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Phytochemical Analysis and Anti-Inflammatory Activity of *Nepeta cataria* Accessions

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ABSTRACT

A method was developed for the identification and quantification of polyphenols in the flowers, upper leaves and lower leaves of *Nepeta cataria* using HPLC/UV/MS. This led to the identification of two phenolic acids including caffeic and rosmarinic acid along with eight flavonoids including luteolin, apigenin and their glucosyl and glucuronyl derivatives. The results indicated that total polyphenols averaged 11.32 ± 3.95 mg/g, 12.31 ± 2.5 mg/g and 11.29 ± 2.88 mg/g for the flowers, upper leaves and lower leaves, respectively. The antioxidant capacities of the extracts from *N. cataria* were determined using the $ABTS^+$ radical scavenging assay and averaged 30.35 ± 3.56 µM, 30.56 ± 4.71 µM and 29.7 ± 4.61 µM equivalent / g DW for the flowers, upper leaves and lower leaves, respectively. Anti-inflammatory activity was assayed by measuring nitric oxide concentrations after treating RAW 264.7 murine macrophages stimulated by *E. coli* lipopolysaccharide and the IC_{50} values averaged 81.96 ± 37.49 µg/mL, 95.99 ± 85.55 µg/mL and 91.28 ± 47.71µg/mL for the flowers, upper leaves and lower leaves, respectively. All extracts from the different plant tissues demonstrated radical scavenging capabilities and dose dependent activity in the inhibition of nitric oxide similar to other members of the Lamiaceae family.

INTRODUCTION

Catnip, *(Nepeta cataria*; Family, Lamiaceae), has long been recognized for its phytochemical and medicinal properties (Lewis and Elvin-Lewis, 1982; Sastry et al., 1972). Members of this plant family are highly aromatic with many species renown for culinary uses such as basil, mints, oregano, rosemary, sage and thyme and others such as peppermint, spearmint, clary sage and others are grown to distill their essential oils for use in foods, flavors, personal hygiene and industrial products (Ellis and Stevenson, 1950; Lewis and Elvin-Lewis, 1982). While catnip is well known for producing volatile monoterpene iridoid nepetalactones, the compounds responsible for eliciting euphoria in felines (Waller et al., 1969), members of this genus have been reported to produce a wide array of non-volatile medicinally bioactive polyphenols that are

often overlooked (Jamzad et al., 2003). Less recognized is that catnip is consumed as an herbal tea purported in traditional cultures to be useful for treating medical disorders such as inflammation, digestive ailments, infantile colic, toothaches, used as a cold remedy, anxiety and as a blood depurative (Gilani et al., 1993; Lewis and Elvin-Lewis, 1982; Naghibi et al., 2010). Native American tribes made catnip tea and would drink this as a beverage multiple times a day to help treat their ailments even after the introduction of western medicine (Cichoke, 2001). African American slaves as well as their owners used catnip for the same purposes in the southern Appalachian Mountains and it is still used by African Americans to treat infantile colic and digestive issues (Smitherman et al., 2005). Europeans and the Chinese have previously used catnip leaves in their cooking and herbal infusions (Liu et al., 2009). Catnip while rich in volatile aromatic oils may also contain other water-soluble bioactive compounds as do other members of the Lamiaceae (Shen et al., 2010; Shen et al., 2011).

Polyphenols are characterized by the presence of more than one aromatic hydroxyl group and are commonly consumed from natural sources such as herbs, fruits and vegetables. Individuals that frequently consume polyphenol rich foods have been reported to have a reduced risk towards getting cancer, cardiovascular diseases, neurodegenerative diseases and inflammation (Embuscado, 2015). Polyphenols biosynthesized in members of the Lamiaceae family have been shown to exhibit medicinal bioactivity for a broad range of ailments such as acetocholinesterase inhibitory activity indicating that they could be useful in enhancing the chlorogenic transmission of messages for people suffering from Alzheimer’s Disease (Vauzour et al., 2010). Polyphenols from oregano and other Lamiaceae species inhibited DPP-IV, which is responsible for insulin secretion, warranting further investigation into Lamiaceae derived polyphenols for diabetes management (Vladimir-Knežević et al., 2014). Anti-oxidant activity is well reported within the Lamiaceae plant family with lavender, mint, oregano and lemon balm being just some of the species reported to contain high concentrations of bioactive polyphenols and other compounds exhibiting radical scavenging capabilities (Bower et al., 2014; Shen et al., 2010; Shen et al., 2011).

