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Original article

Modulation of redox homeostasis by Lamiaceae herbs in seminal vesicles of *Lumbricus terrestris*

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ABSTRACT

Background/Aims: Health benefits of Lamiaceae herbs are attributed to the presence of antioxidant phytochemicals. However, mechanism of *in vivo* functionality is not very well understood. We determined the *in vivo* effect of dietary Lamiaceae herbs on modulating redox-nitric oxide (NO) homeostasis and sperm quality in oxidation prone environment of seminal vesicles in *Lumbricus terrestris*.
Methods: Animals fed ad libitum on Lumbricus growth medium supplemented with 0% (control), 0.1% or 0.5% (w/v) of different herbs. The seminal vesicles of the animal were dissected out on day 2 and day 6, and gently disrupted. Levels of malondialdehyde (MDA), DNA fragmentation (DNAF), glutathione (GSH), nitrates/nitrites (NOx), superoxide dismutase (SOD), catalase (CAT) were determined using standard assays. Sperm maturity and deformation (DFO) was quantified microscopically.
Results: Overall, the herb treatments decreased MDA levels by (60–90%), increased SOD activity (15–50%), and decreased DNAF (6–11%). Treatments with basil and oregano at 0.1% (w/v) were most effective.
Conclusion: Our suggests that phytochemicals from Lamiaceae herbs modulate redox stress response, and protect against oxidative stress in seminal vesicles.

1. Introduction

Cells are constantly exposed to free radicals as part of normal metabolic processes. However, in certain conditions the homeostatic balance that exists between free radicals and cellular antioxidants can be altered, resulting in oxidative stress. In this state, excess free radicals can damage surrounding macromolecules, dysregulate signaling and subsequently inhibit normal cellular function which can lead to the progression of many chronic diseases. Emerging evidence from epidemiological studies has suggested that plant based diets rich in foods such as fruits, vegetables, herbs, spices, and nuts are valuable in disease prevention and treatment.[1–4] These foods are abundant sources of plant secondary metabolites such as polyphenols and carotenoids that can function as effective antioxidants.[5] Lamiaceae herbs, like many other herbs, are historically well known for their health benefits. It is now known that these herbs contain substantial amounts of phenolic compounds such as rosmarinic acid, caffeic acid, vanillic acid, and ferulic acid that may contribute to their health promoting properties.[6] Lamiaceae herbs are popular worldwide because of their natural antibacterial and antiviral properties which research has been shown to be associated with their unique phytochemical makeup. Most of these herbs are generally regarded as safe (GRAS) and antioxidants from these herbs may someday replace synthetic antioxidants in all food products and thus impact human health.[7] However, *in vivo* mechanistic evaluation of their biological functionality against different oxidative stress related pathologies are scarce in literature. Therefore, in this study, five Lamiaceae herbs (basil, oregano, rosemary, sage, and thyme) were evaluated for their ability to modulate antioxidant enzyme activity, nitric oxide biomarkers, and markers for oxidative induced damages in *Lumbricus terrestris*. This organism was allowed to feed ad libitum on a diet supplemented with Lamiaceae herbs, and biochemical tests were performed in seminal vesicles tissues. Seminal vesicles were selected as the target organ of study as this environment is especially sensitive to oxidative stress and it participates in many ROS mediated processes such as spermatogenesis and sperm development and maturation.[8] Additionally we also determined the modulatory effect of Lamiaceae herbs on hydrogen peroxide induced oxidative stress in seminal vesicles of *L. terrestris*. Specifically, the effect of Lamiaceae phytochemicals on redox status, antioxidant enzymes (superoxide dismutase, catalase), oxidative damage to DNA, and NO mediated spermatogenesis was evaluated.

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2. Materials and methods

2.1. Animals

Selection and treatment worms and priming: Sexually mature *L. terrestris* worms with a fully developed clitellum were purchased from *DMFB Company (Waterford, MI)* (and washed in distilled to remove soil and debris. For priming, worms were transferred to petri plate with Lumbricus growth medium (LGM) (1.25% agar, 0.31% gerber oatmeal, single grain (Nestle, Switzerland)) and allowed to feed for 48 h at 10 °C and gently massaged along the length of the body to clear the gut of any digestive content. Worms were then weighed and six worms within a range of 5.0–6.0 g were selected.

2.2. Treatment

Treatment plates were prepared by directly mixing dried Lamiaceae herbs (basil, oregano, rosemary, sage, and thyme) to LGM prior to pouring them into plates to obtain a concentration of 0.5% w/v or 0.1% w/v. One worm was transferred on to each treatment and control (LGM only) plate and allowed to feed on for 6 days at 18–20 °C in the dark. After 4 days of treatment, worms were transferred to a fresh plate.

