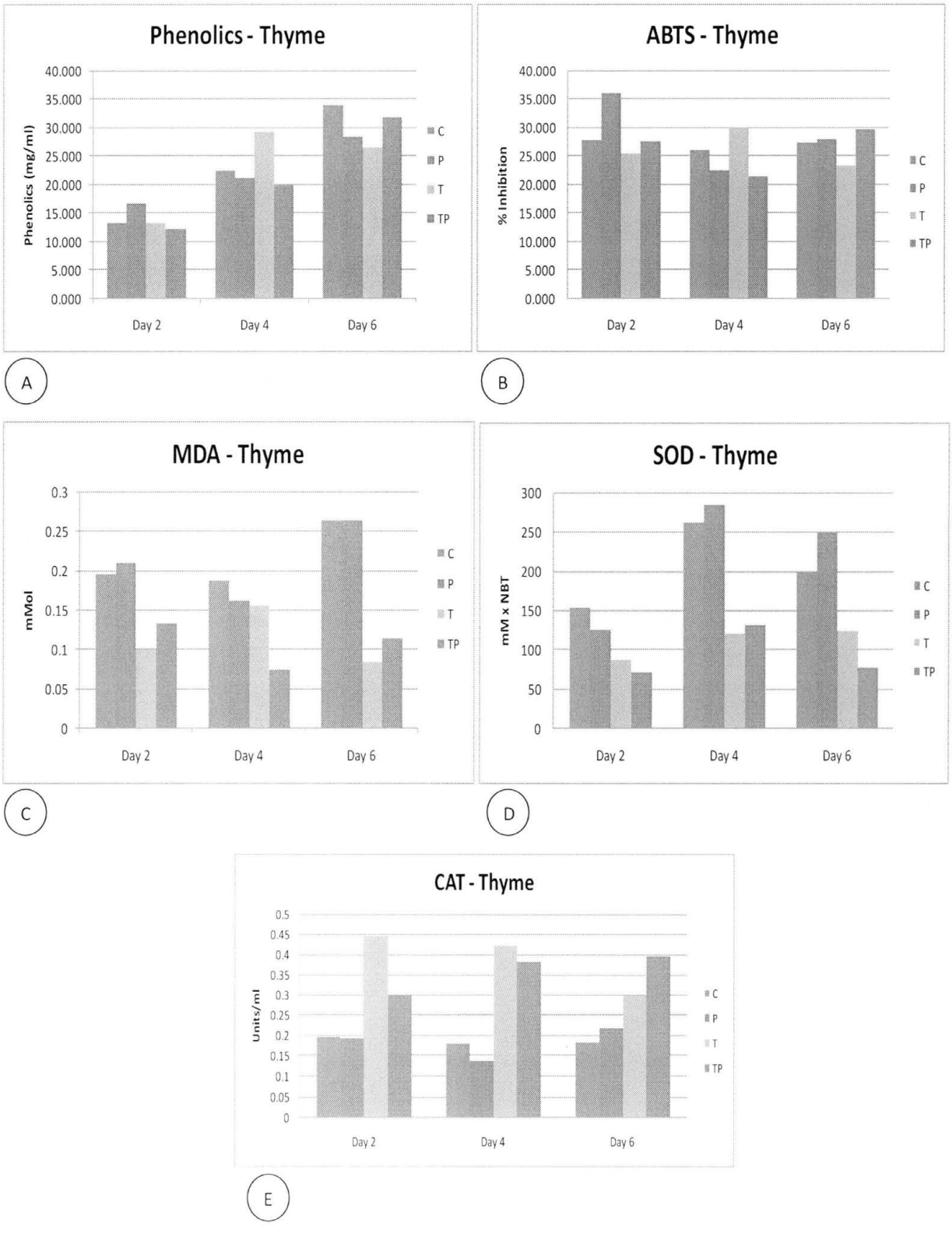

Figure 36. Effect of thyme on oxidative stress in *Lumbricus terrestris*. A) Phenolic content, B) ABTS radical inhibition AOX activity, C) MDA formation, D) SOD activity by NBT formation, E) CAT activity.