Research supporting that *N. cataria* may have health and a nutritional or medicinal application is emerging. Medicinally bioactive phenolic acids such as rosmarinic acid, caffeic acid, p-coumaric acid, chlorogenic acid and cinnamic acid have been detected in *N. cataria* (Spiridon et al., 2011). Glucuronidated and glycosylated apigenins and luteolins have been identified in *N. cataria* var. *citriodora* (Mihaylova et al., 2013). Anti-oxidant capabilities essential for reducing inflammation and hazardous free radical formations have been confirmed in *N. cataria* with one study showing three separate assays (ABTS, DPPH radical scavenging, ferric reducing power) to confirm the activity of ethanol extracts containing luteolin and apigenins (Spiridon et al., 2011).

Growing usage of flavonoids such as luteolin and apigenin in herbal products and dietary supplements and recognizing the role catnip plays as a medicinal herb in the United States, led us to investigate several genetic lines of *N. cataria* for their polyphenol profiles. Plant material that is highly concentrated in polyphenols as well as the presence of a diverse array of actual flavonoids could both partially explain the possible health benefits from traditional uses as well as identify potential applications in the nutraceutical market. Florae that are members of the same family that produce secondary metabolites may contain the same or often have different chemical profiles and bioactivity due to different genetic predispositions, environmental influences, growth stages and the timing of harvest (Modnicki et al., 2007). Other Lamiaceae family members such as lavender, mint, oregano and thyme, have shown broad chemical diversity in the production of non-volatile compounds which has resulted in non-uniform marketable products (Bower et al., 2014; Delgado et al., 2014; Shen et al., 2010; Shen et al., 2011; Simona et al., 2015; Voirin et al., 1999). Evaluating the uniformity in the production of secondary metabolites within a population of plants is crucial when first examining the chemical characteristics of

a plant product and more so when introducing a new natural product since so much environmental and inherent variability can alter the product. When such variation exists in plants, it provides an opportunity to improve and enrich targeted phytochemicals that are responsible for the medicinal bioactivity. For these reasons we screened a population of *N. cataria* plants for polyphenol content, anti-oxidant capacity and anti-inflammatory activity to evaluate the medicinal potential and phytochemical variability with respect to an intraspecies population.

MATERIALS AND METHODS

Chemicals and cell lines. HPLC grade acetonitrile (ACN), methanol (MeOH), ethanol (EtOH), formic acid (FA), acetic acid (AcOH), hydrochloric acid (HCl) and HPLC grade water (H_2O) were obtained from Fisher Scientific Co. (Fair Lawn, NJ). Trolox® (a water soluble vitamin E derivative), ABTS (2, 2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid)), potassium persulfate ($K_2S_2O_8$) and sodium carbonate (Na_2CO_3) were obtained from Sigma Aldrich (St. Louis, MO). Commercial standards for caffeic acid, rosmarinic acid, luteolin and apigenin were obtained from Sigma Aldrich (St. Louis, MO). RAW 264.7 cells were procured from the American Type Culture Collection (Rockville, MD). RPMI-1640 cell culture medium, penicillin, streptomycin, dimethyl sulfoxide (DMSO), lipopolysaccharide (LPS) and the Griess Reagent was obtained from GIBCO (Grand Island, NY).

Nepeta cataria cultivation. The clonal *N. cataria* populations used in this study included six Rutgers University lines selected from the USDA germplasm (Reichert et al., 2016) as well as four seeded commercial catnip sources that were initially grown at the Rutgers University Research Greenhouses. Once mature, the flowers, the leaves closest to the shoot apical meristem but below the flowers and the leaves near the basal portion of the plant were harvested separately and dried at 37°C until no moisture was present with an onsite Powell walk-in forced air heat dryer. After the leaves and flowers had dried and lost no further weight, the plant samples were carefully manually separated from the stems for further analyses.