2.3. Removal of seminal vesicles

Worms were collected on Day 6 and gently massaged along to clear the gut of any contents. Worms were then sacrificed by holding them at −20 °C for 30 min. Using a surgical knife, a cut was carefully made along the dorsal side of the immobile worm from the prostomium to the segments. Seminal vesicles were was gently removed using a fine-tip forceps and immediately transferred to a microcentrifuge tube containing 1 mL of Ca-free Lumbricus balanced salt solution (LBSS) at 4 °C and homogenized with a tissue tearor at low shear speeds.

2.4. Evaluation of sperm quality parameters

The sperm of *L. terrestris* was evaluated for abnormalities in morphology and the stages of developmental maturation as previously described by Zang et al.[9] 5 μL of the seminal vesicle homogenate was transferred onto a clean microscope slide and a cover slip placed on top avoiding air bubbles. 200 spermatozoa were counted under an inverted microscope at 40× objective. The number of mature (developed head and tail), immature (lack tail), and deformed sperm (apical loops, head bending) was noted. The percentage (%) of sperm deformity, maturity, and immaturity was calculated: % (deformed) = Average number of deformed sperm/total number of sperm counted × 100. The same formula was used for calculating the percent of mature and immature sperm.

2.5. Preparation of tissue homogenate for biochemical analysis

The tissue homogenate is centrifuged at 1100 rpm for 2 min at 4 °C. The supernatant was then transferred to a clean microcentrifuge tube and stored −80 °C for future analyses. The remaining pellet is used for DNA fragmentation analysis.

2.6. Malondialdehyde assay

This assay measures the lipid peroxidation by-product, malondialdehyde (MDA), through its reaction with thiobarbutyric acid.[10] The resulting pink MDA/thiobarbituric acid adduct can be measured spectrophotometrically. 150 μL of the seminal vesicle extract supernatant was transferred to a test tube. The blank contained distilled water in place of the seminal vesicle extract supernatant. 100 μL of 20% (w/v) trichloroacetic acid was then transferred to the test tube. 200 μL of 10 mM thiobarbutyric acid was also added to the test tubes. The test tubes were then covered and incubated in a hot water bath at 100 °C for 30 min. The content of the tubes was then transferred into a microcentrifuge tubes and centrifuge at 13000 rpm for 10 min at 4 °C. 250 μL of the supernatant was transferred to a microplate and measured spectrophotometrically at an absorbance of 532 nm. Calculation of the concentration of MDA is derived from its molar extinction coefficient, 156 μmol^{-1} cm^{-1}. The concentration of MDA was expressed as mmol/mg of protein.

2.7. Protein assay

This colorimetric protein assay is based on differential color changes of a dye in response to varied protein concentration derived from the method of Bradford.[11]

2.8. SOD-riboflavin-NBT assay

The superoxide dismutase activity was assayed by quantifying the superoxide mediated oxidation of nitroblue tetrazolium (NBT) to diformazan as a result of the photooxidation of riboflavin.[12] The concentration of diformazan was determined using its molar extinction coefficient, 26478 mol^{-1} cm^{-1} and expressed as μmol/mg of protein.

2.9. Catalase assay

Catalase activity in the tissue homogenate was measured using a standard spectrophotometric microplate assay at 570 nm using potassium chromate–acetic acid reagent described previously.[13]

2.10. Reduced glutathione determination

The seminal vesicle extract supernatant is placed in a precipitant solution to denature proteinaceous compounds. After a centrifugation step, the supernatant is assayed for nonprotein thiols, reduced glutathione.[14] A reduced glutathione-dithiobis nitrobenzoic acid (GSH-DTNB) complex can be measured spectrophotometrically at 412 nm. 100 μL of seminal vesicle extract supernatant is transferred to microcentrifuge tubes. 0.75 mL of precipitant solution is then added to the microcentrifuge tube. The mix is vortexed and incubated at room temperature for 5 min. The microcentrifuge tubes are then centrifuged at 3000 ×*g* for 15 min at 4 °C. 500 μL of the supernatant is transferred to a test tube. 2.0 mL of 0.2 M sodium phosphate (Na_2HPO_4) buffer (pH 8) is added to a small test tube. 250 μL of 0.5 mM DTNB solution (Ellmans Reagent) is transferred to the test tube and vortexed. 250 μL of the mixture was immediately transferred to a microplate. The blank consisted of 500 μL of 0.2 M Na_2HPO_4 buffer (pH 8), 500 μL DH_2O, 250 μL of precipitant solution, 125 μL of DTNB. The absorbance was reading spectrophotometrically at 412 nm. The reduced glutathione concentration was calculated using the extinction coefficient $E = 13.7$ mol^{-1} cm^{-1}. Molecular weight of GSH = 307. The concentration of GSH was expressed as mmol/mg of protein.

