Chemical composition, antioxidant and anti-inflammatory properties of *Monarda didyma* L. essential oil

Daniele Fraternale [1], **Hanh Dufat** [2], **Maria Cristina Albertini** [1], **Chouaha Bouzidi** [2], **Rossella D'Adderio** [1], **Sofia Coppari** [1], **Barbara Di Giacomo** [1], **Davide Melandri** [3], **Seeram Ramakrishna** [4], **Mariastella Colomba** [Corresp. 1]

[1] Department of Biomolecular Sciences, University of Urbino, Urbino, PU, Italy

[2] Produits Naturels, Analyse et Synthèse, CITCOM-UMR CNRS 8038 - Faculté de Santé, Pharmacie, Université de Paris, Paris, France

[3] U. Burns Center, Dermatology and Emilia Romagna RegionalSkinBank, M. Bufalini Hospital, Cesena, (FC), Italy

[4] Center for Nanofibers and Nanotechnology, National University of Singapore, Singapore, Singapore

Corresponding Author: Mariastella Colomba
Email address: mariastella.colomba@uniurb.it

The chemical composition of the essential oil of *Monarda didyma* L. aerial parts from Urbino (Central Italy, Marche region) was examined for the first time. In addition to the quali-quantitative analysis, the associated antioxidant and anti-inflammatory activities of the essential oil were also investigated. Different analyses (TLC, GC-FID, GC-MS and ^{1}H-NMR) were performed to identify twenty compounds among which thymol, carvacrol, and p-cymene are the most representative. The antioxidant effect was assessed by DPPH assay. Moreover, the essential oil showed *in vitro* anti-inflammatory effect in LPS-stimulated U937 cells. The decreased expression of pro-inflammatory cytokine IL-6 and the increased expression of miR-146a were found to influence the Toll-like receptor-4 signaling pathway. Our results showed that *M. didyma* essential oil is rich in bioactive compounds that may be considered, after normalization, in herbal medicinal products or food supplements and could be proposed as a natural anti-inflammatory agent in further applications.

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[1] Department of Biomolecular Sciences, University of Urbino Carlo Bo, 61029, Urbino (PU), Italy
[2] Produits Naturels, Analyse et Synthèse, CITCOM-UMR CNRS 8038/Université de Paris, Faculté de Santé, Pharmacie, 75006 Paris, France
[3] U. Burns Center, Dermatology and Emilia Romagna RegionalSkinBank, M. Bufalini Hospital, Viale Ghirotti n.286, Cesena (FC), Italy
[4] Center for Nanofibers and Nanotechnology, National University of Singapore, Singapore 119077

Corresponding Author:
Mariastella Colomba
Department of Biomolecular Sciences, University of Urbino Carlo Bo, 61029, Urbino (PU), Italy
Email address: mariastella.colomba@uniurb.it

Abstract

The chemical composition of the essential oil of *Monarda didyma* L. aerial parts from Urbino (Central Italy, Marche region) was examined for the first time. In addition to the quali-quantitative analysis, the associated antioxidant and anti-inflammatory activities of the essential oil were also investigated. Different analyses (TLC, GC-FID, GC-MS and ^{1}H-NMR) were performed to identify twenty compounds among which thymol, carvacrol, and p-cymene are the most representative. The antioxidant effect was assessed by DPPH assay. Moreover, the essential oil showed *in vitro* anti-inflammatory effect in LPS-stimulated U937 cells. The decreased expression of pro-inflammatory cytokine IL-6 and the increased expression of miR-146a were found to influence the Toll-like receptor-4 signaling pathway. Our results showed that *M. didyma* essential oil is rich in bioactive compounds that may be considered, after normalization, in herbal medicinal products or food supplements and could be proposed as a natural anti-inflammatory agent in further applications.

Introduction

Aromatic plants play a fundamental role in investigations on natural sources of secondary metabolites due to their essential oils (EOs) and their potential use. In the last decades, the

