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Inhibitory effects of cranberry polyphenol and volatile extracts on nitric oxide production in LPS activated RAW 264.7 macrophages

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Cranberry volatiles have received little attention for health-promoting properties. In this study, we compared the inhibitory effects of cranberry polyphenol and volatile extracts and volatile standards on nitric oxide (NO) production in lipopolysaccharide (LPS) activated RAW 264.7 macrophages. Polyphenols were analyzed by HPLC/HPLC-MS and volatiles were analyzed by GC/GC-MS. The inhibition of NO production of the fresh cranberry polyphenol and volatile extracts and α-terpineol, linalool, linalool oxide, and eucalyptol standards at 2, 4, and 8-fold dilutions of their original concentrations in fresh cranberries was evaluated by treating these extracts/standards for 1 hr before or after LPS application for 24 hr. After inducing inflammation with LPS, the polyphenol treatments (317.8 and 635.7 μg/g) and 1.8 μg/g volatile treatment lowered NO levels 46-62% compared to the positive control ($P<0.05$). When the cells were treated with polyphenol and volatile extracts before inducing inflammation, the 635.7 μg/g and 317.8 μg/g polyphenol treatments and 1.8 μg/g and 0.9 μg/g volatile treatments lowered NO levels (13-52%) compared to the positive control ($P<0.05$). Polyphenol and volatile extracts from cranberry were effective in reducing NO production whether applied before or after the

application of LPS. α-terpineol at a concentration found in fresh cranberries (1.16 μg/mL) was also found to be effective in reducing NO production whether cells were treated before or after application of LPS. Future studies are needed to reveal the mechanisms by which volatile compounds, especially α-terpineol act to mitigate inflammation and to determine the bioavailability of terpenes.

KEYWORDS: α-terpineol**,** Anti-inflammatory, Cranberry, Polyphenols, Nitric oxide, Volatiles

1 Introduction

Cranberries (*Vaccinium macrocarpon*) have received much attention as a functional food. The berries contain a complex composition of phenolic compounds including anthocyanins, proanthocyanidins (PACs), flavonols and phenolic acids.[1] The bioactive phenolics present in cranberries are reported to improve urinary tract health, boost immune function, and reduce cardiovascular disease and cancer.[2] Cranberry phenolics have been shown to exhibit many biological properties in in vitro studies including anticarcinogenic, anti-inflammatory, antioxidant, antiviral, antibacterial, anti-adhesion, antimutagenic and antiangiogenic properties.[3] Additionally, in animal studies, cranberry extracts were shown to ameliorate production of C-reactive protein and inflammatory markers and improve insulin sensitivity.[3] Cranberry phenolic compounds are suggested to be responsible for the majority of beneficial health effects, including their antioxidant and anti-inflammatory activities.

A class of phytochemicals found in cranberries that has received little attention for bioactive properties and health-promoting effects are volatile compounds. Volatile compounds including esters, alcohols, monoterpenes, acids, sesquiterpenes, C_{13} isoprenoids and others are responsible for the aroma of cranberries.[4-7] Although volatiles in berries, including cranberries have never been studied for their antioxidant and anti-inflammatory effects, studies on essential oils reported that monoterpenes have both chain breaking antioxidant activity[8,9] and anti-inflammatory properties.[8,10] Protection against oxidative stress and inflammation is important since these two processes are related and if not controlled by a healthy diet are thought to contribute to many chronic diseases including cardiovascular disease, type 2 diabetes, various types of cancer and neurological diseases such as Alzheimer's and Parkinson's disease.[11] It is well established that

berry polyphenols, especially anthocyanins can protect against oxidative stress[12] and inflammation[11], but the role of volatiles in ameliorating these factors is unknown.

Due to their tart and astringent taste, cranberries are commonly consumed in processed products such as juice, dried and canned sauce where significant amounts of sugar are added to the products to improve taste. There is little information available on how different processing techniques affect volatile composition of cranberries, but drying conditions are reported to alter the volatile composition of cranberries with loss of some volatiles and formation of new volatiles observed based on the degree of thermal treatment applied.[13]

We hypothesized that the volatile compounds, and not just the previously reported phenolic compounds, also have anti-inflammatory capabilities. Therefore, the objectives of this study were to compare the antioxidant and anti-inflammatory activities of phenolic and volatile fractions isolated from fresh cranberries, and to determine the anti-inflammatory activity of major monoterpene standards found in the volatile fraction. Nitric oxide was selected as a maker for inflammation since it regulates pro-inflammatory conditions in epithelium and early stages of inflammation.[14] Nitric oxide is generated by nitric oxide synthase (NOS) enzymes through oxidation of L-arginine to L-citrulline. Constitutively produced low concentrations of NO have physiological functions that regulate biological homeostasis.[15] However, when some stimuli such as lipopolysaccharide (LPS) and interferon-gamma (IFN-γ) activate transcription factor (nuclear factor-kappa B) excessively produce NO by inducible isoform of NOS (iNOS), tissue damage and inflammation can occur through production of reactive nitrogen/oxygen species (RNS/ROS) and DNA damage.[14,16] Increased RNS and ROS such as peroxynitrite and superoxide, respectively, can lead to chronic inflammation, diabetes, inflammatory bowel

disease, atherosclerosis and septic shock.[16-18] Thus, it is important to maintain low levels of cellular NO to decrease cytotoxic RNS and ROS effects.[19]

2 Materials and methods

Materials

Cranberries (Stahlbush Island Farm brand) were purchased from Harps Foods, Fayetteville, AR. The packaged cranberries were stored frozen at -20° C until analysis. RAW 264.7 mouse macrophage cells were purchased from the American Type Culture Collection (ATCC, Rockville, MD) and cultured in Dulbecco's modified eagle's medium (DMEM) enriched with 1% penicillin-streptomycin, 2 mM L-glutamine, and 10% fetal bovine serum (FBS). The components for the DMEM media and all cell culture experimental reagents were purchased from Gibco® through Life Technologies (Carlsbad, CA). CellTiter 96 AQ $_{ueous}$ One Solution Cell Proliferation Assay (MTS) and the Griess Reagent System kit were purchased from Promega Corporation (Madison, WI). 6-hydroxy-2,5,7,8-tertamethylchroman-2-carboxylic acid (Trolox), 2,2-diphenyl-1-picrylhydrazyl (DPPH), Tween-80, α-terpineol, α-phellandrene, linalool, α-ocimene, β-ocimene, linalool oxide, nonanal, heptanal, octanal, carvone, terpinen 4-ol, eucalyptol, rutin, chlorogenic acid, gallic acid, Folin-Ciocalteu reagent, 4-dimethylaminocinnamaldehyde (DMAC) and catechin were purchased from Sigma Aldrich (St. Louis, MO). A standard mixture of delphinidin, cyanidin, petunidin, peonidin, pelargonidin and malvidin glucosides was purchased from Polyphenols (Sandnes, Norway).