2.11. Total nitrate and nitrite determination

The assay described by Miranda et al is used to reduce nitrate to nitrite by vanadium(III) chloride, followed by a spectrophotometric analysis of total nitrite using Griess reagent.[15] Colorimetric methods based on the Griess reaction fundamentally detect NO_2^-

that, under acidic conditions, reacts with sulfanilamide and NEDD to produce an azo compound, which strongly absorbs in the visible region with a peak around 545 nm. 50 μL of seminal vesicle extract supernatant was transferred to a well of a microplate. The blank contains Ca-free LBSS in place of seminal vesicle extract supernatant. 50 μL of 0.8% (w/v) vanadium chloride was added to the well. 50 μL of Griess reagent (if premade) was transferred to the well of the microplate. If Griess reagent isn't available, use 25 μL sulfanilamide (2%) and 25 μL N-(1-Naphthyl) ethylenediamine dihydrochloride (0.1%). The mixture was incubated in a microplate reader at 37 °C for 30 min and the absorbance measured at 540 nm. Calculation of NOx in serum samples was determined from linear standard curve established by 0–150 umol/l sodium nitrate. Concentration (μmol/L) = (A540 − 0.0344)/0.0057. The concentration of NOx was expressed as μm/L/mg of protein.

2.12. Statistical analysis

Statistical analysis of data was performed using a two tailed Student's t test. The data is presented as means + standard error mean (SEM). p-Values < 0.05 were considered statistically significant. Additionally, to measure the overall effectiveness of treatment over the duration of 6 days the area under the curve (AUC) was calculated.

3. Results

3.1. Effect of Lamiaceae herbs on intrinsic lipid oxidation

The extent of lipid peroxidation in seminal vesicles was measured by assessing the amount of malondialdehyde formed. In control worms (LGM diet) the levels of MDA over the course of 6 days was 6.9 mmol/mg of protein. Among all the Lamiaceae herbs tested, inclusion of basil in LGM was most effective in lowering the extent of lipid oxidation. In worms that fed on basil at a concentration of 0.1% (w/v) a 12-fold decrease in MDA ($p = 0.0013$) was observed. At a concentration of 0.5% (w/v) basil in LGM the MDA values were not significantly different from control (Fig. 1A). In worms that fed on rosemary, a 10-fold and 8-fold decrease in MDA was observed at 0.1% (w/v) and 0.5% (w/v) respectively. In seminal vesicles of worms that fed on oregano, a significant decrease in MDA values was observed at both doses. Compared to control, decrease in MDA values at 0.1% (w/v), was 4.9-fold and at 0.5% (w/v) of oregano, it was 3.3-fold (Fig. 1A). Feeding with sage also resulted in a significant decrease in MDA values compared to control at both doses. At a concentration of 0.1% (w/v), the MDA values decreased by 3.2-fold and at 0.5% (w/v) compared to control a 5.5-fold reduction in MDA values was noted (Fig. 1A). Treatment with thyme at 0.1% (w/v) decreased MDA values by 4.8-fold and at 0.5% (w/v) a 3.1-fold reduction in MDA was noted (Fig. 1A).

3.2. Effect of Lamiaceae herbs on total catalase

In control worms which fed only on LGM, the catalase activity in the seminal vesicle homogenates was 130.4 mU/mg of protein (Fig. 1B). Among all the herbs tested, addition of basil at both the dosages caused a significant increase in catalase activity compared to control (Fig. 1B). At 0.1% (w/v) the catalase activity increased by 1.3-fold ($p = 0.0233$) and at 0.5% (w/v), the activity increased by 1.2-fold; $p = 0.0410$. Compared to control worms, addition of Oregano, rosemary, sage and thyme at 0.1% (w/v) and 0.5% (w/v) did not have a significant effect on the total catalase activity in seminal vesicles (Fig. 1B).