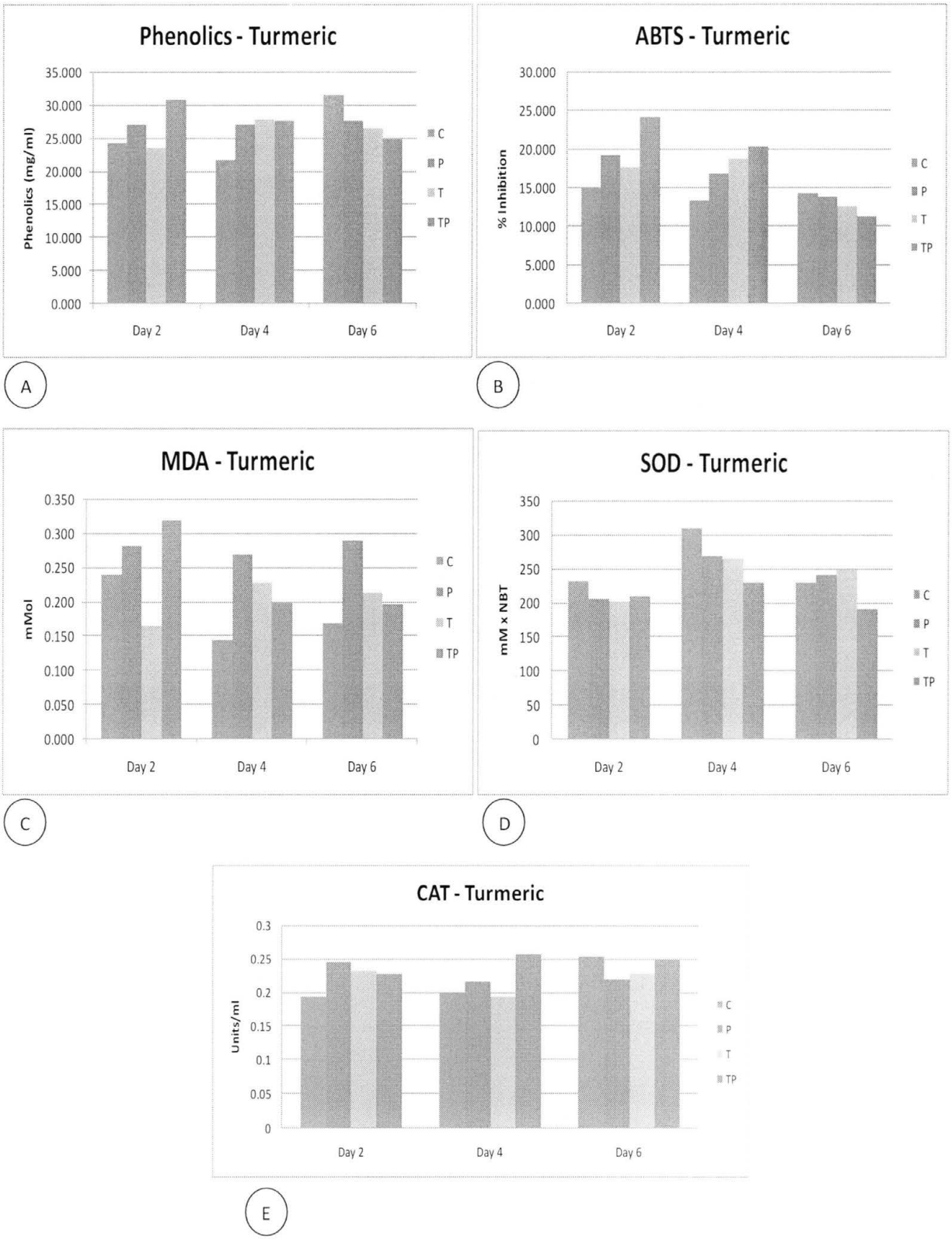

Figure 37. Effect of turmeric on oxidative stress in *Lumbricus terrestris*. A) Phenolic content, B) ABTS radical inhibition AOX activity, C) MDA formation, D) SOD activity by NBT formation, E) CAT activity.