2.1 Extraction of phenolic compounds

After thawing frozen berries three separate phenolic extracts were prepared. Approximately 30 g of the rinsed cranberries were homogenized with 250 mL of methanol/ water/formic acid (60:37:3 v/v/v) using a Euro Turrax T18 Tissuemizer (Tekmar-Dohrman

Corporation, Mason, OH) for approximately 60 sec. The homogenate was then vacuum filtered through Miracloth (CalBiochem, LaJolla, CA) and the resulting filtrate collected. The resulting residue was homogenized using the Euro Turrax Tissuemizer with 250 mL of acetone/water/acetic acid (70:29.3:0.5 v/v/v) for approximately 60 sec. The homogenate was vacuum filtered through Miracloth and combined with the previously collected filtrate. The filter cake was then collected and the extraction process repeated twice until no color was visible in the filter cake. The pooled filtrate was evaporated to dryness using a Buchi Rotary Evaporator R-114 (Buchi, Flawil, Switzerland) set at 40 °C and then the dried residue was re-solubilized in 30 mL of water. Next, the phenolic extract was centrifuged for 5 min at 10,864 x g (Beckman-Coulter Allegra X-22R) and the supernatant was collected. The extract (5 mL) was then loaded onto a preconditioned Sep-Pak® C_{18} column cartridge (Waters Corporation, Milford, MA). After eluting interfering compounds with water, phenolics were eluted with 70-100% ethanol until no color was visible in the cartridges. The samples were passed through 0.45 μm filters (Whatman) prior to HPLC analysis.

2.2 High-performance liquid chromatography (HPLC) analysis of phenolic compounds

The individual phenolic compounds from the cranberry extract were separated by HPLC on a 250 X 4.60 mm Symmetry 5 μm C_{18} column (Waters Corp, Milford, MA) as described in Cho *et al.* 2004.[13] Detection wavelengths of 320, 360, and 510 nm were used to monitor hydroxycinnamic acids, flavonols and anthocyanins, respectively. Individual anthocyanin monoglucosides and acylated anthocyanin derivatives were quantified as Cyd (cyanidin), Pnd (peonidin), and Mvd (malvidin) glucoside equivalents using external calibration curves of a mix of the three anthocyanin glucosides. Hydroxycinnamic acids were quantified as chlorogenic acid equivalents using external calibration curves of chlorogenic acid and flavonols were quantified

as rutin equivalents using external calibration curves of rutin. Results are expressed as mg of anthocyanin-3-glucoside equivalents, chlorogenic acid equivalents, and rutin equivalents per kg of fresh weight.

2.3 Total phenolic content assay

The total phenolic content of phenolic and volatile extracts was measured using the Folin-Ciocalteu (FC) assay.[14] Gallic acid was used as standard using a calibration curve ranging from 3.12 to 100 mg/kg. Sample absorbance was measured at 760 nm with results expressed as mg of gallic acid equivalents (GAE) per kg fresh weight.

2.4 DMAC (4-dimethylaminocinnamaldehyde) total procyanidins assay

Total procyanidins present in the phenolic extract were measured using the DMAC assay following the method of Payne *et al.* 2010.[15] Catechin was used as a standard using a calibration curve ranging from 2 to 64 mg/kg. Sample absorbance was measured at 640 nm with results expressed as mg of catechin equivalents per kg fresh weight.

2.5 DPPH (2,2-diphenyl-1-picrylhydrazyl) total antioxidant capacity assay

Total antioxidant capacity of phenolic and volatile extracts was measured using a modified DPPH method published by Akkari et al. 2016.[16] 1.4 mL of a solution of DPPH in methanol was added to 0.1 mL of phenolic and volatile extracts at 10x, 20x, 40x, 80x, and 160x dilutions. The combined solutions sat in the dark for 30 minutes, then absorbances on a spectrophotometer at 517 nm were measured. Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) was used as the standard (50, 100, 200, 400, 800 μM) with results expressed as μM of Trolox per kg of sample.

2.6 Extraction of volatile compounds

Volatile extracts were prepared from thawed cranberries. Volatiles were extracted by homogenizing 300 g of berries, 300 mL deionized water, and 100 g NaCl in a Waring blender for 1 min. The homogenate was vacuum distilled at 28 in. Hg, 50 °C water bath, 0 ºC condenser for 30 min using a Buchi rotary evaporator (Buchi, Flawil, Switzerland). The first 200 mL was collected in a flask contained in an ice water bath. Additional ice packs were strapped to the condenser to aid in condensation and collection of volatiles. The volatile extracts were stored in sealed glass jars at -20 ºC until use.

2.7 Solid phase micro-extraction (SPME) of volatiles

An 85µm, CAR/PDMS, Stableflex, 24 Ga, Manual Supelco (Bellefonte, PA) SPME fiber was used in this study. Vials containing 4 mL of volatile extract were placed on a stir plate set at 65° C with the SPME fiber inserted into the headspace above the sample. Adsorption was performed for 30 min.

2.8 Gas chromatography quantification of volatiles

Volatiles adsorbed to the SPME fibers were desorbed at 270 °C for 5 min in the injection port of a Varian 3800 GC (Agilent Technologies, Santa Clara, CA) equipped with a HP-5 (5% phenyl-methylpolysiloxane) column (30 m x 250 um x 1 um) (Agilent Technologies, Santa Clara, CA). The Flame Ionization Detector (FID) was at 280 °C. GC runs were 29 min, and the fiber remained in the injection port for 10 min after each run. The injection port was operated in splitless mode with a constant He flow of 25 psi. The initial oven temperature was 25° C, held for 4 min, ramped up at 12 °C/ min to 289° C, and held for 3 min. Heptanal, octanal, α-phellandrene, limonene, eucalyptol, α-ocimene, β-ocimene, nonanal, terpinen 4-ol, α-terpineol and carvone were quantified using SPME standard curves of authentic standards. Compounds

where standards were unavailable were quantified as equivalents of standard compounds eluting in close proximity; 1-butanol, 1-pentanol, hexanol, 1–heptanol, benzaldehyde and 1 octen-3-ol were quantified as heptanal equivalents, 2-octanal, linalool, and linalool oxide were quantified as limonene equivalents, and trans-2-decanal was quantified as α-terpineol equivalents.