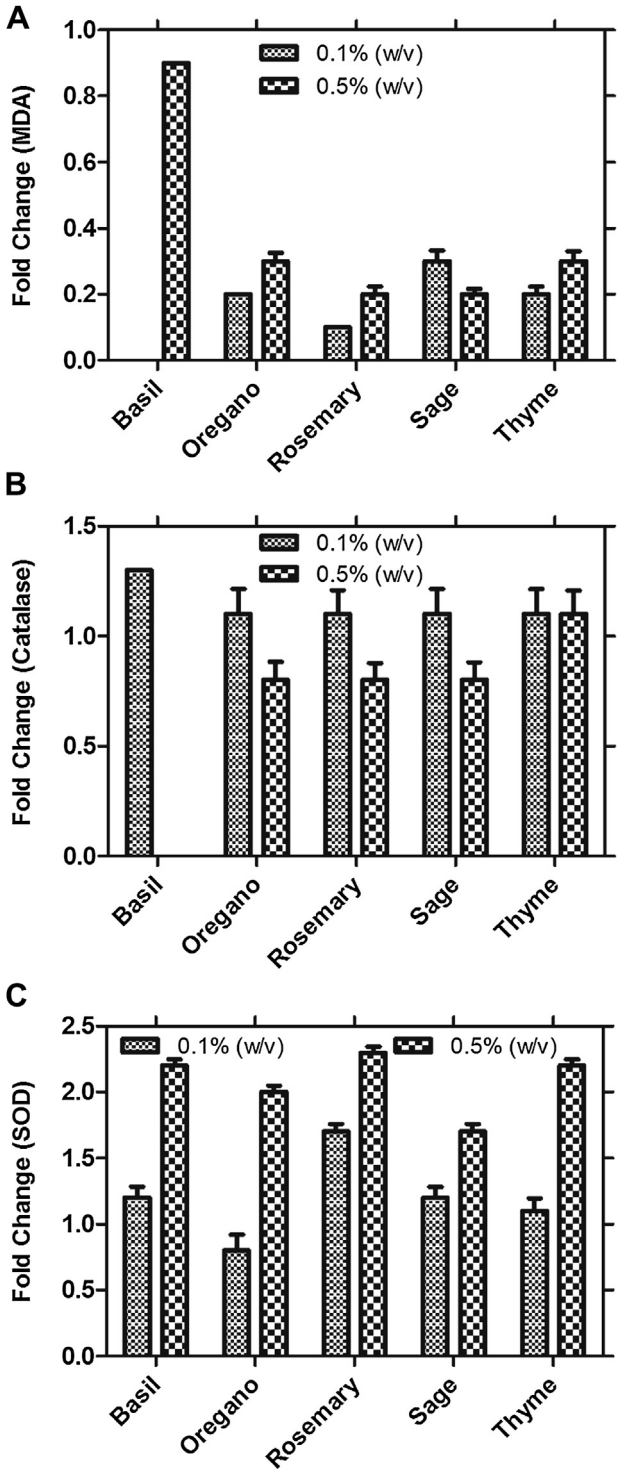

Fig. 1. (A) Fold change (vs. Control) in malondialdehyde (MDA) (B) catalase (C) superoxide dismutase (SOD) in *L. terrestris* in response to treatment with Lamiaceae herbs. Mean + SEM. 6 days, $n = 36$. Concentration of herbs: 0.1% (w/v) and 0.5% (w/v).

3.3. Effect of Lamiaceae herbs on total SOD

At a dosage of 0.1% (w/w), compared to control, SOD activity increased by 1.7-fold in the seminal vesicles of rosemary fed worms. A significant increase in SOD activity by 1.2-fold was also noted in worms that consumed basil and rosemary (Fig. 1C). At 0.1% (w/w) oregano and thyme treatments did not significantly affect

SOD activity. At a dosage of 0.5% (w/w), a 2.3-fold increase in SOD activity was noted in rosemary fed worms (Fig. 1C). Both basil and thyme at 0.5% (w/w) increased the SOD activity in seminal vesicles by 2.2-fold. Compared to control, a 2-fold and 1.7-fold increase in SOD activity was noted in worms that consumed oregano and sage respectively (Fig. 1C).

3.4. Effect of Lamiaceae herbs on total nitrates

In control worms that consumed LGM, the total nitrates over the duration of 6 days were determined to be 196.9 μmol/L/mg of protein. At a dosage of 0.1% (w/w), basil, oregano and rosemary significantly increased nitrate levels by 1.2-fold. In worms that fed on sage and thyme at a 0.1% (w/w), the seminal vesicle nitrate levels were not significantly different than control (Fig. 2A). At a dosage of 0.5% (w/w), a significant increase in total nitrates by 1.5-fold and 1.3-fold was observed only in worms that consumed sage and thyme respectively. Basil, rosemary and oregano did not significantly affect seminal vesicle nitrate levels (Fig. 2A).

3.5. Effect of Lamiaceae herbs on reduced glutathione

In control worms that consumed LGM, the total glutathione over the duration of 6 days were determined to be 71.1 mmol/mg of protein. Irrespective of the dosage, compared to control, levels of glutathione did not change in response to any herbs, except with rosemary at 0.1% (w/w), where a 1.2-fold increase was noted (Fig. 2B).

3.6. Effect of Lamiaceae herbs on sperm deformity

In control worms that consumed LGM, 20.1% of all spermatozoa evaluated had some type of deformation. The proportion of defective sperms significantly decreased 2-fold in worms that consumed 0.1% (w/w) of basil (Fig. 2B). A 1.6-fold decrease in defective sperms was noted in worms that consumed either rosemary or thyme at 0.1% (w/w). In worms that fed on oregano at 0.1% (w/w), the proportion of defective spermatozoa decreased by 1.4-fold compared to control. A 2.5-fold decrease in defective spermatozoa was noted in worms that consumed either 0.1% (w/w) rosemary or thyme. In worms that fed on basil and sage, compared to control worms, the proportion of defective sperms decreased by 1.6-fold and 1.4-fold respectively.

