Journal of Medical Sciences

ISSN 1682-4474

science alert

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Research Paper

J. Med. Sci., 13 (7): 514-525
1st October, 2013
DOI: 10.3923/jms.2013.514.525

Effect of Dietary Bioactives on *in vivo* Peroxide Induced Stress in *Lumbricus terrestris*

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Oxidative stress has been linked to several chronic diseases, including heart disease and cancer. Phenolic phytochemicals abundant in natural products have high antioxidant activity and can potentially reduce the effects of oxidative stress. However, their antioxidant response mechanism *in vivo* is not very well understood. Present objective was to investigate the effect of several natural products on modulating the antioxidant response *in vivo* in Hydrogen Peroxide (HP) stressed *Lumbricus terrestris*. Sexually mature *L. terrestris* were randomly divided into 4 treatment groups and were allowed to feed *ad libitum* on 1.25% Gerber-oatmeal Agar (GA). The animals in control, peroxide, treatment and treatment-peroxide groups, fed on GA, GA+0.13% HP, GA+10% extract and GA+0.13% HP+10% extract, respectively. Two animals from each group were euthanized after 2 d, 4 d and 6 d and 1 inch of circular muscle posterior to the clitellum was dissected and extracted. Malondialdehyde (MDA) levels were determined to assess peroxide induced lipid oxidation. The antioxidant response was determined by measuring the radical quenching activity of muscle, the levels of Superoxide Dismutase (SOD) and Catalase (CAT) using standard methods. Present results indicated that compared to controls, animals on grapefruit, grape, ginger and cranberry diet had the lowest MDA levels and high radical quenching activity. Animals on grapefruit, ginger, raspberry and cranberry diet had 3-7 fold more muscle CAT levels. SOD levels in animals on turmeric, mace, blackberry and oregano diet was almost 100-200 fold higher. Our results suggest that dietary phytochemicals can manage acute oxidative stress *in vivo* by inducing antioxidant enzyme response mediated by SOD and CAT.

Key words: Oxidative stress, antioxidant activity, superoxide dismutase, catalase, lumbricus terrestreis

JMS (ISSN 1682-4474) is an International, peer-reviewed scientific journal that publishes original article in experimental & clinical medicine and related disciplines such as molecular biology, biochemistry, genetics, biophysics, bio-and medical technology. JMS is issued eight times per year on paper and in electronic format.

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INTRODUCTION

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 *et al*., 2004). Under certain conditions, however, free radical production increases to the point that it exceeds the detoxification capacity of cellular antioxidants (Konig *et al*., 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 *et al*., 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 *et al*., 1997; Sastre *et al*., 1992; Thompson *et al*., 2001) and/or long duration (Kanter, 1998; Mastaloudis *et al*., 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 *et al*.,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 *et al*., 2005; Gomez-Cabrera *et al*., 2005; McAnulty *et al*., 2005; Vassilakopoulos *et al*., 2005). The antioxidants most commonly investigated in athletes have been vitamins E and C (Alessio *et al*., 1997; Buchman *et al*., 1999; Goldfarb *et al*., 2005; Itoh *et al*., 2000; Simon-Schnass and 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 *et al*., 1979; Itoh *et al*., 2000; Nieman *et al*., 2004; Simon-Schnass and Pabst, 1988; Satoshi *et al*., 1989; Surmen-Gur *et al*., 1999). For example, Helgheim *et al*. (1979) reported that muscle damage following heavy exercise was not decreased in subjects who ingested 300 mg day^{-1} of vitamin E for 6 weeks. In contrast, after a comparable bout of exercise, Satoshi *et al*. (1989) found that supplementing athletes with 300 mg day^{-1} 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 *et al*., 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^{-1}) for 8 weeks increased antioxidant enzyme response and attenuated EIOS following 45 min of cycling at 70% of VO2peak, compared to a placebo. However, when a higher dosage of vitamin C was ingested regularly (i.e., 2 g day^{-1}) 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 *et al*., 2007; Urso and 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 *et al*., 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 *et al*., 2005). Polyphenolics are products of the secondary metabolism of plants and exhibit a wide range of antioxidant properties (Urquiaga and Leighton, 2000). The hydrogen donating substituents attached to the aromatic rings of the polyphenolic compounds allow polyphenolics to effectively scavenge free radicals (Pari and Suresh, 2008). While a few studies have shown actual *in vivo*

functionality of polyphenolics (Dunlap *et al*., 2006; Nakazato *et al*., 2007; O'Byrne *et al*., 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 animals is primarily muscle; (2) The immune function of the earthworm is sufficiently analogous to that of vertebrates; (3) Animals are inexpensive and easy to maintain in the laboratory and (4) Animals can be quickly exposed to a test substance and rapidly harvested for analysis (Cooper *et al*., 2002; Goven *et al*., 1994). Based on previous research regarding polyphenolic supplementation (O'Byrne *et al*., 2002; 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.