exploration of new green bioactive compounds has attracted the attention of the scientific community aiming at avoiding the harmful effects of chemically synthesized compounds. *Monarda didyma* L. (bergamot or beebalm) (Lamiaceae family) is a perennial herbaceous aromatic plant, with verdant coarse leaves and scarlet-red flowers in terminal tufts ("*Monarda didyma* - Wikipedia"). The plant flowers have been recently included in the group of edible flowers suitable for human consumption (Grzeszczuk et al., 2018; Stefaniak & Grzeszczuk, 2019; Marchioni et al., 2020). Moreover, *M. didyma* leaves and flowers are also used in the preparation of the "Oswego tea" a beverage by the Oswego tribe of American Indians and imported in Great Britain for traditional English tea. The Native Americans didn't use Oswego tea only as a beverage especially for digestive disorders but also for a wide range of medicinal purposes, including treatment of fever, headache and cough, heart ailments, bee stings, skin and mouth infections ("*Monarda didyma* - Wikipedia"; - Nrcs). There are a number of studies dealing with *M. didyma* essential oil extracted from flower or aerial parts (Mattarelli et al., 2017; Wróblewska et al., 2019; Marchioni et al., 2020; Côté et al., 2021) (Fig. 1). First phytochemical studies on *Monarda didyma* reported the presence of flavonoids such as didymin (Brieskorn & Meister, 1965; Scora, 1967) and linarin (Ch & Carron, 2007) from leaves; a bis-malonylated anthocyanin: monardaein ("Structure of monardaein, a bis-malonylated anthocyanin isolated from golden balm, *Monarda didyma*") from flowers; and interesting content in thymoquinone and thymohydroquinone from aerial parts, flowers and leaves (Taborsky et al., 2012). The chemical composition of *M. didyma* essential oil is complex and may vary considerably, depending on several parameters leading to different chemotypes with a specific qualitative and quantitative chemical profiles.

The major compounds representing the chemotype could be thymol (Shanaida et al., 2021), linalool ("Antifungal action of the essential oil of *Monarda didyma*"), carvacrol (Di Vito et al., 2021), geraniol ("*Monarda*: A Source of Geraniol, Linalool, Thymol and Carvacrol-rich Essential Oils") or borneol (Gwinn et al., 2010). The phytochemical studies on each *M. didyma* essential oil were generally completed by *in vitro* or *in vivo* biological and/or physicochemical studies in order to justify a potential application in various fields. In particular, most studies have focused on antifungal/antibacterial activities, especially to target resistant strains (Shanaida et al., 2021) and to search safe alternatives to pure chemical products, such as preservative for food (Wróblewska et al., 2019). Moreover, several authors reported about the usage of *M. didyma* essential oil for human health as topical application (Di Vito et al., 2021). The antioxidant activity of *M. didyma* essential oil was also reported by physicochemical analysis or cell-based assay (Fraternale et al., 2006; Côté et al., 2021). The anti-inflammatory activity of essential oils may be attributed not only to their antioxidant properties but also to their interactions with signaling pathway that involves the expression of pro-inflammatory genes and consequently the cytokines production (Miguel, 2010). Some authors found that essential oils may suppress the protein and mRNA expression of the cytokines in different cells after induction by lipopolysaccharide (LPS), assuming that this inhibitory effect seems to be mediated mainly at a transcriptional level (Yoon et al., 2010; Yadav & Chandra, 2017; Gandhi et al., 2020).

In this study, the chemical composition of *M. didyma* essential oil from the flowering aerial parts obtained from plants collected in Urbino area (Central Italy), was explored by analytical methods to establish for the first time its quali-quantitative chemical profile (i.e., the Urbino chemotype). Additionally, after measuring the antioxidant activity, we aimed to investigate whether *M. didyma* essential oil presented anti-inflammatory effects and whether the effect was mediated through TLR4 signaling pathway.

Materials & Methods

Chemicals and Reagents

Thymol methyl ether, carvacrol methyl ether, (+)-2-carene, 1-octen-3-ol, myrcene, trans-anethole, α-terpineol were purchased from Sigma-Aldrich (St. Louis, MO, USA). Carvacrol, γ-terpinene, 3-carene, p-cymene, camphene, α-terpinene, terpinolene, (+/-)-α-pinene, (-/-)-β-pinene, R-(+)-limonene, S-(-)-limonene, were purchased from Extrasynthese (Genay, France). Thymol, linalool, α-phellandrene were purchased from Merck Millipore (Massachusetts, USA). All reagents and solvents have European Pharmacopoeia quality.

Plant material

The plants were grown in the city of Urbino at 450 m a.s.l. (Marche Region, Central Italy) and identified as *M. didyma* L. by Professor Daniele Fraternale from the Department of Biomolecular Sciences of the University of Urbino Carlo Bo. The plant flowering aerial parts were collected in October 2017. A specimen of the plant has been preserved in the herbarium of the Botanical Garden of University of Urbino Carlo Bo with accession number: Md 19-63.