2.9 GC-MS identification of volatiles

SPME-collected volatiles were analyzed by GC-MS using a Hewlett-Packard HP 5890 series gas chromatograph equipped with a mass selective detector (MSD) and a HP-5 capillary column (Agilent, 30 m x 0.25 mm, film thickness 1 μm). Working conditions included: injector temperature 270 ^{o}C; MSD interface temperature 280 ^{o}C; oven temperature programmed from -10 ^{o}C (1 min) to 280 ^{o}C at 12 ^{o}C/min; carrier gas (He) at a flow rate of 0.78 mL/min; injection port operated in splitless mode. MSD acquisition parameters included full scan mode, scan range 20-300 *m/z* and scan speed 3.2 scans/sec. Volatiles were identified by comparing their mass spectra with the spectral library (Wiley7NIST0.5), literature data, and alkane retention indices C5-C20.

2.10 *In vitro* anti-inflammatory activity

Treatment dosage of cranberry phenolic and volatile extracts. Phenolic and volatile extracts were used as experimental treatments on RAW 264.7 cells before measuring the NO levels. The extracts were adjusted to the original starting weight of the fresh cranberries to reflect the natural concentrations of the phenolics and volatiles in a fresh cranberry. To keep the treatment dosage as high as possible, 2x, 4x, and 8x dilutions of the phenolic and volatile extracts were used resulting in phenolic doses of 635.9, 317.8 and 158.9 μg/g and volatile doses of 1.8, 0.9 and 0.45 μg/g.

Cell culture. RAW 264.7 mouse microphage cells were cultured in Dulbecco's modified eagle's medium (DMEM) enriched with 1% penicillin-streptomycin, 2 mM L-glutamine, and 10% fetal

bovine serum (FBS). The cells were maintained in 75 cm^2 cell culture flasks and incubated at 37 °C in a 5% CO_2 environment. All NO assays were conducted between cell passage numbers 4-8.

Cell viability. Cell viability was evaluated using CellTiter 96 AQ $_{ueous}$ One Solution Cell Proliferation Assay (MTS) (Promega Co., Madison, WI). RAW 264.7 cells were seeded into 96-well plate at a density of 1 x 10^3 cells per well and incubated at 37˚C, 5% CO_2 for 16 hr. Cells were treated with different dilutions (2, 4 and 8x) of cranberry phenolic and volatile extract, and four different standard solutions of major volatile standards (α-terpineol, linalool oxide, eucalyptol and linalool) for 1 hr and then the cells were stimulated with LPS (100 ng/mL) for 24 hr. After 24 hr, 20 μL of MTS was added to each well and incubated for 4 hr. Cell viability was determined by measuring the absorbance at 490 nm using a microplate reader (Synergy HT Multi-Mode Microplate Reader, BioTek Instruments, Inc., Winooski, VT).

Nitric oxide assay. Nitric oxide production was analyzed using the Griess Reagent System kit containing nitrite standard, N-1-napthylethylenediamine dihydrochloride (NED) solution, and sulfanilamide solution. RAW 264.7 cells (100 μL) in enriched DMEM media were seeded in a 96 well plate (plate 1) and incubated at 37 °C and 5% CO_2 for 16 hr. After 16 hr, which allowed the cells to attach to bottom of the wells in the plate, the media was removed and treatment media containing a range of phenolic and volatile cranberry extracts, or volatile standards was added. Two control samples were used, one without LPS and one with LPS (100 ng/mL). Tween 80 was added at a concentration of 0.02% to both the volatile and phenolic treatment media. The treatment media was left on the cells for 1 hr, and then removed. Next, lipopolysaccharide (LPS) media, containing 100 ng LPS per mL, was applied to the cells and left on for 24 hr. After 24 hr, the LPS media was removed and the nitric oxide levels were measured. To measure nitric oxide, a nitrite standard reference curve, with concentrations ranging from 0 to 100 μM, was prepared

on a separate 96 well plate (plate 2). Samples on plate 1 were centrifuged for 1 min at 1000 rpm and the supernatants from the samples were added to plate 2. Then, 50 μL of sulfanilamide solution was added to the standard reference curve and the samples on plate 2 and allowed to sit for 10 min protected from light. After ten min, 50 μL of NED solution was added to all wells on plate 2 and allowed to sit protected from light for 10 min. After 10 min, the absorbance was read on a plate reader at 540 nm. After the absorbance readings, corrections were made to account for background absorbance of the sample control media. The absorbance readings were converted into NO levels using the slope value from the NO standard curve.

This experiment was conducted as described above, except LPS media was left on the cells for 24 hr. After 24 hr, the cranberry phenolic and volatile and standard treatment volatile media containing the different concentrations was added, and a final concentration of 0.02% Tween 80 was added to the phenolic, volatile and standard volatile treatment media. Tween 80 was found to be non-cytotoxic at a concentration up to 0.1% in a preliminary experiment. After 1 hr with the cranberry treatment media, the plate was centrifuged, the supernatant transferred to a second plate, and the nitric oxide levels measured in the same way as described above where treatments were applied before LPS.

2.13 Statistical Analysis

All statistical analysis was performed using JMP® Pro Ver. 14 Statistics Software. One replicate was used for volatile analysis, three replicates were used for phenolic analysis and DPPH total antioxidant capacity, three replicates were used for analysis of cell viability and five replicates were used for NO analysis. One-way analysis of variance for all responses for all treatment dilution combinations before and after by each treatment combination was ran in the Fit Y by X platform of JMP Pro Ver. 14. Multiple comparisons were performed using the Tukey-

Kramer HSD test. Paired t-tests were used in the Matched Pair platform of JMP Pro Ver. 14 to compare the before and after LPS results for each treatment.

3 Results

3.1 Phenolic composition and antioxidant activity of cranberry phenolic extract

According to the FC and DMAC assays the phenolic extract had a total phenolic content of 1,271 mg GAE/kg FW and a total proanthocyanidin content of 324 mg/kg FW, respectively. The phenolic extract had a total antioxidant activity (DPPH) value of 4,676 μM Trolox per kg. Anthocyanins and flavonols in the phenolic fraction were identified by LC-MS (Table S1 and S2) and the concentrations of the individual compounds are shown in Table 1. Seven major anthocyanin compounds were identified; peonidin 3-galactoside, peonidin 3-arabinoside, peonidin 3-glucoside, cyanidin 3-galactoside, cyanidin 3-arabinoside, malvidin 3-galactoside and cyanidin 3-glucoside confirming previous studies.[24-26] The extract had a total anthocyanin content of 936.6 ± 8.0 mg ACY-3-glucoside equiv/kg fresh weight, which was lower than the previously reported value of 1400 mg/kg fresh weight.[27] Twelve flavonol compounds were identified, composed of quercetin and myricetin derivatives, with quercetin 3-galactoside and myricetin 3-galactoside predominating. Flavonol composition of the extract was consistent with previous reports.[26,28] The extract had a total flavonol content of 77.0 ± 1.0 mg rutin equiv. per kg fresh weight. Additionally, one hydroxycinnamic acid, chlorogenic acid was present at a concentration of 14.7 ± 0.2 mg/kg fresh weight.