3.7. Effect of Lamiaceae herbs on peroxide induced lipid oxidation

In the seminal vesicles of worms fed on peroxide diet (12 mM hydrogen peroxide + LGM), a 2.9-fold increase in MDA values was observed compared to control. In worms that consumed basil (BA) and peroxide, the MDA values were 1.3-fold higher than in control worms (Fig. 3A). MDA values in worms that consumed both oregano (OA)-peroxide and thyme (TH)-peroxide at 0.1% (w/v) added to peroxide medium, did not significantly increase with respect to control (Fig. 3A). When compared to MDA values in the seminal vesicles of worms that consumed H_2O_2, a 3.3-fold decrease in MDA value was observed in worms that fed on OR + PER and a 2.5-fold decrease was observed with both BA + PER and TH + PER respectively (Fig. 3A).

3.8. Effect of Lamiaceae herbs on catalase in response to peroxide stress

Catalase activity in worms that consumed PER did not significantly differ from control. Consumption of BA, OR or TH at 0.1% (w/v) did not result in any significantly change the catalase activity in worms (Fig. 3B).

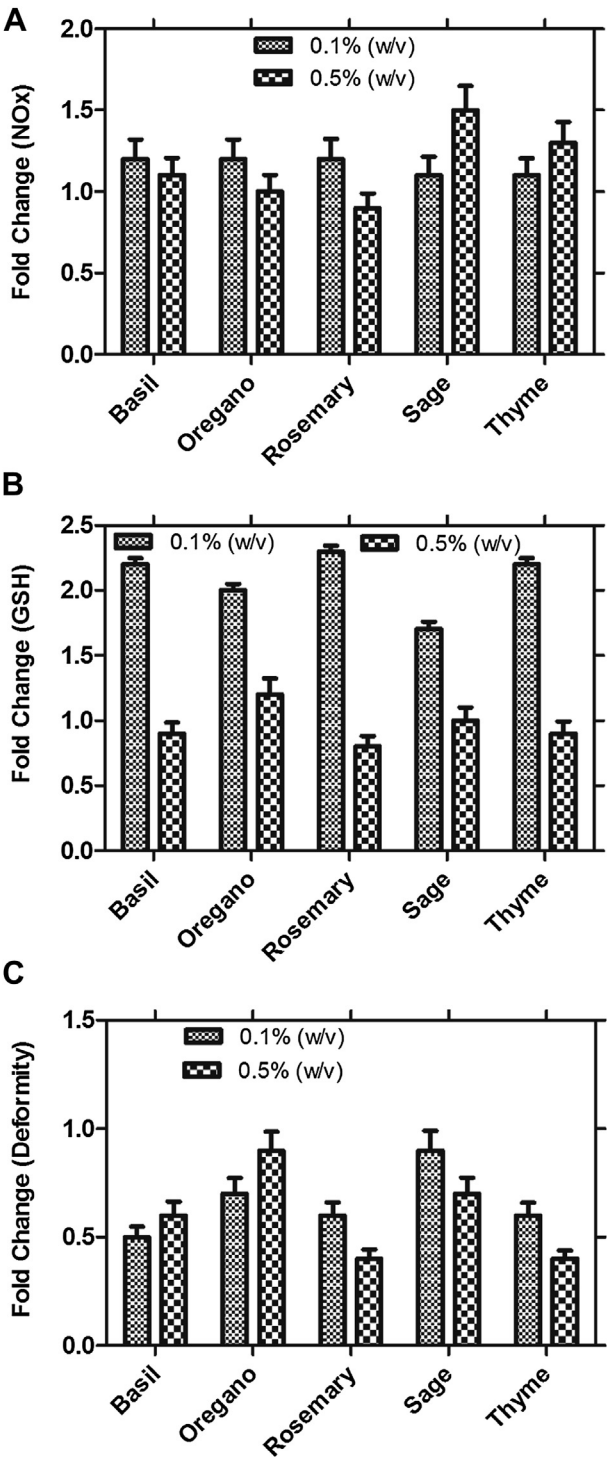

Fig. 2. (A) Fold change (vs. Control) in total nitrates (NOx) (B) glutathione (GSH) (C) sperm deformity in *L. terrestris* in response to treatment with Lamiaceae herbs. Mean + SEM. 6 days, $n = 36$. Concentration of herbs: 0.1% (w/v) and 0.5% (w/v).

3.9. Effect of Lamiaceae herbs on SOD in response to peroxide stress

The activity of SOD in seminal vesicles of the worms consuming LGM with 12 mM PER significantly increased by 1.2-fold compared