Essential Oil Isolation

The flowering aerial parts (5.0 Kg fresh weight) of the plants were steam distilled by hydro-distillation method with a yield (v/w) of 2.55 mL/Kg dry weight. The oil was dried and, after filtration, stored at 4 °C until use.

Essential Oil Analyses

Thin Layer Chromatography (TLC)

TLC analyses were performed using the following indications of paragraph 2.2.27 ‘Thin-Layer Chromatography’ of the European Pharmacopoeia 7.0 (“European Pharmacopoeia 7.0 Thin-layer chromatography”), and the guidelines of the Technical Guide for the Elaboration of monographs (“EDQM 7 Edition 2015”). The TLC fingerprint profile was obtained using the following conditions; stationary phase: TLC pre-coated silica gel 60 F254 and HIRSCHMANN® ring caps® 1.2.3.4.5 µL with reproducibility ≤ 0.6% and accuracy ≤ ±0.30% (REF 960 01 05, LOT 861744); essential oil: 0.1 mL/mL in toluene; reference substances: 6-7 mg/mL; mobile phase: toluene/ethyl acetate (93:7 v/v); spotted volume: 3 µL; start position: 15 mm from the plate edge. The visualization of spots on TLC plates was performed under UV light at 254 nm (UV lamp Benda, NU-8 KL, SN: 6001003): compounds containing at least two conjugated double bonds

quench fluorescence and appear as dark zones against the light-green fluorescent background of the TLC plate. We used a vanillin-sulphuric acid solution equal volume of 1% vanillin in anhydrous ethanol w/v and 1% sulphuric acid in anhydrous ethanol v/v as spray reagent and heat for 1 min at 120 °C.

GC-FID analysis

GC-FID analysis was carried out in an Agilent GC-7820A (Agilent Technologies, USA) equipped with a Flame Ionization Detector (FID) and coupled to an electronic integrator. The column used was HP-5 column, ref. 19091J-413, lot n° USA563455H (5% Phenyl Methyl Siloxane, 30 m x 0.32 mm i.d. x 0.25 µm); Temperature limits: from -60 °C to 325 °C. The carrier gas was helium (1 mL/min); the injector and detector temperatures were 250 °C and 270 °C, respectively. The samples for the analysis were prepared by diluting 10 µL of essential oil in 1 mL of heptane.

We used two different methods for GC-FID analysis: Method A, the analysis of the *M. dydyma* essential oil samples and the reference substances was carried out in sequence for a preliminary identification of the peaks of each compound and to observe their possible presence in the sample by comparing their retention time in the spectra. Method B, the essential oil was co-injected with the various reference substances to confirm their presence in the samples by observing an increase in the intensity of the relative peaks.

In the method A the column temperature was programmed from 40 °C to 220°C (from 40 °C to 78 °C at a rate of 4 °C/min; from 78 °C to 106 °C at 2 °C/min; from 106 °C to 220 °C at 26 °C/min; then 220 °C for 5 min). Running Time: 32 min 54 sec, and 5 min (Post Run) at 40 °C.

In the method B the column temperature was programmed from 40 °C to 300 °C (from 40 °C to 78 °C at a rate of 4 °C/min; from 78 °C to 106 °C at 2 °C/min; from 106 °C to 220 °C at 26 °C/min; from 220 °C to 300 °C at 40 °C/min; then 300 °C for 3 min). Running Time: 34 min 54 sec, and 3 min (Post Run) at 40 °C.

Compounds identification was carried out by comparison of calculated retention indices with those reported in the literature (Khan et al., 2018).

The quantitation of the nine main compounds (ca 72%) identified in *M. didyma* essential oil was carried out by external calibration from the areas of the chromatographic peaks obtained by GC-FID analysis (method A). The choice was based on the verified presence of these compounds by GC-MS analysis and on the availability of the standards. A stock solution of α-terpinene, p-cymene, γ-terpinene, linalool, 1-octen-3-ol, thymol methyl ether, carvacrol methyl ether, thymol and carvacrol was serially diluted with the same solvent to prepare calibration curves ranging from 20-170 mg/mL. The R^2 coefficients for the calibration curves were > 0.99. We did not consider some minor compounds. We performed two repetitions for each sample at each concentration level (50, 100 and 150%). The analyses were repeated for 3 days, each time the samples were prepared by the same operator before starting the analysis.