3.2 Volatile composition and antioxidant activity of cranberry volatile extract

As expected, the volatile fraction contained no total phenolics or proanthocyanidins as determined by the FC and DMAC assays. The volatile fraction had a total antioxidant capacity (DPPH) value of 40.2 μM Trolox per kg. Monoterpenes possess antioxidant activity due to their

conjugated double bond structure.[9] The volatile fraction had a 116-fold lower DPPH value than the phenolic fraction (4,676 μM Trolox per kg), which reflects the low concentration of volatiles present in cranberry (3.6 mg/kg) verses high levels of phenolics in the phenolic fraction (1271 mg/kg), a 353-fold difference.

Following GC-MS analysis, 23 volatiles were identified in the volatile extract (Table 2). All of the volatiles have previously been identified in cranberries with the exception of α-phellandrene, α and β-ocimene, carvone, and trans-2-decanal.[4-7] Monoterpenes were the major constituent of the extract accounting for 84% of total volatiles, followed by aldehydes (8%), and alcohols (3%). In terms of individual compounds, α-terpineol was the predominant volatile compound accounting for 65% of total volatiles, other major volatile compounds included linalool oxide (6%), eucalyptol (5%), trans-2-decanal (4%) and 2-octanal (3%). Other studies have reported α-terpineol to be the major volatile in American cranberries[4,5] while another study found benzyl alcohol to predominate.[6] GC analysis revealed that the volatile extract had a total volatile concentration of 3,551 μg/kg, which agrees with the value of 3,700 μg/kg previously reported for American cranberry.[6] Based on volatile analysis, the four major monoterpene volatiles, α-terpineol, linalool oxide, eucalyptol, and linalool present in the extract were evaluated for anti-inflammatory activity at concentrations similar to those found in Table 2.

3.3 Inhibitory effects of phenolic and volatile extracts on nitric oxide production

The goal of this study was to compare the inhibitory effects of phenolics and volatiles at concentrations present in fresh cranberry on nitric oxide production produced 1 hr before and 24 hr after application of LPS. Nitric oxide (NO) concentrations produced by RAW 264.7 cells treated with phenolic and volatile extracts before and after applying LPS are shown in Fig. 1 and 2, respectively. In Fig.1 it is clear that application of cranberry phenolic and volatile extract

treatments before applying LPS decreased NO levels compared to the positive control. The phenolic extract treatments at 2x and 4x dilutions (635.7 μg/g and 317.8 μg/g) reduced NO production by 52% and 26%, respectively, while the volatile extract treatments at 2x and 4x dilutions (1.8 μg/g and 0.9 μg/g) reduced NO production 47% and 13%, respectively compared to the positive control. Interestingly, the results of the 2x and 4x dilutions of the phenolic and volatile extracts were not significantly different from one another (P = 0.894 and 0.0529, respectively), but were all significantly different from the positive control. Hence, even though volatile extract dilution concentrations were 353-fold lower than the respective phenolic extract dilution concentrations, they worked just as effectively in mitigating the production of NO.

The results in Fig. 2 show that application of more concentrated cranberry phenolic (635.7 μg/g and 317.8 μg/g) and volatile (1.8 μg/g) extract treatments after applying LPS decreased NO levels compared to the positive control. The phenolic extract treatments at 2x and 4x (635.7 μg/g and 317.8 μg/g) reduced NO production by 62% and 46%, respectively, while the volatile extract treatments at 2x (1.8 μg/g) reduced NO production by 50% compared to the positive control. Both the phenolic 635.7 μg/g and volatile 1.8 μg/g treatments showed comparable ability to mitigate the pre-existing NO produced by LPS (P=0.9948). The phenolic extract treatment at 635.7 μg/g had comparable levels of NO as the negative control, i.e. cells without any exposure to LPS, indicating phenolics at high concentration brought NO levels back to baseline levels.

Upon statistical comparison of the two treatment strategies, applying the treatments before inducing inflammation with LPS versus applying the treatments after inducing inflammation with LPS, it was clear that applying the treatments before inducing inflammation with LPS resulted in lower NO levels in all cases. Phenolic treatments (635.7 μg/g, 317.8 μg/g

and 158.9 μg/g) applied before LPS addition had mean differences of -5.57 (Prob > [t] = 0.0012), -9.37 (Prob > [t] = 0.0003) and -9.53 (Prob > [t] = 0.0022), respectively compared to the same treatments applied after addition of LPS. Volatile treatments (1.8 μg/g, 0.90 μg/g, and 0.45 μg/g) applied before LPS addition had mean differences of -5.15 (p > [t] = 0.0065), -10.51 (P > [t] = 0.0003), and -11.46 (p > [t] = 0.0005), respectively compared to the same treatments applied after addition of LPS. This indicates that the phenolic and volatile extract treatments worked better when applied before application of LPS as opposed to treatment after the application of LPS.

3.4 Inhibitory effects of standards of major volatiles found in cranberries and volatile extracts on nitric oxide production

Nitric oxide concentrations (NO) produced by RAW 264.7 cells treated with standard solutions of major volatiles at concentrations present in fresh cranberries; α-terpineol, linalool, linalool oxide, and eucalyptol and volatile extracts before and after applying LPS are shown in Fig. 3 and 4, respectively. The application of α-terpineol standard at 2x (1.16 μg/mL) and volatile extracts at 2x and 4x (1.8 and 0.9 μg/mL) before applying LPS decreased NO levels compared to the positive control, demonstrating a potential preventative effect (Fig. 3). The α-terpineol treatments at 2x (1.16 μg/mL) reduced NO production by 43%, while the volatile extracts at 2 and 4X dilution (1.8 and 0.9 μg/mL) reduced NO production by 55% and 27%, respectively compared to the positive control. The mitigation of NO production by α-terpineol at 2x (1.16 μg/mL) was similar to the volatile extract at 2x (1.8 μg/mL) indicating there was no synergy among α-terpineol and other volatile compounds in the volatile extract. This suggests that most of the anti-inflammatory effect of the volatile extract is due to the predominant volatile α-terpineol.

The results in Fig. 4 show the same results for the major cranberry volatiles when applied after LPS treatment. The α-terpineol treatments at 2x and 4x (1.16 μg/mL and 0.58 μg/mL) reduced NO production by 43% and 29%, respectively compared to the positive control, while the volatiles extracts at 2 and 4X dilution (1.8 and 0.9 μg/mL) reduced NO production by 52% and 36%, respectively compared to the positive control. Similar to results obtained when treatments were applied before application of LPS there was no difference between volatile extracts and α-terpineol standard treatments (diluted 2x and 4x) applied after application of LPS indicating no synergy among α-terpineol and other volatile compounds in the volatile extract. α-terpineol, which accounted for 65% of total volatiles in the volatile extract appeared to be the major contributor to the anti-inflammatory effect of the volatiles extracts.