Analytical equipment. Sample separation was performed on a Prodigy Phenomenex ODS3 5µm, 150 x 3.2 mm, 5 micro column (Phenomenex Inc., Torrance, CA). For all LC/ESI-MS analysis, a Hewlett Packard Agilent 1100 Series LC/MSD (Agilent Technologies, Waldbronn, Germany) equipped with an autosampler, quaternary pump system, diode array detector, thermostated column compartment, degasser, MSD trap with an electrospray ion source (ESI) and HP ChemStation, Data Analysis 4.2 was used for data analysis.

HPLC/UV/MS conditions for the identification and quantification of polyphenols. The solvent conditions for sample separation had the mobile phase containing solvent A (0.1% FA in H_2O) and B (0.1% FA in ACN) in gradient: 0-30 min, linear gradient from 10% B to 40% B. The flow rate was set at 1.0 mL/min. The eluent was monitored by an electrospray ion mass spectrometer (ESI-MS) and scanned from *m/z* 100 to 1200. ESI was conducted in negative mode by using a needle voltage of -3.5 KV. High purity nitrogen (99.999%) was used as dry gas at a flow rate of 12 L/min and capillary temperature was at 350°C. Nitrogen was used as nebulizer at 60 psi, and helium as collision gas set to 80% collision energy. For UV detection, phenolic acids were detected at 320 nm and flavonoids at 370 nm.

Sample preparation. N. cataria samples were extracted from ca. 200 mg of dried ground plant material that was sonicated for 10 minutes in 25 mL of 70% MeOH in H_2O with 0.1% AcOH. Extracts were then conditioned at room temperature overnight, after which they were filtered through a 0.45-µm filter for HPLC separation and antioxidant capacity determination. The samples for the anti-inflammatory analysis were prepared by extracting the plant materials using 70% MeOH in H_2O with 0.1% AcOH. These extracts were allowed to condition for 24 hours. Once the MeOH was removed by rotary evaporation, the samples were cooled to -50˚C, lyophilized and prepared with DMSO at different concentrations.

Hydrolysis of apigenin and luteolin glycosides was done by weighing ca. 60 mg of *N. cataria* powder and placing it in a volumetric flask with 2 ml of a 2.7 M HCl solution in a water bath at 90°C for 60 min. After hydrolysis, the volume was filled up to 5 ml and filtered through a 0.45-µm filter before injection into the HPLC.

All standards were measured on an analytical balance and dissolved in 70% MeOH in H_2O with 0.1% AcOH to prepare the stock solution. The stock solution was serially diluted with 70% MeOH in H_2O with 0.1% AcOH. The range of quantifiable concentrations for each standard as well as their calibration curve and determination coefficient are as follows: caffeic acid (0.80-51.50 µg/mL, y = 30.03x + 2.6422, R^2 = 0.9999), rosmarinic acid (0.78-50.00 µg/mL, y = 13.145x + 0.7892, R^2 = 0.9999), luteolin (0.39-25.00 µg/mL, y = 19.204x-0.5329, R^2 = 0.9999), apigenin (0.41-26.00 µg/mL, y = 13.022x + 0.5061, R^2 = 0.9999).

Trolox equivalent absorption capacities. The method for the determination of total anti-oxidants was used as described (Zulueta et al., 2009) with minor modifications, based on the capacity of a sample to inhibit the ABTS radical compared to Trolox®. The ABTS radical was generated by single electron oxidation by $K_2S_2O_8$, accomplished by preparing a solution containing 7.46 mM ABTS and 2.44 mM $K_2S_2O_8$, in deionized H_2O. This solution was allowed to stand in darkness at room temperature for 12–16 h (the time required for formation of the radical). The working solution was prepared by taking a volume of the previous solution and diluting it in EtOH until its absorbance at λ = 734 nm was 0.70 ± 0.02. The measuring was done using an Agilent G1111AA UV-Vis spectrophotometer. For this purpose, 990 µl of the ABTS radical was added to the cuvette; the absorbance was measured, and 10 µl of sample or standard solution were added immediately, followed by a 20 min reaction time in which the final absorbance was measured. There is a quantitative relationship between the reduction of the absorbance and the concentration of anti-oxidants present in the sample against a blank solution of ethanol. A calibration curve was prepared using Trolox at a range of 0.0359 mM - 1.65 mM in EtOH. Trolox Equivalent Anti-oxidant Capacity (TEAC) was expressed as the content in µM Trolox/ g dry weight (DW) required to achieve the same response as measured in the sample.