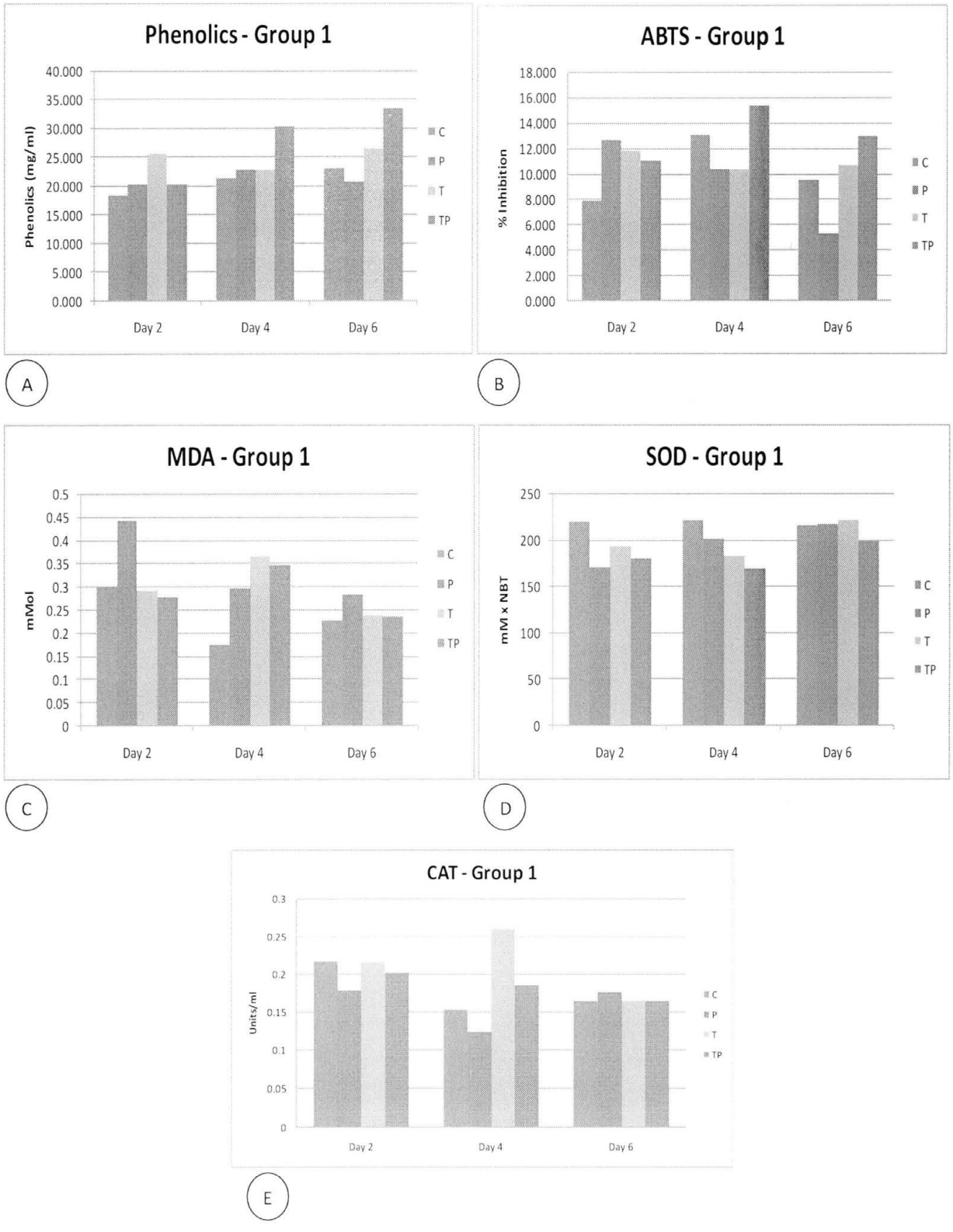

Figure 38. Effect of Group 1 polyphenolics on oxidative stress in *Lumbricus terrestris*. A) Phenolic content, B) ABTS radical inhibition AOX activity, C) MDA formation, D) SOD activity by NBT formation, E) CAT activity.