However, none of the other volatile treatments were effective in reducing the production of NO. This was likely due to the lower concentrations of eucalyptol, linalool and linalool oxide tested, which simulated levels of the volatiles present in fresh cranberries. Upon statistical comparison of the two treatment strategies, applying the treatments before inducing inflammation with LPS versus applying the treatments after inducing inflammation with LPS, applying the treatments before inducing inflammation with LPS resulted in lower NO levels in all cases. α-terpineol treatments (1.16 μg/mL, 0.58 μg/mL, and 0.29 μg/mL) applied before LPS addition had mean differences of -7.06 ($p > [t] = 0.0001$), -12.64 ($P > [t] = 0.0001$), and -12.80 ($p > [t] = 0.0001$), respectively compared to the same treatments applied after addition of LPS. Eucalyptol treatments (0.095 μg/mL, 0.047 μg/mL, and 0.024 μg/mL) applied before LPS addition had mean differences of -11.46 ($p > [t] = 0.0001$), -11.98 ($P > [t] = 0.0001$), and -12.88 ($p > [t] = 0.0001$), respectively compared to the same treatments applied after addition of LPS. Linalool treatments (0.021 μg/mL, 0.010 μg/mL, and 0.005 μg/mL) applied before LPS addition

had mean differences of -11.94 ($p > [t] = 0.0001$), -12.44 ($P > [t] = 0.0001$), and -12.30 ($p > [t] = 0.0001$), respectively compared to the same treatments applied after addition of LPS. Linalool oxide treatments (0.112 μg/mL, 0.056 μg/mL, and 0.028 μg/mL) applied before LPS addition had mean differences of -12.27 ($p > [t] = 0.0001$), -12.51 ($P > [t] = 0.0001$), and -12.01 ($p > [t] = 0.0001$), respectively compared to the same treatments applied after addition of LPS. These results indicated that volatile standard treatments were more effective in reducing NO production when applied before the application of LPS as opposed to application after LPS. This finding was consistent with results from the previous experiments involving phenolic and volatile extracts.

3.5 Effects of phenolic and volatile extracts, and standards of major volatiles on cell viability

Cell cytotoxicity of different dilutions (2, 4, and 8x) of phenolic and volatile extracts, and standards of major volatiles (α-terpineol, linalool oxide, eucalyptol and linalool) in cranberry were evaluated by using the MTS assay to exclude the possibility that their NO inhibitory effect was due to their cytotoxicity. The results in Fig. 5 show that all samples regardless of concentration had no effect on viability of RAW264.7 cells, thus showing no cytotoxic effect. Therefore, their inhibitory effects on NO production were not due to cell death.

4 Discussion

Phenolic and volatile extracts isolated from cranberries showed comparable ability to inhibit NO production both before and after LPS application. Inhibition of NO production is important since the compound is associated with cytotoxic RNS and ROS effects.[19] The results of the anti-inflammatory effect of the phenolic extract agreed with previous studies where the total cranberry phenolic fraction or a specific portion of the total phenolic fraction tested on other

cell lines, all exhibited an anti-inflammatory effect.[29-31] A potential mechanism for cranberry phenolic compounds to exhibit anti-inflammatory effects is their ability to inhibit cells from producing pro-inflammatory cytokines including interleukin-8 (IL-8) and chemokine ligand 5 (CCL5) and to reduce the activation of the nuclear factor-κB (NF-κB) p65 pathway when inflammation is induced by LPS.[30] The anti-inflammatory capabilities of cranberry extract have also been attributed to its' high antioxidant capacity, additionally the extract inhibited κB kinase β, which plays a major role in inflammatory signal transduction.[32]

There has been little to no research conducted on volatile compounds in berries and their anti-inflammatory capabilities *in vitro,* so there is no direct literature available for comparison. However, there is ample evidence from essential oil research that volatiles, especially monoterpenes exhibit potent anti-inflammatory activity. In an epithelial buccal cell model, α-terpineol from orange juice was found to have an anti-inflammatory effect by reducing IL-6 production through inhibition of gene expression of the IL-6 receptor.[33] In another study on U937 human macrophage cells, tea tree oil steam distilled from *Melaleuca alternifolia* that contained α-terpineol, suppressed production of the inflammatory markers IL-β1, IL-6, and IL-10 induced by LPS, by interfering with the NF-κB, p38, extracellular signal-regulated kinase (ERK), or mitogen-activated protein kinase (MAPK) pathways.[34]

Anti-inflammatory effects of eucalyptol have been reported in both *in vitro* and *in vivo* studies. In a study on mice, eucalyptol was found to inhibit the production of TNF-α, IL-β1, and IL-6 induced by injection of complete Freund's adjuvants, by a mechanism involving transient receptor potential cation channel subfamily M member 8, TRPM8.[35] In a mouse lung cell study, LPS was used to induce inflammation, and after 24 hr, the lungs and bronchoalveolar lavage of eucalyptol treated and untreated TRPM8 knockout and TRPM8 wild-type were isolated and

analyzed. The authors found that only the TRPM8 wild-type mice exhibited anti-inflammatory effects, showing that TRPM8 was essential for eucalyptol to exhibit its anti-inflammatory capabilities.[36] In another *in vivo* study, mice were injected intraperitoneally with eucalyptol one hr before being challenged with LPS and compared to controls. The eucalyptol injected mice had lower levels of TNF-α, IL-6, NO, and NF-κB than control mice.[37]

The standard linalool treatment exhibited an anti-inflammatory effect in the present study only when applied to the RAW 264.7 cells as a treatment before inducing inflammation with LPS, lowering NO levels by 21.4% compared to the positive control. Huo *et al.* 2013[38] investigated the preventative effect of linalool *in vitro* on RAW 264.7 cells and *in vivo* using a lung injury model and found that linalool lowered the production of TNF-α, and IL-6 *in vitro* and *in vivo* compared to controls. In a study involving *Cinnamomum osmophloeum Kanehira*, a Taiwan native plant, mice administered linalool at 2.6 and 5.2 mg per kg of body weight before injection with endotoxin to induce inflammation, were found to have decreased levels of the inflammatory markers including peripheral nitrate and nitrite, IL-1β, IL-18, TNF-α, and IFN-λ.[39]

The doses of the cranberry phenolic and volatile extracts used to treat the RAW 264.7 cells were lower than the actual concentration in a cranberry. However, the dilutions were necessary due to needs of the RAW 264.7 cells for media and nutrients. Even at these dilutions, some of the phenolic and volatile extract treatments showed a significant anti-inflammatory effect on the RAW 264.7 cells compared to the positive control, when treated both before and after applying LPS, especially at the 2x dilution (635.7 μg/g phenolic and 1.8 μg/g volatile) of both extracts. Taking into consideration that cranberries can be consumed fresh or without the dilutions required for this study, it is reasonable to hypothesize an even stronger anti-inflammatory effect could be observed in an *in vivo* study. The mechanisms of how the phenolic

and volatile treatments work to prevent and treat inflammation are unclear. The volatile compounds could be reducing the aforementioned pathways and cytokines in the same way, but further research is necessary to uncover that information. Interestingly, the antioxidant activity of the volatile extract 40 μM Trolox per kg was much lower than that of the phenolic fraction, 4,676 μM Trolox per kg, indicating that suppression of oxidative stress may not play an important role in prevention of inflammation by the volatile fraction.