Anti-inflammatory conditions. As described (Kim et al., 1995), RAW 264.7 cells, derived from murine macrophages, were cultured in RPMI-1640 (without phenol red) supplemented with 10% endotoxin-free, heat-inactivated fetal calf serum, 100 mg/mL penicillin, and 100 mg/mL streptomycin. Once the cells reached a density of (2-3) x 10^6 cells/mL, they were activated by *E. coli* LPS at 100 ng/mL to signal the production of nitric oxide to destroy bacterial cells. Catnip extracts dissolved in DMSO at concentrations of 20, 40 and 80 µg/mL were combined with LPS. The cells were cultured in 100 mm tissue culture dishes and incubated with 100 ng/mL of LPS for 12 h. The cells were treated with 0.05% DMSO without LPS as vehicle control. The cells were then harvested and plated in a 24-well plate and treated with LPS only or with different concentrations of test compounds for a further 12 h. Indomethacin was defined as 100% inhibition and used as positive control for the experiment due to its ability to completely inhibit NO production.

At the end of incubation time, 100 µL of the culture medium was collected for the nitrite assay. The amount of nitrite, an indicator of NO synthesis and subsequent iNOS expression, were measured using the Griess reaction. The supernatants (100 µL) were mixed with the same volume of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylenedia- mine dihydrochloride in H_2O) in duplicate on 96-well plates. After incubation at room temperature for 10 min, absorbance at 570 nm was measured with a 96-well plate reader (Thermo Labsystems, Multiskan Ascent, Finland). The values are expressed as means (standard error of triplicate tests).

Statistical analysis. Values are presented as means ± standard deviation (SD). Data was analyzed by an unpaired, two-tailed student's t-test to identify significant difference $P < 0.05$.

RESULTS

Qualitative identification. Using HPLC/UV/MS under negative ion mode, a total of 10 major polyphenols were identified based on retention time, UV absorption spectrum, mass spectrum and by comparison to co-injected authenticated standards. The aglycones of flavonoid glycosides were further confirmed by comparison with the authenticated standards of luteolin and apigenin in an acidic hydrolyzed material. The retention time and detected mass ions under negative mode for each compound are summarized in Table 1. Representative UV chromatograms are shown in Figure 1 and the mass spectra of the major compounds in Figure 2. Chemical profiling of the different tissues of catnip revealed that the aglycones were present largely in the flowers. The leaves did not produce the aglycone apigenin but three lines upper leaf samples produced the luteolin aglycone. The other eight compounds were frequently present in the flowers, upper leaves and lower leaves though their concentrations differed significantly statistically.

Quantitative analysis of polyphenols. Quantification of polyphenols was performed on the HPLC with UV detection at 370 nm and 320 nm for flavonoids and phenolic acids, respectively.

Table 1. Polyphenols identified in catnip (*Nepeta cataria*).

Compound ID #	t_R (min)	Identities	$[M-H]^-$ (m/z)
1	7.2	Caffeic Acid*	179
2	7.9	Luteolin diglucuronide	637
3	9.7	Apigenin diglucuronide	621
4	10.7	Luteolin glucoside	447
5	11.3	Luteolin glucuronide	461
6	13.0	Apigenin glucoside	431
7	13.9	Apigenin glucuronide	445
8	14.2	Rosmarinic Acid*	359
9	18.6	Luteolin*	285
10	22.3	Apigenin*	269