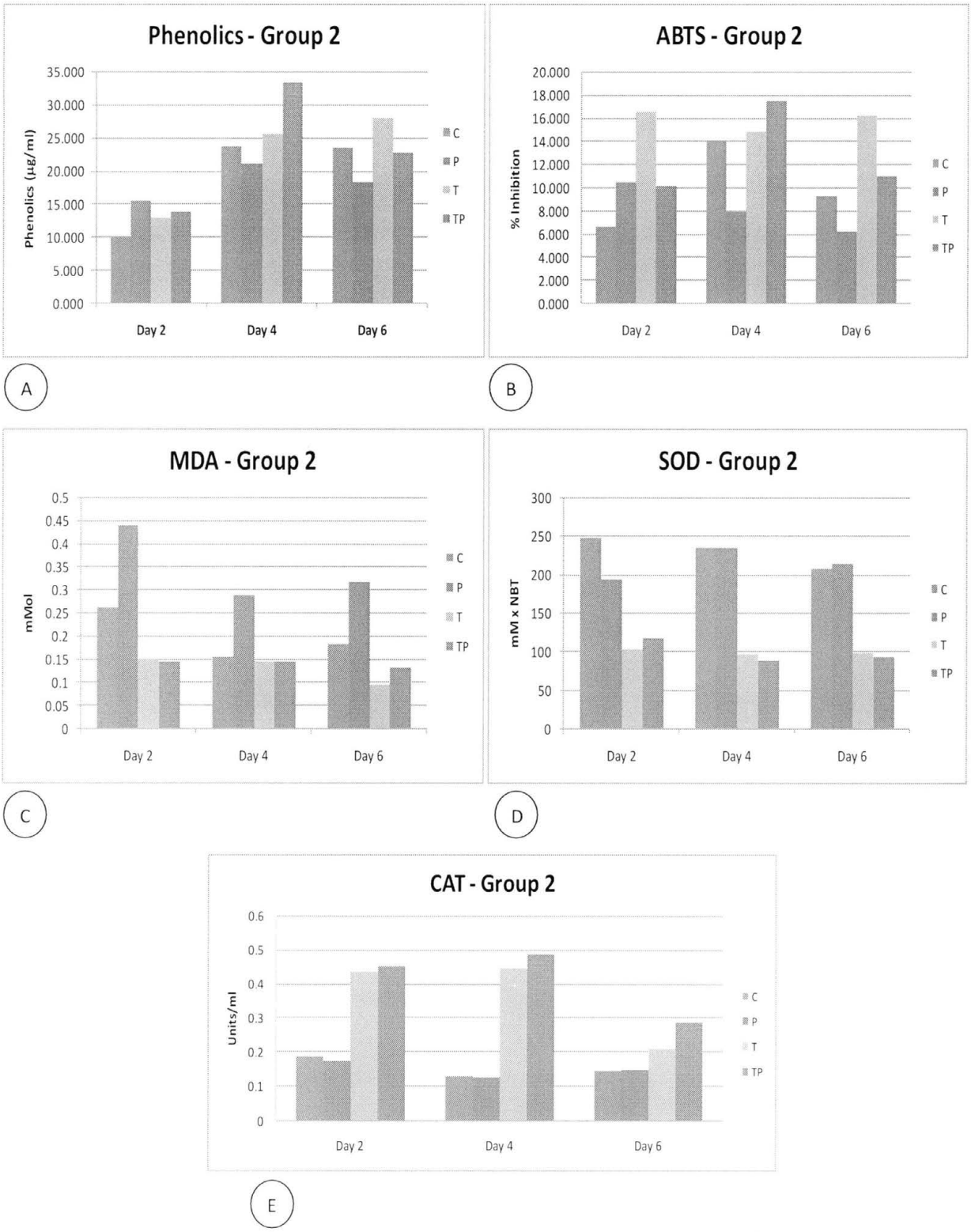

Figure 39. Effect of Group 2 polyphenolics on oxidative stress in *Lumbricus terrestris*. A) Phenolic content, B) ABTS radical inhibition AOX activity, C) MDA formation, D) SOD activity by NBT formation, E) CAT activity.