The *in vivo* absorption and metabolism of cranberry volatiles is unknown, but i*n vivo* metabolism of R-limonene one of the volatiles present in cranberries has been studied. When human subjects were fed a single limonene dose of 100-130 μg/kg body weight, six metabolites formed through oxidation were identified in blood and urine.[40] These metabolites reached maximum concentrations 1-2 hours post-exposure and were eliminated within 0.7-2.5 hr.[40] The concentrations of the six urinary metabolites accounted for <0.1% to 32% of the orally administered dose.[40]

5 Conclusions

The volatile fraction isolated from cranberries showed comparable ability to inhibit NO production as the polyphenol fraction. This inhibition was found whether volatile and polyphenol treatments were applied before or after application of LPS for 24 hr. However, both volatile and polyphenol fractions showed greater ability to inhibit NO production when treatments were applied before application of LPS as opposed to after application of LPS. α-terpineol the major volatile found in the volatile extract was also found to be an effective inhibitor of NO production when applied to cells at concentrations close to those present in fresh cranberries. Our results demonstrate that although volatiles are present at much lower concentrations than polyphenols in fresh cranberries, they show comparable anti-inflammatory

activity. Additional research is needed to determine the bioavailability of cranberry volatiles and to identify the mechanisms by which they may play a role in the prevention and mitigation of pre-existing inflammation.

Conflicts of interest

There are no conflicts of interest to declare.

Acknowledgments

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Table 1 Anthocyanin, flavonol and chlorogenic acid concentrations in the cranberry phenolic fraction

Anthocyanins[1]	Concentration (mg ACY-3-Glu Equiv./kg)
Cyanidin 3-galactoside	148 ± 1.0
Cyanidin 3-glucoside	2.7 ± 0.1
Cyanidin 3-arabinoside	93.1 ± 1.0
Peonidin 3-galactoside	489.8 ± 4.0
Peonidin 3-glucoside	30.2 ± 0.3
Malvidin 3-galactoside	18.6 ± 0.2
Peonidin 3-arabinoside	195.0 ± 2.0
Total anthocyanins	936.6 ± 8.0
Flavonols[2]	Concentration (mg Rutin Equiv./kg)
Myricetin 3-galactoside	16.9 ± 0.09
Myricetin 3-xyloside	0.42 ± 0.02
Myricetin 3-arabinoside	6.67 ± 0.14
Quercetin 3-galactoside	25.22 ± 0.14
Quercetin 3-glucoside	0.83 ± 0.15
Quercetin 3-xyloside	2.51 ± 0.02
Quercetin 3-arabinopyranoside	3.01 ± 0.02
Quercetin 3-arabinofuranoside	8.86 ± 0.08
Quercetin 3-rhamnoside	3.10 ± 0.02
3-methoxyquercetin-3-pentoside	2.69 ± 0.02
Quercetin 3-coumaroylgalactoside	6.18 ± 0.28
Quercetin 3-benzoylgalactoside	0.67 ± 0.02
Total flavonols	77.0 ± 1.0
Hydroxycinnamic acids	Concentration (mg CLA Equiv./kg)
Chlorogenic acid[3]	14.7 ± 0.2

[1]Anthocyanins were quantified using an external calibration curve of a mix of the three predominant anthocyanin glucosides found in cranberries; cyanidin, peonidin and malvidin and are expressed as glucoside equivalents per kg of fresh weight. [2]Flavonols were quantified using an external calibration curve of rutin and are expressed as mg of rutin equivalents per kg of fresh weight. [3]Hydroxycinnamic acids were quantified using a chlorogenic acid external calibration curve and are expressed as mg of chlorogenic acid equivalents per kg of fresh weight.

Table 2 Volatile composition of cranberry distillate

Compound	Retention time (min)	Retention Index	Concentration (μg/kg)	% of Total volatiles
1-Butanol	4.22	675	18.2	0.51
1-Pentanol	8.12	760	24.6	0.69
Hexanol	9.10	869	24.7	0.70
Heptanal	11.97	903	6.8	0.19
Benzaldehyde	13.45	960	17.9	0.51
1-heptanol	13.65	970	14.6	0.41
1-octen-3-ol	13.87	982	16.9	0.47
Octanal	14.40	1006	10.9	0.31
α-phellandrene	14.57	1006	27.2	0.76
D-Limonene	14.98	1031	22.7	0.64
Benzyl alcohol	15.06	1031	4.2	0.12
Eucalyptol	15.17	1030	189.0	5.32
α-ocimene	15.21	1038	40.3	1.13
β-ocimene	15.36	1038	7.8	0.22
2-octanal	15.63	1064	110.3	3.10
Linalool oxide	15.88	1070	224.4	6.32
Linalool	16.35	1095	41.3	1.16
Nonanal	16.50	1104	15.5	0.44
α-dimethylstyrene	16.58	1118	46.7	1.32
Terpinen-4-ol	18.09	1179	74.4	2.09
α-terpineol	18.45	1195	2320.6	65.35
Carvone	19.46	1254	45.9	1.29
Trans-2-decanal	19.61	1265	134.2	3.78
Unknown	22.59	1414	54.7	1.54
Unknown	23.21	1455	58.0	1.63