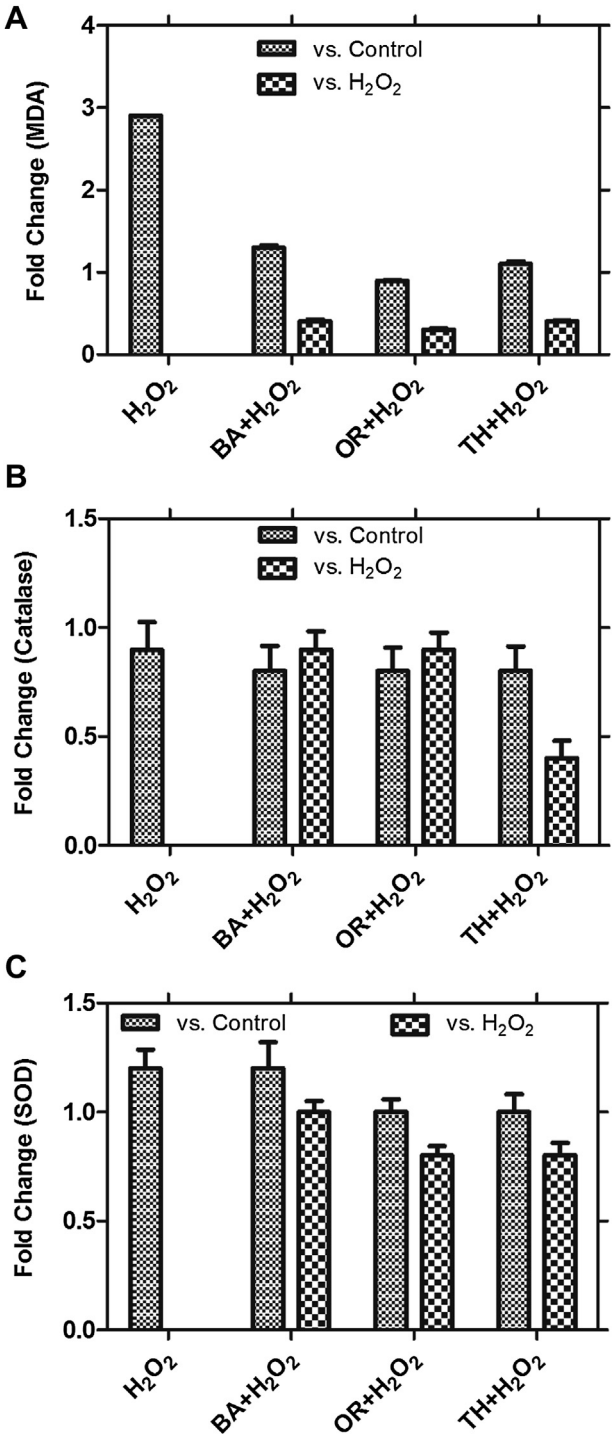

Fig. 3. (A) Fold change (vs. Control) and (vs. H_2O_2) in malondialdehyde (MDA) (B) catalase (C) superoxide dismutase (SOD) in *L. terrestris* in response to treatment with basil (BA), oregano (OR), thyme (TH) and H_2O_2-herb (BA + H_2O_2, OR + H_2O_2, TH + H_2O_2) co-treatment. Mean + SEM. 6 days, $n = 36$. Concentration of herbs: 0.1% (w/v); H_2O_2: 12 mM.

to control (Fig. 3C). In worms that consumed BA + PER diet, the SOD activity was 1.2-fold higher than in control worms. SOD activity in seminal vesicles of worms that fed on OR + PER and TH + PER was not statistically different from control worms, but was 1.25-fold lower than in control worms (Fig. 3C).

3.10. Effect of Lamiaceae herbs on total nitrates in response to peroxide stress

In the seminal vesicles of worms fed on peroxide diet (12 mM hydrogen peroxide + LGM), the total nitrates decreased by 1.6-fold compared to control worms (Fig. 4A). In worms that consumed OR + PER a 1.4-fold reduction in nitrates was noted. Worms that fed