MATERIALS AND METHODS

Fruit, herb and spice samples: Freeze dried powders of Basil (BA), Blackberry (BK), Blueberry (BU), Clove (CL), Cranberry (CR), Ginger (GI), Grape (GP), Grapefruit (GPF), Mace (MA), Oregano (OR), Raspberry (RA), Rosemary (RO), Thyme (TH), Turmeric (TU) were gifts from Nutrafuncfoods LLC (Champaign, IL). Five hundred milligrams of samples were suspended in 10 mL of distilled water and extracted on a rotary shaker for 15 min. The samples were then centrifuged and supernatants were used as treatments.

Total polyphenolics assay: The increase in polyphenolic content of the muscle was measured to determine the overall absorption and bioavailability. Total polyphenolics were measured using a protocol described previously (Vattem *et al*., 2005). Briefly, polyphenolics was measured as gallic acid equivalents using the Folin Ciocalteu method. Two hundred and fifty microliters 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 min, 0.25 mL of 5% Na_2CO_3 was added, again vortexed and the solution were kept in the dark for 1 h. The absorbance of the samples was measured at 725 nm using a UV spectrophotometer (Thermo Biomate 3; Houston, TX).

ABTS (2, 2'-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid)) 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 min 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:

$$\text{Inhibition (\%)} \frac{\text{AB-AS}}{\text{AB}} \times 100$$

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 min. 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 min at 100°C. After incubation, tubes were centrifuged at 13,000 g for 10 min 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.

Lumbricus terrestris Culture and Maintenance Sexually mature worms (identified based on the presence of clitellum) and thoroughly washed in DH_2O to ensure their skin was free from any soil or debris. The worms were then transferred to a Gerber-Agar (GA) petri plates (1.25% Agar, 0.31% gerber oatmeal, single grain) for 24 h to clear the digestive tract of any soil. After 24 h, twenty eight worms were divided into 4 groups and placed on different GA plates labeled as a C (Control), P (Peroxide; 0.13% H_2O_2), T (Treatment; 10% v/v extract)

and TP (Treatment+Peroxide). The worms were allowed to feed on the plates at 20°C in dark and sampled every 48 h for 6 days. Worms for sampling were cleaned by washing and their gut was cleared by gently massaging the worm along its length. The worms were then euthanized at -20°C for 30 min and a 1 inch muscle sample excised distal to the clitellum and homogenized in 5 mL of 0.1 M Sodium Phosphate Buffer using a mortar and pestle and centrifuge at 5000 g for 30 min. The total phenolic content of the tissue extracts was determined as described above.

Malondialdehyde (MDA) assay: The MDA concentration in homogenate was determined by its reaction with thiobarbituric acid (TBA). In a test tube 0.8 mL of the tissue homogenate was mixed with 500 μLof 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 min. 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}.

Superoxide dismutase (SOD)-riboflavin-nbt assay: Two hundred microliters of the tissue homogenate was mixed with 1.5 mL of the reaction mixture (2 mM riboflavin, 50 mM KH_2PO_4 buffer (pH 8.0), 0.1 mM EDTA, 200 μM DTPA and 57 μM NBT). The test tubes were incubated in a fluorescent light chamber for 20 min and absorbance was measured at 560 nm. The reduction in the oxidation of NBT by the superoxide radicals generated by photooxidation of riboflavin was measured in homogenates and compared to the controls.

Catalase (CAT) assay: One hundred microlitter of the tissue homogenate was added to a reaction mixture containing 1.9 mL of DH_2O, 1 mL of 0.059 M H_2O_2 in 0.05 M potassium phosphate, pH 7.0. The disappearance of H_2O_2 was followed at A_{240} nm for 3 min.