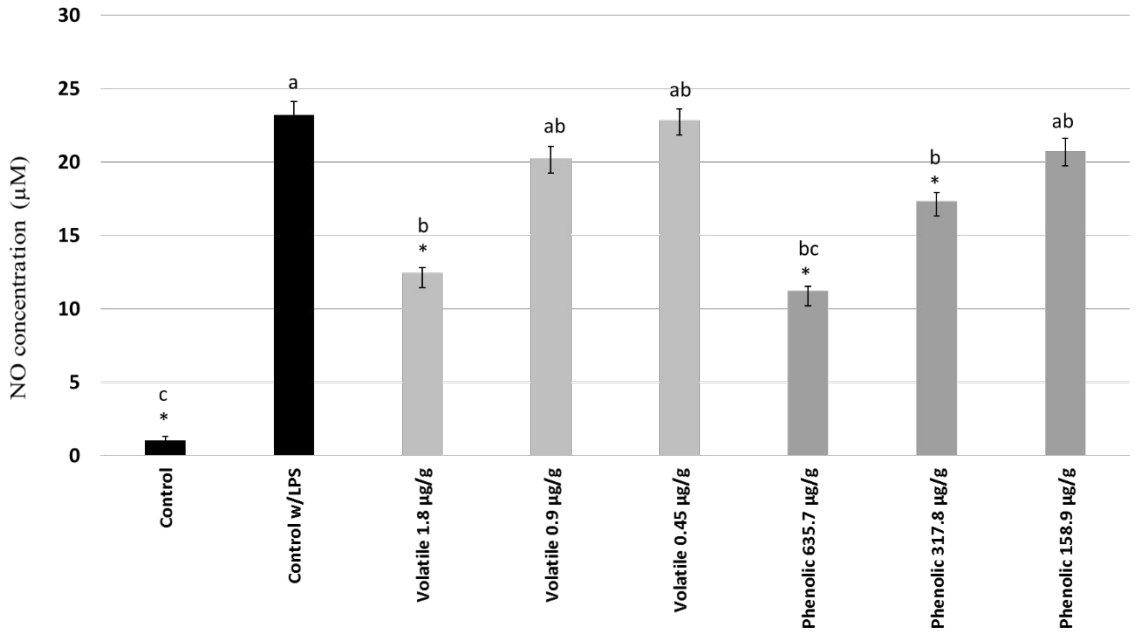

Fig. 1 Average NO concentration (n=5, ± SEM) produced by RAW 264.7 cells treated with phenolic and volatile extracts before applying LPS. * indicates a significant difference from the positive control (CTR w/ LPS). Bars with different letters are significantly different ($P < 0.05$).

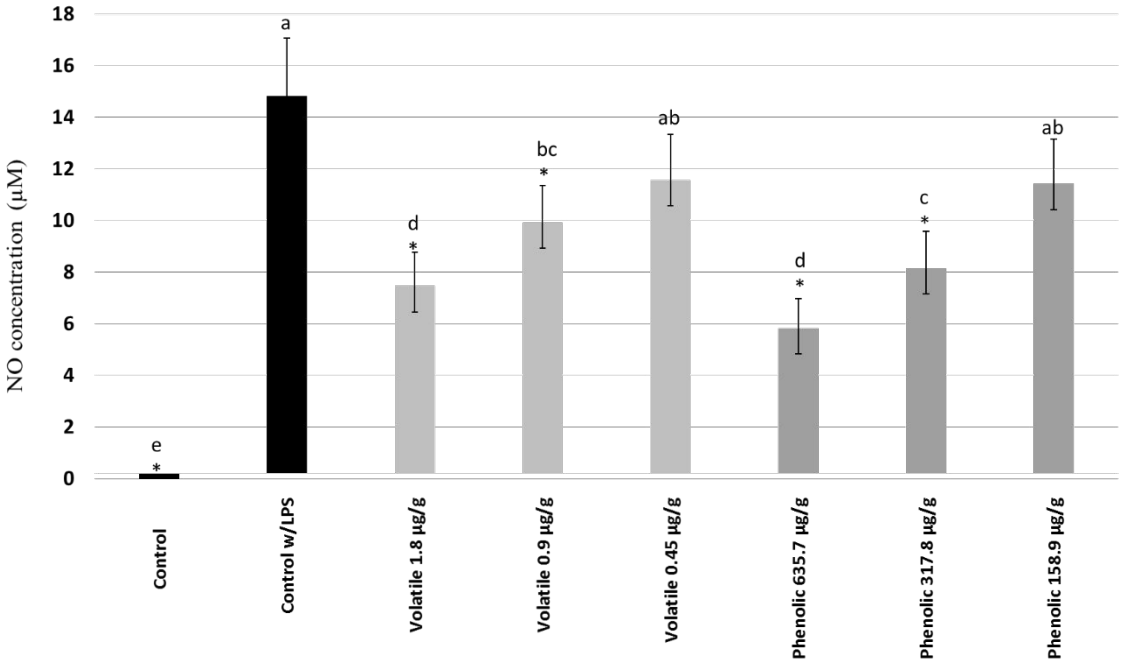

Fig. 2 Average NO concentration (n=5, ± SEM) produced by RAW 264.7 cells treated with phenolic and volatile extracts after applying LPS. * indicates a significant difference from the positive control (CTR w/ LPS). Bars with different letters are significantly different ($P < 0.05$).

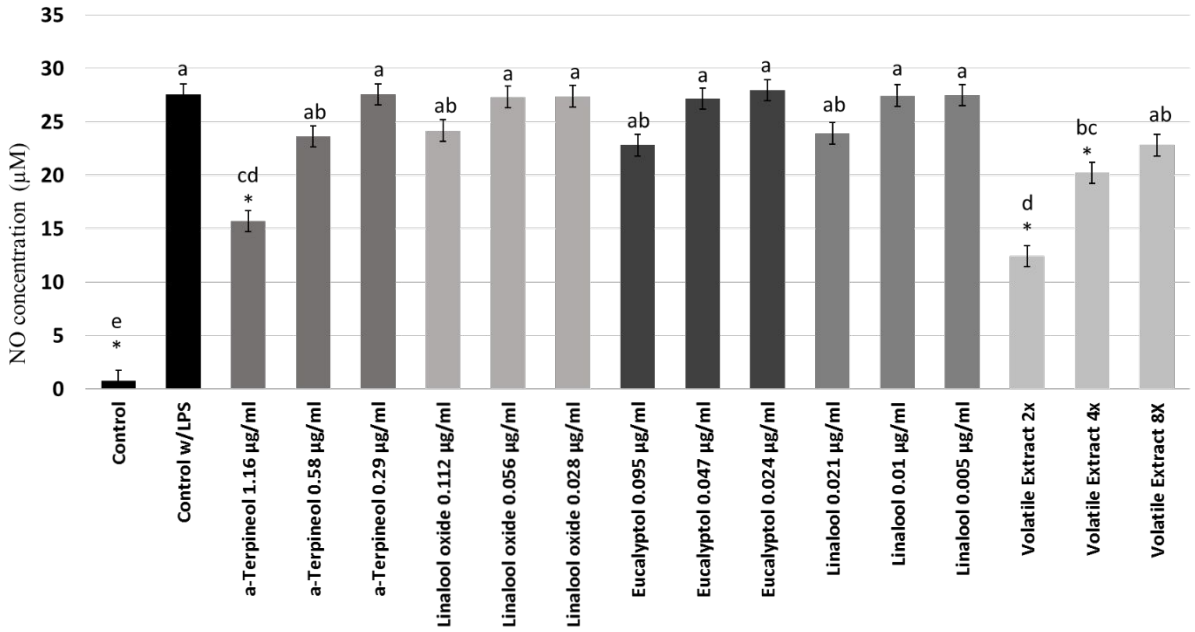

Fig. 3 Average NO concentration (n=5, ± SEM) produced by RAW 264.7 cells treated with α-terpineol, linalool, linalool oxide, and eucalyptol standards and volatile extracts before applying LPS. * indicates a statistically significant difference from the positive control (CTR w/ LPS). Bars with different letters are significantly different ($P < 0.05$).