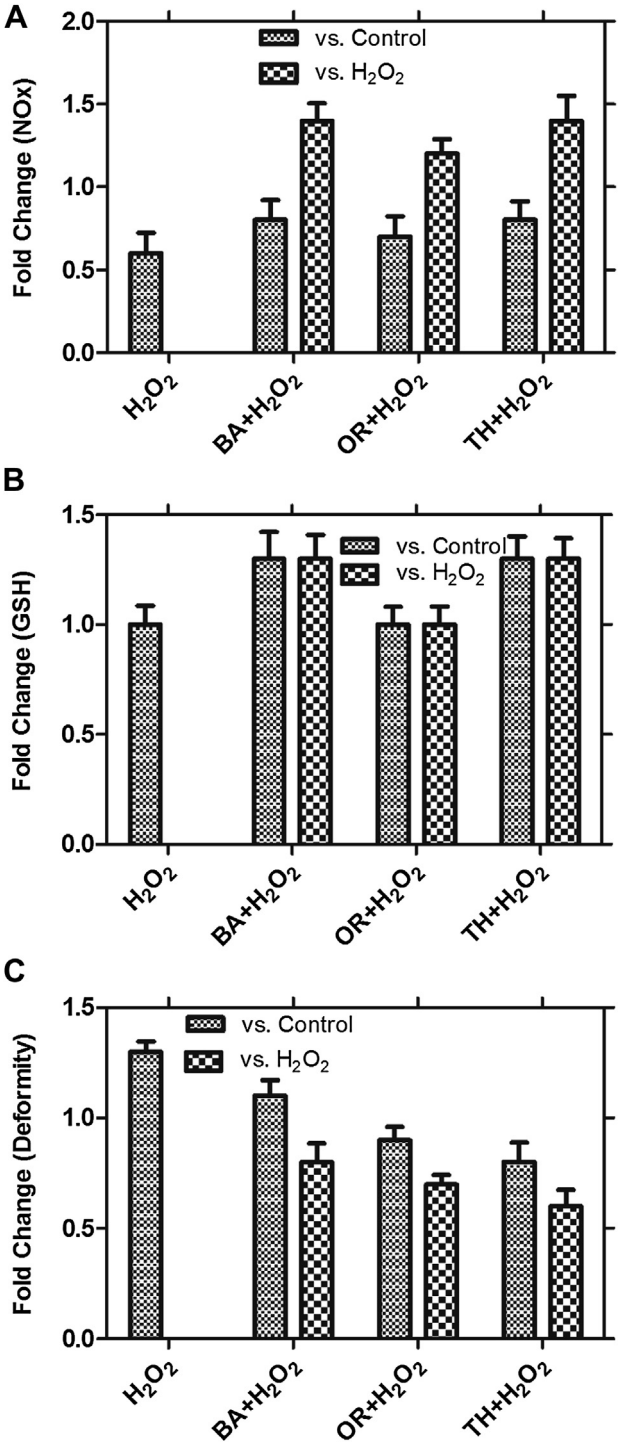

Fig. 4. (A) Fold change (vs. Control) and (vs. H_2O_2) in total nitrates (NOx) (B) glutathione (GSH) (C) sperm deformity in *L. terrestris* in response to treatment with basil (BA), oregano (OR), thyme (TH) and H_2O_2-herb (BA + H_2O_2, OR + H_2O_2, TH + H_2O_2) co-treatment. Mean + SEM. 6 days, $n = 36$. Concentration of herbs: 0.1% (w/v); H_2O_2: 12 mM.

on BA + PER or TH + PER, the nitrates levels were not significantly different from control (Fig. 4A). When compared to the nitrate levels worms that consumer PER, the nitrate levels increased by 1.4-fold in response to BA + PER and TH + PER intake at 0.1% (w/w). In worms that fed on OR + PER, the total nitrates increased by 1.2-fold compared to nitrate levels in seminal vesicles of worms on a PER diet (Fig. 4A).

3.11. Effect of Lamiaceae herbs on total reduced glutathione in response to peroxide stress

The total glutathione levels in seminal vesicles of worms on a PER diet were not significantly different than control (Fig. 4B). In worms that consumed a diet of BA + PER or TH + PER at 0.1% (w/w), the total glutathione values were 1.3-fold higher than in both control and PER diet worms. Consumption of OR + PER did not significantly affect the glutathione levels in worms (Fig. 4B).

3.12. Effect of Lamiaceae herbs on sperm deformity in response to peroxide stress

The proportion of deformed spermatozoa in seminal vesicles of PER diet fed worms increased by 1.3-fold compared to control (Fig. 4C). In BA + PER, OR + PER and TH + PER diet fed worms the fraction of deformed spermatozoa was not statistically different from control (Fig. 4C). However, when compared to PER diet fed worms, a 1.25-fold, 1.4-fold and 1.6-fold reduction in sperm deformity was noted in response to BA + PER, OR + PER and TH + PER treatments (Fig. 4C).

4. Discussion and conclusion

In the current study, five Lamiaceae herbs (basil, oregano, rosemary, sage, and thyme) were evaluated for their ability to modulate antioxidant enzyme activity, nitric oxide biomarkers, and markers for oxidative induced damages *in vivo* in *L. terrestris*. The results of this study indicated a modulatory effect of Lamiaceae herb treatment on two important antioxidant enzymes, superoxide dismutase (SOD) and catalase (CAT). *In vivo* treatment with Lamiaceae herbs resulted in an overall increase in SOD activity compared to controls. Rosemary was most effective, but basil, sage, thyme, and oregano were also able to increase SOD activity. Previous studies have also shown inductions of endogenous antioxidant enzyme activity upon consumption of various dietary and medicinal plants.[16] SOD, catalase, and glutathione peroxidase (GPx)/glutathione are the predominate antioxidants which are involved in the metabolism of superoxide and hydrogen peroxide.[17] The overall decrease in glutathione levels in herb treated worms, may suggest a very rapid utilization of GSH perhaps by GPx. Previous studies have shown glutathione peroxidase to be abundant in the testis, epididymis, and spermatozoa with levels peaking during the time of puberty and spermatogenesis.[18] With the noted induction of SOD and antioxidant activity in seminal vesicle tissues, treatment with antioxidant rich herbs could possibly render these tissues more resistant to oxidative stress, and therefore facilitate healthy sperm development and maturation.[19,20] We have previously shown that *L. terrestris* treated with various herbs, fruits, and spices were able to manage an acute hydrogen peroxide initiated oxidative stress in muscle tissues by inducing an antioxidant enzyme response.[21] An increase in superoxide dismutase and antioxidant activity in seminal vesicle tissue by Lamiaceae herb treatment may indicate an important pathway for protecting developing spermatozoa against oxidative induced damages.