GC-MS analysis

GC-MS analysis was performed using a Shimadzu gas chromatograph, model GC-MS-QP2010SE, equipped with a quadrupole analyzer ionization mode with electronic impact and DB-5 capillary column (30 m x 0.25 mm i.d. x 0.25 µm, ref. 122-5032, lot n° USR146513H; Agilent Technologies, USA). The oven temperature was programmed from 40 °C to 220 °C (from 40 °C to 78 °C at a rate of 4 °C/min; from 78 °C to 106 °C at 2 °C/min; from 106 °C to 220 °C at 10° C/min; then 220 °C for 5 min). Running time: 39 min 54 sec. Helium was used as carrier gas (constant flow rate 36.1 cm/sec). The temperature of ion source and interface were maintained at 220 °C. The injection volume was 1 µL. Prior to injection, the essential oil was diluted (10 µL /1 mL heptane). The acquisition data and instrument control were performed by the GC-MS Solution software. The identity of each compound was assigned by comparison with the mass spectra characteristic features obtained with the NIST library spectral data bank. For semi-quantification purpose the normalized peak area abundances without correction factors were used. Compounds can be identified by a comparison of their retention index, relative to a standard mixture of n-alkanes (Adams, 2009).

Proton Nuclear Magnetic Resonance (^{1}H-NMR)

The ^{1}H-NMR spectra were recorded at 300 MHz for ^{1}H experiments on a Bruker AC 300 MHz spectrometer and 400 MHz for ^{1}H experiments on a Bruker Avance 400 MHz spectrometer. NMR Fourier transform, integration and peak picking were done with Bruker Top Spin software. Chemical shifts (δ) are reported in ppm. The one-dimensional spectra were performed in CDCl3. The residual solvent was used as a reference (δ = 7.26 ppm).

Determination of anti-oxidant activity: DPPH radical assay

The anti-oxidant activity was measured by DPPH (diphenyl picrylhydrazyl) radical-scavenging method described by (Cuendet et al., 1997) with slight modification. For the DPPH assay, 80 µL of 0.5 mM DPPH ethanol solution (Sigma-Aldrich, Missouri, US) and 40 µL of *M. didyma* essential oil were dissolved in ethanol at final concentrations ranging from 0.10 to 0.50 µL/mL (final volume 400 µL). Mixtures were vigorously shaken and left for 1 hour in the dark. Absorbance was measured at 517 nm using UV/Vis spectrophotometer. Eighty microliters of 0.5 mM DPPH diluted in 400 µL of ethanol was used as control, ethanol without DPPH was used as a blank. Lower absorbance of the reaction mixture indicated higher free radical scavenging activity. The following equation was used to calculate the Inhibitory activity (I) of DPPH radical:

$I\ (\%) = 100 \times (A0-As)/A0$

Where A0 is the absorbance of the control and As is the absorbance of the tested sample.

To assess the antioxidant effect, different amounts of *M. didyma* essential oil ethanol solution (0.5 µL/mL) ranging from 20 to 320 µL were employed. All tests and analyses were run in triplicate. EC50 L-ascorbic acid (Sigma-Aldrich, Missouri, US) served as positive control (40 µg/mL final concentration). *M. didyma* essential oil EC50 (50% DPPH scavenging activity) was calculated by regression analysis.

Biological Assays

Cell culture and treatments

U937 cells (purchased at Euroclone, Milan, Italy) were cultured in RPMI 1640 medium (Sigma Aldrich, Missouri, US) supplemented with 10% fetal bovine serum (FBS), penicillin (50 U/mL) and streptomycin (50 µg/mL) (Euroclone, Milan, Italy). Cells were maintained at 37 °C in T-75 tissue culture flasks (Corning, NY) gassed with an atmosphere of 95% air to 5% CO_2. To evaluate the anti-inflammatory properties of the essential oil, U937 cells were differentiated into macrophages with 100 nM Phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich, Missouri, US) treatment for 48 h. The cells were incubated for 24 hours without PMA. To stimulate U937 cells, 1 µg/mL lipopolysaccharide (LPS; Sigma-Aldrich, Missouri, US) final concentration was added to the medium for 6 hours. The essential oil (0.5 µL/mL final concentration) was added 1 hour before LPS stimulation. Three replicates were performed.