* Compared with authenticated standard

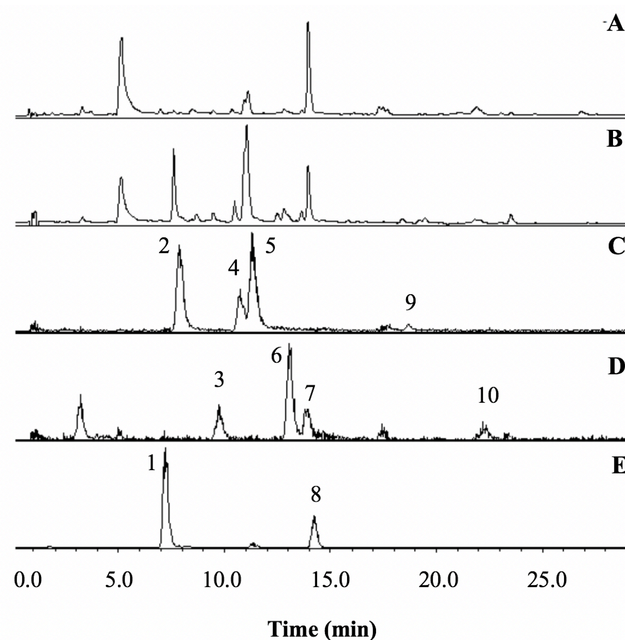

Figure 1. Representative LC/UV/MS chromatograms of a *Nepeta cataria* extract. (A) UV chromatogram at 370 nm for detection of flavonoids; (B) UV chromatogram at 320 nm for detection of phenolic acids; (C) Extracted ion chromatogram for luteolin and its derivatives; (D) Extracted ion chromatogram for apigenin and its derivatives. (E) Extracted ion chromatogram for caffeic acid and rosmarinic acid. The identities, retention time (t_R) and MS of each peak are listed in Table 1.

The individual glycosides of the flavones were estimated against the respective aglycones of apigenin and luteolin using the correction factor of molecular weight ratio.

The results indicated that individual compound concentrations differed statistically within plant tissues: caffeic acid and luteolin diglucuronide were more concentrated in the leaves of the plants while all of the other compounds were more concentrated in the flowers. ($P < 0.05$) (Table 2). Total polyphenols averaged 11.32 ± 3.95 mg/g for the flowers, 12.31 ± 2.5 for the upper leaves and 11.29 ± 2.88 for the lower leaves. There was no significant difference in the production of polyphenols between the different plant tissues (Figure 3).

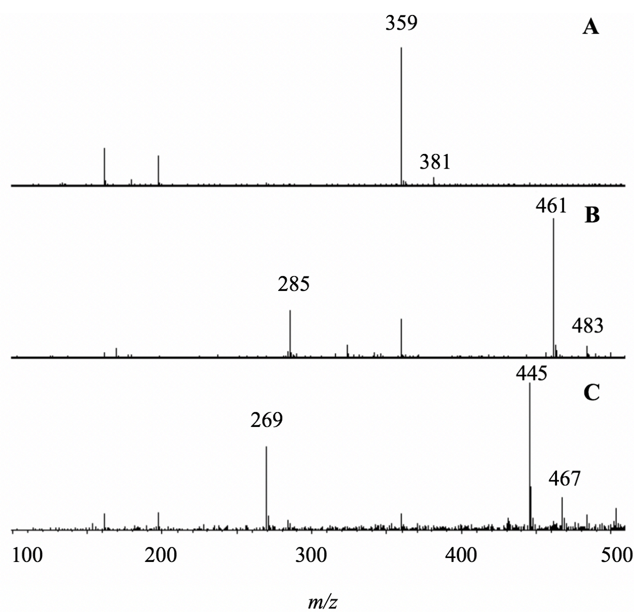

Figure 2. Mass spectra of major peaks identified in *Nepeta cataria*. (A) MS spectrum of rosmarinic acid; (B) MS spectrum of luteolin glucuronide; (C) MS spectrum of apigenin glucuronide.

Total flavonoids averaged 6.48 ± 2.98 mg/g for the flowers, 5.39 ± 1.40 mg/g for the upper leaves and 4.60 ± 1.26 mg/g for the lower leaves; their levels of production did not differ statistically by plant tissues ($P > 0.05$). Total phenolic acid content averaged 4.84 ± 1.22 mg/g for the flowers, 6.92 ± 1.18 mg/g for the upper leaves and 6.70 ± 1.72 mg/g for the lower leaves; these compounds did differ statistically with the upper leaves, and lower leaves having significantly more phenolic acid production than the flowers ($P < 0.05$). The total content of the apigenin aglycone and its derivatives differed significantly between each plant organ ($P < 0.05$). On average, the concentration for apigenins and the derived analogues in the flowers, upper leaves, and lower leaves was 1.35 ± 0.73 mg/g, 0.57 ± 0.18 mg/g and 0.40 ± 0.14 mg/g, respectively. For the luteolins, the average concentration of the aglycone and the derived analogues in the flowers, upper leaves and lower leaves was 5.13 ± 2.27 mg/g, 4.82 ± 1.33 mg/g and 4.20 ± 1.19 mg/g respectively and they did not significantly differ ($P > 0.05$). Total flavonoids in our samples ranged from 0.23% to 1.10% of dry mass while similar members of this species were reported to produce under 0.50% of dry mass (Modnicki et al., 2007). Total phenols ranged from 0.26% to 0.88% of dry matter which is lower than what was reported in a separate *N. cataria* study using different genetic lines that reported up to 1.4% of dry matter to be phenolic acids (Modnicki et al., 2007).