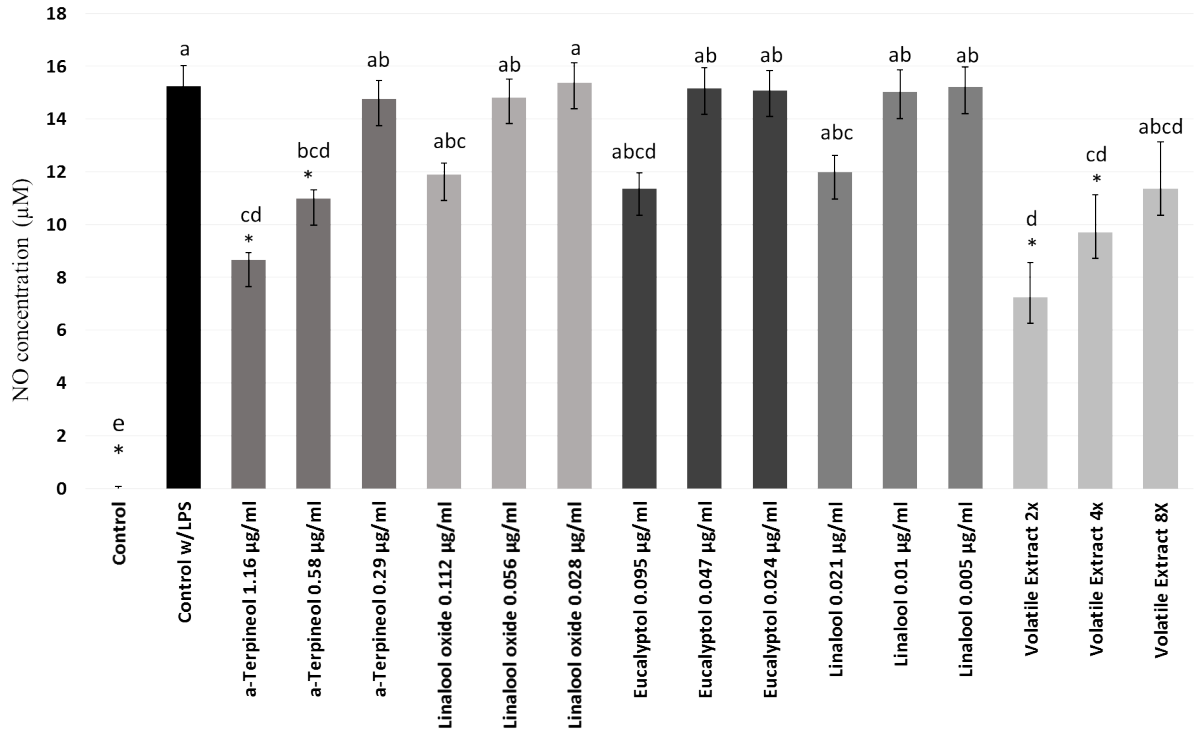

Fig. 4 Average NO concentration (n=5, ± SEM) produced by RAW 264.7 cells treated with α-terpineol, linalool, linalool oxide, and eucalyptol standards and volatile extracts after applying LPS. * indicates a statistically significant difference from the positive control (CTR w/ LPS). Bars with different letters are significantly different ($P < 0.05$).

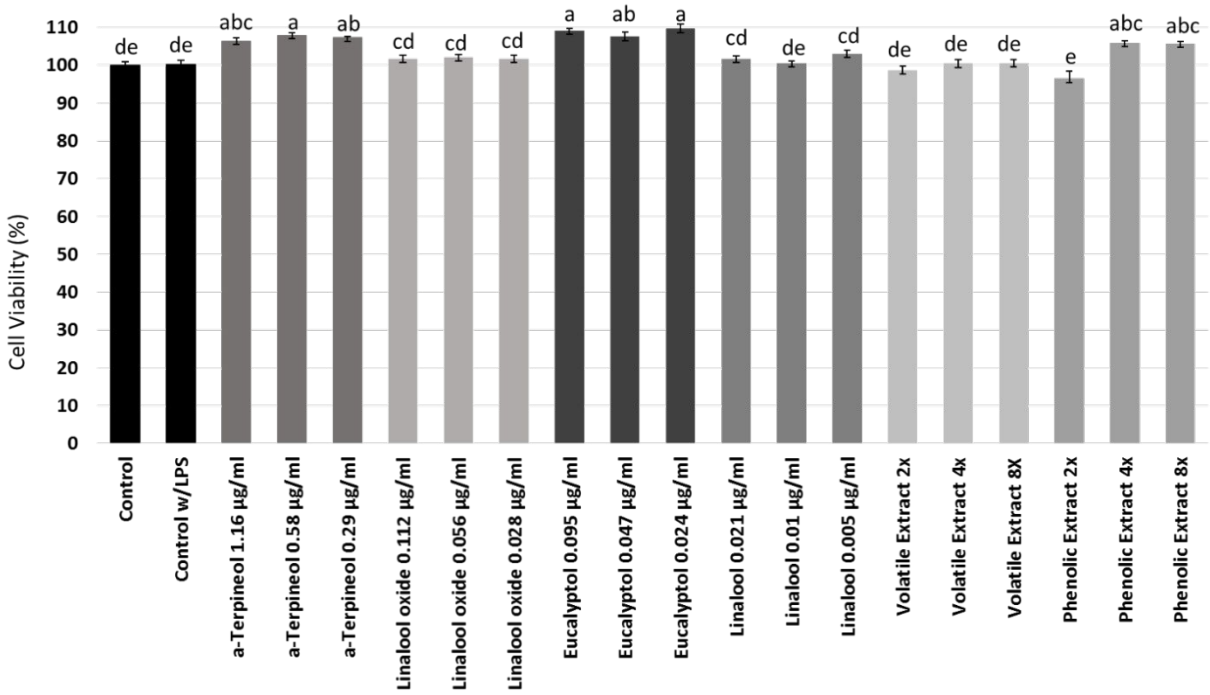

Fig. 5. Percent viability of RAW 264.7 cells (n =3, ± SEM) treated with α-terpineol, linalool, linalool oxide, and eucalyptol standards and phenolic and volatile extracts before applying LPS. Bars with different letters are significantly different ($P < 0.05$).