RESULTS

Total phenolics content and antioxidant activity of different extracts: 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

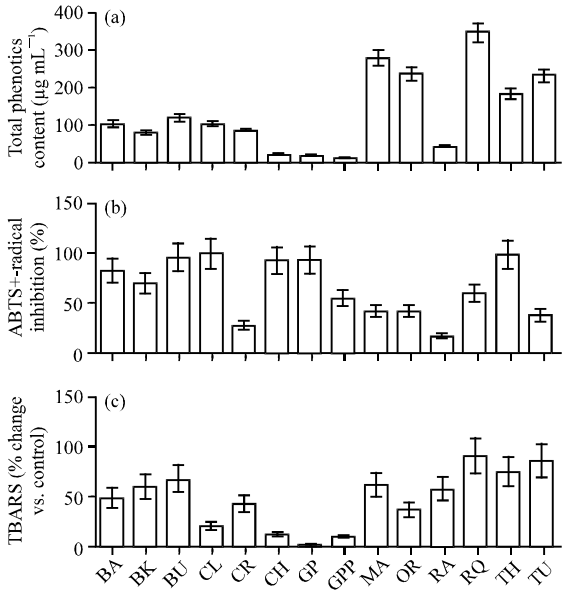

Fig. 1(a-c): Antioxidant activity determined by (a) Total phenolic content (b) ABTS and (c) TBRS of various dietary extracts

(Fig. 1a). 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 (Fig. 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 (Fig. 1b). 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 (Fig. 1b). 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 (Fig. 3). This was followed by BK, RA, BA, CR and OR, which inhibited TBARS formation by 60, 58, 48, 43 and 37%, respectively (Fig. 1c). CL, GI, GP and GPF all decreased TBARS formation by less than 20% (Fig. 1c).

***In vivo* bioavailability of extracts**

Polyphenolic content: After the ingesting the single extracts the amount of polyphenolics absorbed into the

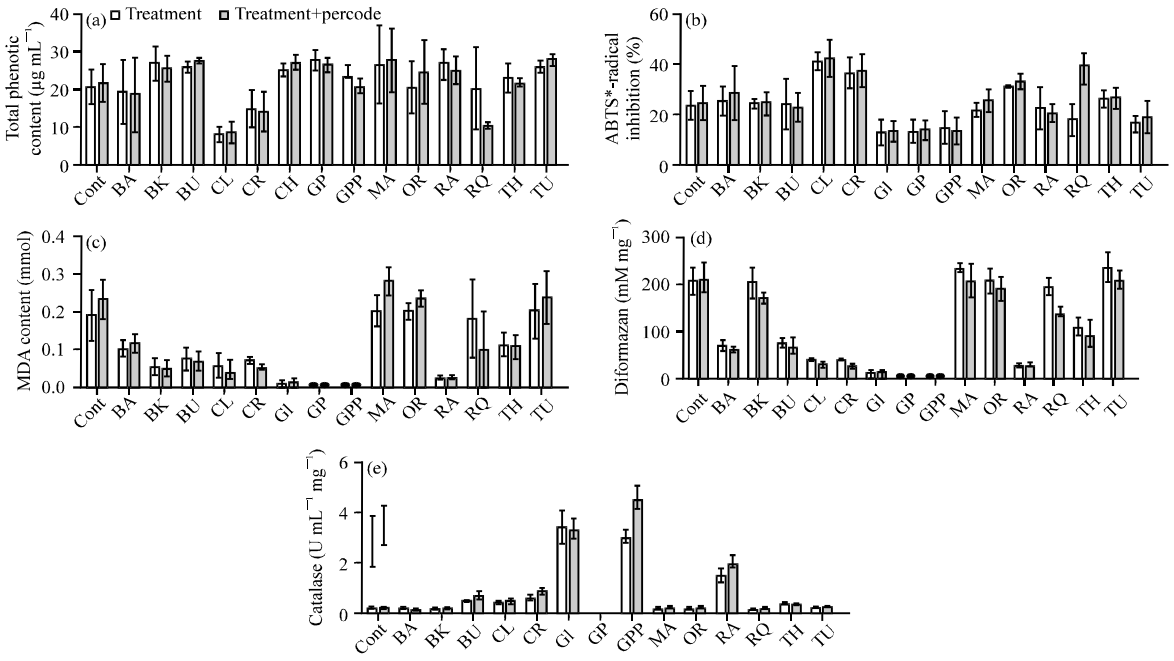

Fig. 2(a-e): Total phenolic content, (a) Antioxidant activity ABTS, (b) MDA formation, (c) SOD activity, (d) CAT activity in *lumbricus* muscle tissue post treatment with H_2O_2 (CONT) and (e) extracts with H_2O_2

animals 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) animals ingesting the TU extract had the highest total amount of bioavailable polyphenolics. This was followed in polyphenolic bioavailability by the TP animals 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 (Fig. 2a).