Table 2. Quantity and bioactivity of compounds in the extracts of *N. cataria* flowers, upper leaves, and lower leaves.

Quantified Compounds (mg/g)	**Flowers**	**Upper Leaves**	**Lower Leaves**
Caffeic acid	3.18 ± 1.44 B*	6.62 ± 1.24 A	6.37 ± 1.82 A
Luteolin diglucuronide	0.82 ± 0.40 B	2.87 ± 1.32 A	2.60 ± 1.03 A
Apigenin diglucuronide	0.36 ± 0.28 A	0.28 ± 0.08 A	0.22 ± 0.08 A
Luteolin glucoside	0.63 ± 0.40 A	0.09 ± 0.06 B	0.03 ± 0.04 B
Luteloin glucuronide	3.57 ± 1.82 A	1.84 ± 0.66 B	1.56 ± 0.69 B
Apigenin glucoside	0.63 ± 0.36 A	0.14 ± 0.07 B	0.07 ± 0.05 B
Apigenin glucuronide	0.32 ± 0.18 A	0.14 ± 0.06 B	0.10 ± 0.04 B
Rosmarinic acid	1.65 ± 0.93 A	0.29 ± 0.20 B	0.32 ± 0.30 B
Luteolin	0.09 ± 0.06 A	0.01 ± 0.12 B	0.00 ± 0.00 B
Apigenin	0.03 ± 0.04 A	0.00 ± 0.00 B	0.00 ± 0.00 B
Bioactivity Data			
TEAC (µM Trolox® Equivalent/g)	30.35 ± 3.56 A	30.56 ± 4.71 A	29.14 ± 4.61 A
NO inhibition IC50 Value (µg/ml)	81.96 ± 37.49 A	95.99 ± 85.55 A	91.28 ± 47.71 A

*Values within rows followed by the different letters are significantly different according to unpaired, two-tailed Student's T-test ($P < 0.05$).

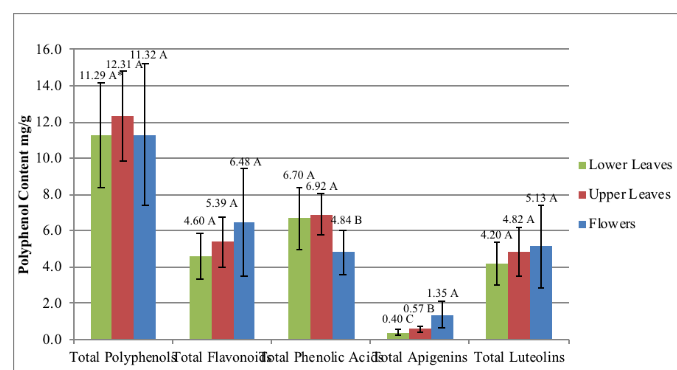

Figure 3. Polyphenol content (mg/g) of catnip (*Nepeta cataria*) in the flowers, upper leaves and lower leaves by HPLC.

*Values within totals followed by the different letters are significantly different according to the unpaired, two-tailed Student's T-test ($P < 0.05$).

Anti-oxidant capacities. Free radical absorption capacity was detected in every sample and the tissues were not statistically different from one another in radical reduction signifying that the bioactivity with respect to anti-oxidant activity did not differ significantly within the different plant tissues ($P > 0.05$). For the flowers, TEAC averaged 30.35 ± 3.56 µM Trolox equivalent / g DW Upper leaves average TEAC was 30.56 ± 4.71 µM Trolox

equivalent / g DW. Lower leaf TEAC averaged 29.7 ± 4.61 µM Trolox equivalent / g DW. The anti-oxidant activity of the *N. cataria* samples was higher than numerous members of unrelated plant species such as pears, apples, melons and bananas with two samples being more active than red grapes (Wang et al., 1996).