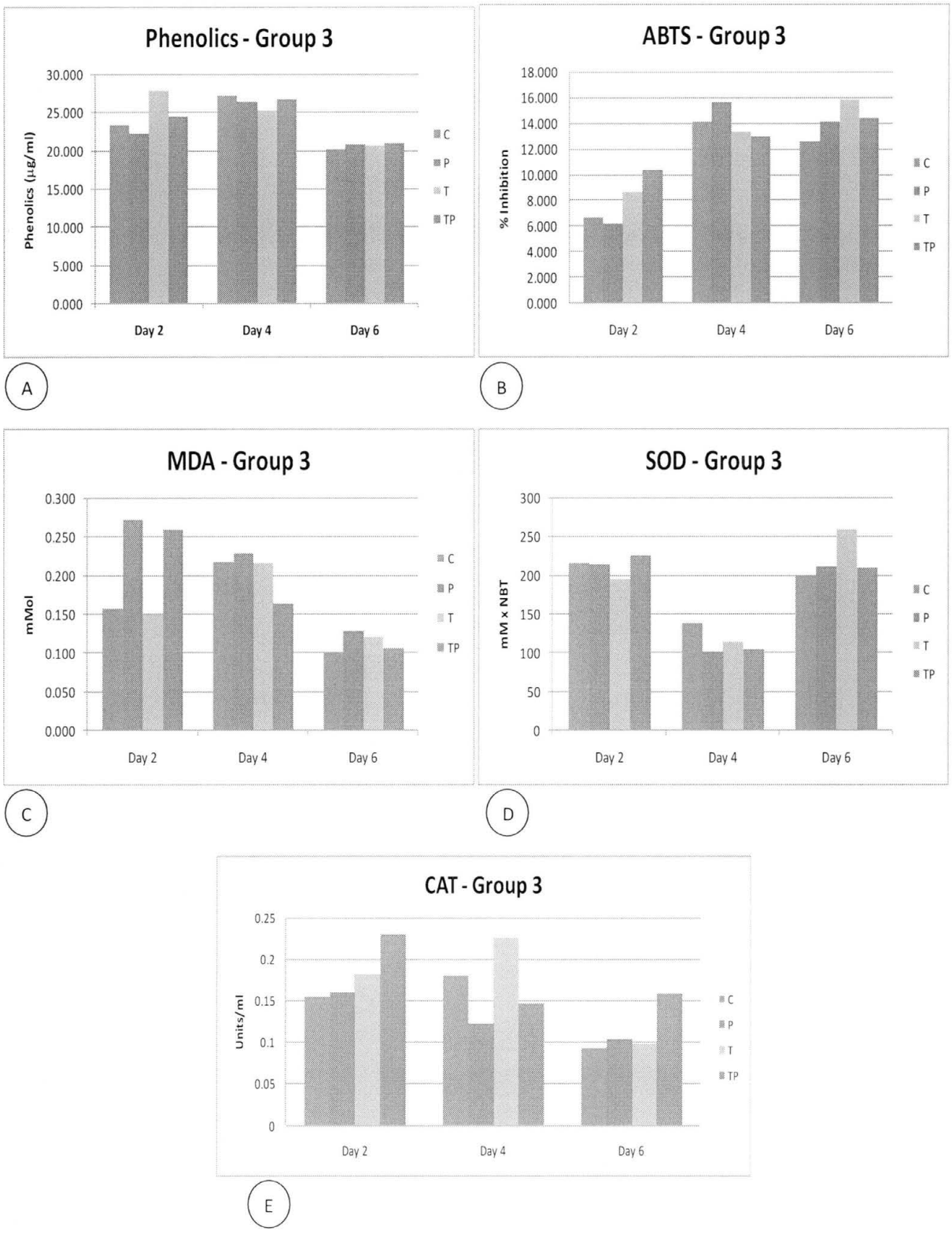

Figure 40. Effect of Group 3 polyphenolics on oxidative stress in *Lumbricus terrestris*. A) Phenolic content, B) ABTS radical inhibition AOX activity, C) MDA formation, D) SOD activity by NBT formation, E) CAT activity.