In the group fed Treatment (T) only, the animals which ingested the GP extract had the highest total amount of bioavailable polyphenolics. This was followed in polyphenolic bioavailability by the animals 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 (Fig. 2a).

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 animals ingesting the CL extract had the most powerful ABTS neutralizing effect. The CL extract inhibited 42% of the ABTS radicals (Fig. 2b). was followed by the TP animals ingesting the RO, CR, OR and BA extracts which reduced ABTS formation by 38, 37, 32 and 28%, respectively (Fig. 2b). TH, MA, BK, BU and RA reduced ABTS radicals by 26, 25, 24, 22 and 20%, respectively (Fig. 2b). TU, GP, GI and GPF all decreased ABTS by less than 20% (Fig. 2b). In the T only group, the animals which ingested the CL extract had the most powerful ABTS neutralizing effect. The CL extract inhibited 40% of the ABTS radicals (Fig. 2b). This was followed in ABTS inhibition by the T animals ingesting the CR, OR, TH and BA extracts which reduced ABTS formation by 36, 31, 26 and 25%, respectively (Fig. 2b). BK, BU, RA, MA and RO reduced ABTS radicals by 24, 23, 22, 21 and 17%, respectively (Fig. 2b). TU, GPF, GP and GI all decreased ABTS by less than 17% (Fig. 2b).

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 animals stressed with H_2O_2 and contained

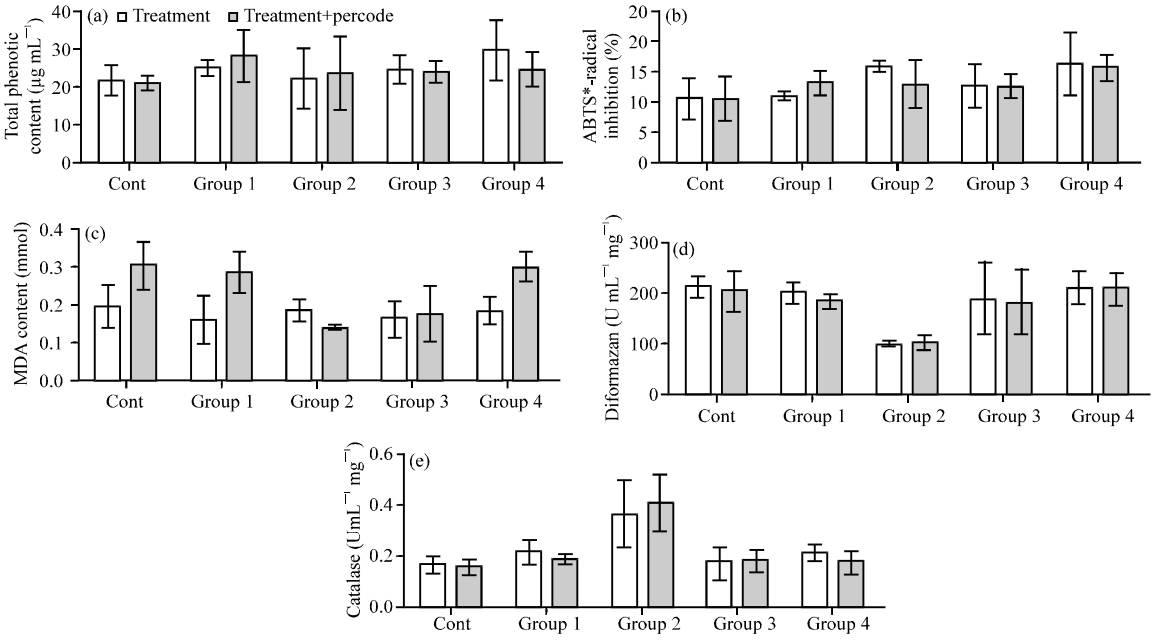

Fig. 3(a-e): Total phenolic content (a) Antioxidant activity by ABTS assay, (b) MDA formation (c) SOD activity (d) CAT activity in *lumbricus* muscle tissue post treatment with H_2O_2 (CONT) and (e) extracts groups and extracts groups with H_2O_2

a polyphenolic treatment (TP group) showed a different trend. Our results indicate that compared to the control, the TP animals ingesting the GP extracts had the lowest total amount of MDA. This was followed by the TP animals 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 (Fig. 2c). In the group fed T only, the animals which ingested the GP extracts had the lowest total amount of MDA. This was followed by the T animals 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 (Fig. 2c).