Anti-inflammatory activity. Inhibition of nitric oxide production (Fig. 4) was measured by evaluating LPS-induced cells with the MeOH extracts of *N. cataria.* All of the samples from the three *N. cataria* tissues exhibited signs of dose-dependent activity in the inhibition of the NO production. There was no statistically significant difference in the plant organ treatment to IC_{50} value ($P > 0.05$) indicating that the compounds essential for anti-inflammatory activity are biosynthesized throughout the plant. The IC_{50} value for the flowers averaged 81.96 ± 37.49 µg/mL. The IC_{50} value from the upper leaves averaged 95.99 ± 85.55 µg/mL. The IC_{50} value from the lower leaves averaged 91.28 ± 47.71µg/mL.

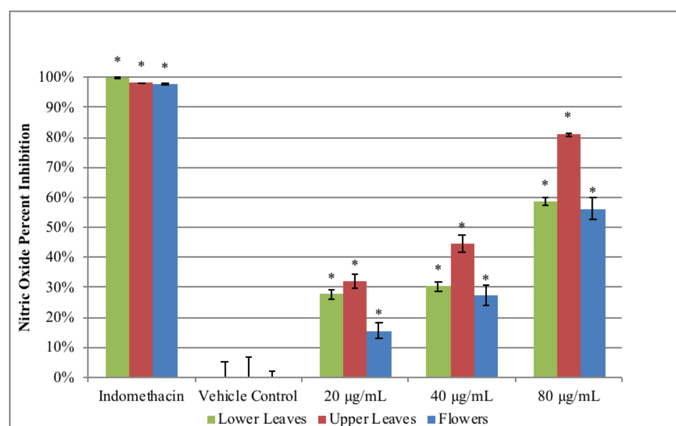

Figure 4. Inhibition of nitric oxide production in LPS-induced RAW264.7 murine macrophages by ethanol extracts of *Nepeta cataria* flowers, upper leaves and lower leaves.
*Differed significantly from vehicle control (unpaired, two tailed Student's T-test, $P < 0.05$)

DISCUSSION

All of the non-glucuronidated compounds contained in the catnip extracts have been identified as being medicinally active *in vivo* with one example being caffeic acid, as it effects inflammatory cell recruitment and subsequent expression of pro-inflammatory chemokines and cytokines in ApoE KO mice (Norata et al., 2007). In DBA/1 mice, rosmarinic acid reduced the arthritic index and COX-2 expression in the mice exhibiting anti-inflammatory activity (Youn et al., 2003). Apigenin consumption also reduced LPS-induced expression of miR-155, a main construct in inflammation initiation (Arango et al., 2015). *In vivo* studies of rats treated intradermally with radical generating enzymes showed that apigenin glucoside can reduce the overall inflammation in the skin in a dose-dependent manner and in other murine models, inhibited IL-6 and TNF-α-production (Fuchs and Milbradt, 1993). *In vivo* studies of luteolin as an anti-inflammatory agent were conducted on mice and they were protected from LPS-induced inflammation by luteolin (Kotanidou et al., 1993). Luteolin glucoside inhibited paw edema in the carrageenan and hind induced murine models (Tatli et al., 2008). The results indicated that catnip is a rich source of bioactive polyphenols including both apigenin and luteolin glycosylated and glurcuronidated derivatives. The *N. cataria* plants analyzed in this study were more effective at inhibiting NO production than apple mint and peppermint and showed comparable activity to oregano (Shen et al., 2010; Shen et al., 2011). As the polyphenols accumulate in each of the three plant tissues, all parts of the plant can be harvested for polyphenols. While the inherent antioxidant and anti-inflammatory activity of *N. cataria* could not be deduced to a single compound, the wide array of flavones and phenolic acids present likely contribute to the associated bioactivity of the plant. Since *N. cataria* is being used as an herbal supplement and as an herbal tea, these results show that both the leaves and flowers should be included in dried products and extracts in order to provide the full battery of bioactive polyphenols. Breeding efforts to increase the yields and concentration of total and selected polyphenols appears most promising given the plant is still largely undomesticated varying in its essential oil composition and polyphenols (Reichert et al.,

2016). Catnip demonstrated strong bioactivity and the holistic use of it as a home remedy appears to be at least partially justified due to the high concentrations of apigenin and luteolin derivatives.

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