The increased antioxidant enzyme activity observed in Lamiaceae herb treated animals was accompanied by decreased levels of lipid peroxidation in seminal vesicles and reductions in morphological sperm deformations. Similar results were seen in another study with aqueous extracts of different Lamiaceae herbs.[22] It was shown that these herbs protect low density lipoprotein (LDL) particles from copper-induced oxidation with the greatest protection coming from rosemary and sage treatments. In our study it was noted that treatment with Lamiaceae herbs reduced MDA levels when compared to controls. Previous studies have also demonstrated that increased levels of MDA are associated with impairment in sperm motility and infertility.[23,24] Along with decreased MDA levels, the results from this study indicated that Lamiaceae herb treatment taken as a whole resulted in a decrease in sperm deformations in comparison to worms only feeding on *Lumbricus* growth medium.

When nitric oxide metabolite levels (NOx) in tissues of the seminal vesicles were measured after supplementation with Lamiaceae herbs resulted in increased levels of NOx. Previous studies on herbs from the Lamiaceae family such as *Salvia miltiorrhiza*, have also increased levels of eNOS mRNA, eNOS, and NO in human umbilical vein endothelial cells.[25] In this study the increase in NOx upon treatment with Lamiaceae herbs occurred with concurrent increases in antioxidant enzyme activity, decreased lipid peroxidation and sperm deformity. This increased production of nitric oxide without an increase in oxidative damage may suggest a novel NOx mediated mechanism of action by Lamiaceae phytochemicals and needs further investigation. The current study has also revealed that herbs from the same family can have different effects on the redox status of tissues. These differences could possibly be attributed to the variation in both phytochemical content and profile among plants which belong to the same family and that share some bioactive compounds.[6]

The addition of Lamiaceae herbs to LGM containing hydrogen peroxide appears to ameliorate the effects of the acute oxidative stress induced in *L. terrestris*. Overall, in comparison to controls it was observed that there was an increase in MDA levels, DNA fragmentation, and sperm deformations. This data suggests that there are slight increases in oxidative damages even with herb treatments; however, the extent of damage in comparison to the peroxide group is drastically reduced. When comparisons are made between the peroxide treatment and the herb + peroxide treatment it was observed that there was a significant decrease in MDA levels, sperm deformations, and DNA fragmentation in worms treated with Lamiaceae herbs. These results are similar to a recent in vitro study that indicated extracts of sage, oregano, and rosemary were able to protect Caco-2 cells against hydrogen peroxide induced DNA damage.[26]

It appears that phytochemicals present in Lamiaceae herbs may protect against oxidative damages induced by hydrogen peroxide by restoring redox homeostasis within the organism. In this study, there was an increase in GSH levels, SOD activity, and a decrease in catalase activity in the seminal vesicles of worms treated with herbs + peroxide relative to controls. Although the antioxidant enzyme activity and GSH levels were increased when compared to controls, the antioxidant enzyme response was not as exaggerated in the herbs + peroxide group as in the peroxide group. This was indicated by an overall decrease in SOD activity and a decrease in catalase activity when compared to the peroxide group. Previous research has shown that extracts of sage, rosemary, basil, oregano, and thyme are effective scavengers of free radicals, able to quench singlet oxygen, and chelate metals capable of participating in the Fenton reaction.[8,27,28] Therefore, the protective benefits of these herbs which were observed in this study could be due, in part, to their free-radical scavenging abilities and reducing power. Interestingly, it was also observed that there were significant increases in GSH levels in the herb + peroxide treatment group. As was predicted

previously, these herbs may activate the antioxidant response element (ARE) via the transcription factor, Nrf2, which could lead to the transcription of γ-glutamylcysteine synthetase and increase levels of GSH.[29] Recently, Masutani et al showed that the Lamiaceae herb, *Perilla frutescens*, was able to activate the ARE via Nrf2 and prevent hydrogen peroxide induced cytotoxicity in vitro.[30]

Moreover, our results indicated that nitric oxide levels increased in response to treatment with herbs when compared to peroxide treatments; however NO levels remained lower than controls. Therefore, this provides further evidence that Lamiaceae herbs can stimulate nitric oxide production even in a situation where NO production is being depressed. Nitric oxide plays a vital role in the tissues of the seminal vesicles where eNOS expression is proposed to play an important role in junction restructuring and differentiation of germ cells into sperm cells.[31] This data suggests that phytochemicals from Lamiaceae herbs may stimulate nitric oxide signaling pathways, and contribute to several of the effects observed.

Conflicts of interest

All authors have none to declare.

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