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 animals ingesting the TU extract were the most effective in increasing enzyme expression. This was followed by the TP animals 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 (Fig. 2d). In the group fed T only, the animals which ingested the TU extract had the highest increase in SOD expression. This was followed in SOD expression by the animals 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 (Fig. 2d).

Catalase (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 animals ingesting the GPF extract was the most effective in increasing CAT enzyme expression. This was followed by the TP animals 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 (Fig. 2e). Of the animals 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 animals 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 animals (Fig. 2e).

Table 1: Arbitrary ranking of extracts based on effectiveness in increasing SOD and CAT activities

Rank	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

Overall effects if extracts *in vivo* and ranking: 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). To form the extract groups, 0.25 g of each extract was used to create the mixture and extracted in 20 mL 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%.

***In vivo* bioavailability and effects of extract combinations:** *Polyphenolic Content.* After the animals 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 animals ingesting the GP1 absorbed the greatest amount of bioavailable polyphenolics. This was followed in polyphenolic bioavailability by the animals ingesting the GP4 (Group 4), GP3 (Group 3) and GP2 (Group 2) extracts (Fig. 3a). The T animals ingesting the GP4 extracts had the highest total amount of bioavailable polyphenolics. This was followed in polyphenolic bioavailability by the T animals ingesting the GP1, GP3 and GP2 extracts (Fig. 3a).

Table 2: Groups of extracts formed by stoichiometric combination of first four extracts from Table 1

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

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 animals 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 animals ingesting the GP1, GP2 and GP3 extracts which reduced ABTS formation by 13.1, 12.9 and 12.5%, respectively (Fig. 3b). The T animals 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 animals ingesting the GP2, GP3 and GP1 extracts which reduced ABTS formation by 15.8, 12.6 and 10.9%, respectively (Fig. 3b).

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 animals 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 animals ingesting the GP2 extracts had the lowest total amount of MDA. This was followed by the TP animals ingesting the GP3, GP1 and GP4 extracts (Fig. 3c). The T animals ingesting the GP2 extracts had the lowest total amount of MDA. This was followed by the T animals ingesting the GP3, GP4 and GP1 extracts (Fig. 3c).

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 animals ingesting the GP4 extract were the most powerful, increasing activity by 207 units mg^{-1} of protein. This was followed by the TP animals ingesting the GP1, GP3 and GP2 which were also effective and increased the activity of the enzyme by 182, 180 and 100 units mg^{-1} of protein, respectively (Fig. 3d). Of the animals which were fed the T extracts, the muscles

Table 3: Arbitrary ranking of extract groups based on effectiveness in increasing SOD, CAT activities and MDA

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

which showed the highest expression of SOD was found in those ingesting the GP4 extract by 207 units mg^{-1} of protein. This was followed in SOD activity by the T animals 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^{-1} of protein, respectively (Fig. 3d). 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 animals ingesting the GP2 extract were the most powerful, increasing activity by 0.409 units mg^{-1} of protein. This was followed by the TP animals 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^{-1} of protein, respectively (Fig. 3e). Within the T animals, ingesting the GP2 extract had the highest increase CAT increasing activity by 0.364 units mg^{-1} of protein. This was followed in CAT activity by the T animals 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^{-1} of protein, respectively (Fig. 3e).

Overall effects if extract combinations in vivo and ranking: 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.

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 *et al*., 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 4th, 7th, 2nd and 12th, 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 13th, 8th, 14th and 3rd, 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 (Fig. 2c). 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 *et al*., 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 (Fig. 2d, e). 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 (Table 1, 2). 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 (Table 3). 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 (Table 3). Thus, the polyphenolics in the combined extracts appeared to be acting synergistically (Chu *et al*., 2002; Sun *et al*., 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*., 2001; 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 *et al*., 2004; Simon-Schnass and Pabst., 1988; Satoshi *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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