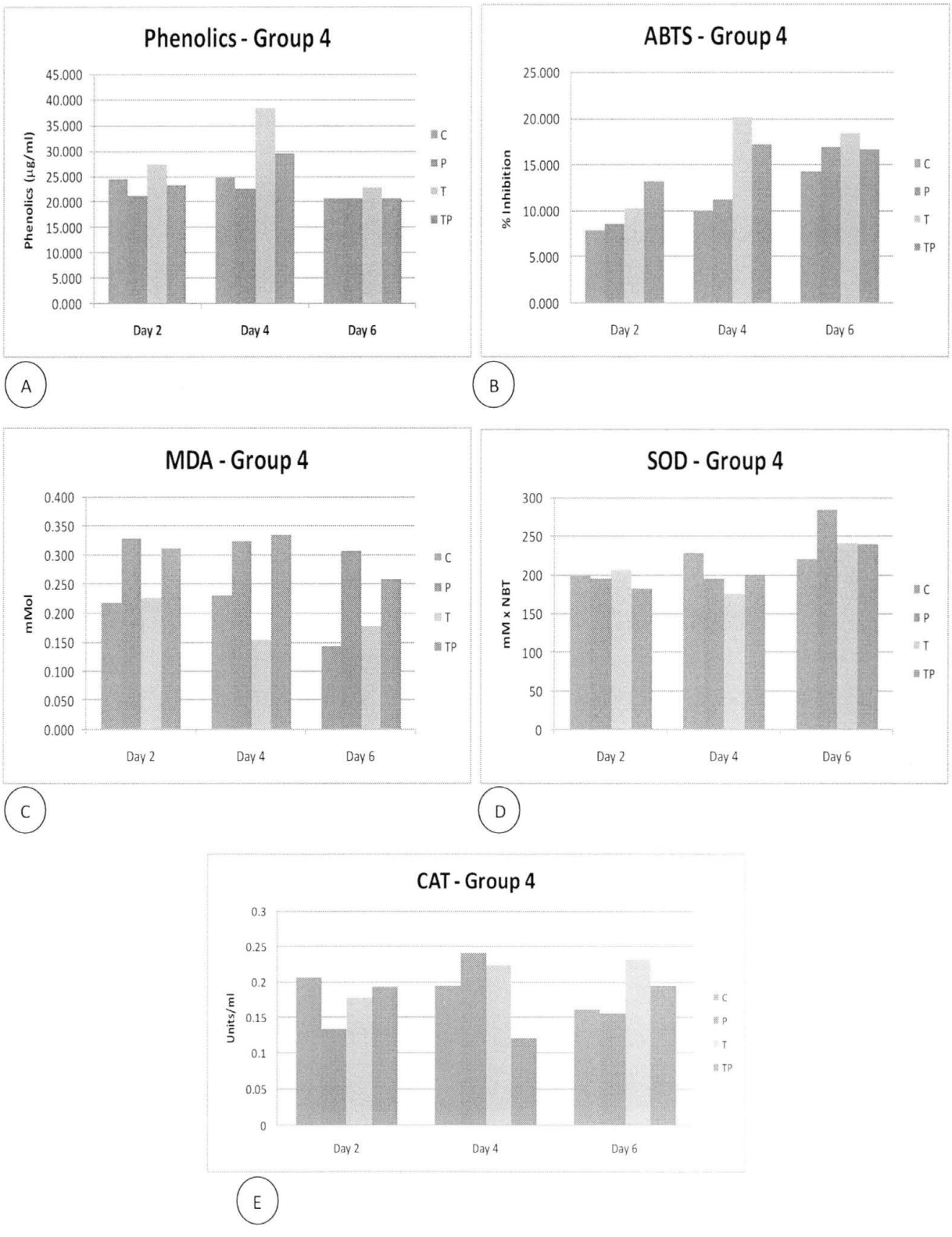

Figure 41. Effect of Group 4 polyphenolics on oxidative stress in *Lumbricus terrestris*. A) Phenolic content, B) ABTS radical inhibition AOX activity, C) MDA formation, D) SOD activity by NBT formation, E) CAT activity.

VITA

Trey Steven Hutton was born in Colorado Springs, Colorado, on August 7th, 1982, the son of Billie Sue Scales and William Steven Hutton. After completing his work at Salado High School, Salado, Texas, in 2000, he entered Texas State University-San Marcos. He received the degree of Bachelor of Science from Texas State in December 2006. In January 2007, he entered the Graduate College of Texas State University-San Marcos.

Permanent Address: 200 Robbie Lane

San Marcos, Texas 78666

This thesis was typed by Trey Steven Hutton.