Quantitative real time PCR (RT-qPCR) of mature microRNAs

The total RNA purification kit (Norgen Biotek Corp., Ontario, Canada) was used to isolate total RNA (including both microRNA and larger RNA species) from 1 x 10^6 U937 cells, following the manufacturer's recommended protocol; samples were evaluated for nucleic acid quality and quantity using the Nano-Drop ND-1000 spectrophotometer (Nano-Drop Technologies, Wilmington, DE, USA). RNA was stored at -80 °C until use. Human miR-146a and human RNU44 (reference miRNA) expressions were quantified using the TaqMan MicroRNA assay (Applied Biosystems), as previously described (Olivieri et al., 2013). Briefly, the TaqMan MicroRNA reverse transcription kit was used to reverse transcribe the total RNA; 5 µL of RT mix contained 1 µL of each miR-specific has-miR-146a stem-loop primers, 1.67 mL of input RNA, 0.4 µL of 10 x buffer, 0.6 µL of RNAse inhibitor diluted 1:10 and 0.55 µL of H_2O. The mixture was incubated at 16 °C for 30 min, at 42 °C for 30 min, and at 85 °C for 5 min. Subsequently RT-qPCR was performed in 20 µL of PCR mix containing 1 µL of 20 x TaqMan MicroRNA assay – which in turn contained PCR primers and probes (5'-FAM) – 10 µL of 2 x TaqMan Universal PCR Master Mix No Amp Erase UNG (Applied Biosystems), and 5 µL of reverse-transcribed product. The reaction was first incubated at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and at 60 °C for 1 min. The qRT-PCR was performed on an ABIPRISM 7500 Real Time PCR System (Applied Biosystems). Data were analyzed by a 7500-system software (1 1.4.0) with the automatic comparative threshold (Ct) setting for adapting baseline. The relative amount of miR-146a was calculated using the Ct method: ΔCt = Ct (miR146a) – Ct (RNU44); $2^{-\Delta Ct}$

Results are expressed as fold change related to control (CTRL).

Quantitative real time PCR (RT-qPCR) of mature mRNAs

Total RNA was extracted as previously using the total RNA purification kit (Norgen Biotek Corp., Ontario, Canada), and samples were evaluated for nucleid acid quality and quantity using the Nano-Drop ND-1000 spectrophotometer (Nano-Drop Technologies, Wilmington, DE, USA).

Isolated RNA was used to synthesize cDNA using a reverse transcription kit (Applied Biosystems) according to the manufacturer's protocol. Quantitative polymerase chain reaction real time (RT-qPCR) was performed with the SYBR Green PCR master mix (Applied Biosystems, Foster City, CA, USA) on an ABI Prism 7500 Real Time PCR System (Applied Biosystems). The primers we used were the ones designed by (Angel-Morales, Noratto & Mertens-Talcott, 2012). TATA binding protein (TBP) has been used as an endogenous reference to determine relative mRNA expression. The pairs of forward and reverse primers were purchased from Sigma-Aldrich. Product specificity was examined by dissociation curve analysis. The following are the sequences of the primers used: TBP, forward primer: TGCACAGGAGCCAAGAGTGAA, reverse primer: CACATCACAGCTCCCCACCA; and IL-6, forward primer: AGGGCTCTTCGGCAAATGTA, reverse primer: GAAGGAATGCCCATTAACAACAA.

The relative amount of IL-6 was calculated using the Ct method:

$\Delta Ct = Ct\ (IL\text{-}6) - Ct\ (TBP)$; $2^{-\Delta Ct}$

Results are expressed as fold change related to control (CTRL).

Statistical analysis

All measurements were expressed as mean values ± standard deviation (SD) from the mean of at least three independent experiments. The two-tailed paired Student's t-test was used for the analyses. The results were considered significant at the level of $p < 0.05$.

Results

TLC analysis

In line with available data, the TLC analysis showed the presence of thymol, carvacrol, thymol methyl ether, carvacrol methyl ether, 1-octen-3-ol, and linalool. On the contrary, the interpretation of the results obtained for p-cymene, α-terpinene, γ-terpinene, α-terpineol and eucalyptol was uncertain.

Qualitative GC-FID analysis

Two different methods (Method A and Method B) were successfully used. GC-FID by method A (analysis, in sequence, of the essential oil samples and the reference substances) resulted in fifteen compounds identified, among which nine were selected as the major ones, whereas the presence of β-pinene, δ-2-carene, δ-3-carene, β-myrcene and α-phellandrene was doubtful (Table 1, see also Fig. 1S).

To have further confirmation of the identified compounds, the essential oil was co-injected with the standards (Method B). Compounds identified are listed in Table 2 (see also Fig. 2S). Using Method B conditions, the absence of β-pinene and δ-2-carene was confirmed; whereas camphene, δ-3-carene and α-terpinolene showed too small peaks to be identified.

GC-MS analysis

GC-MS analysis pointed to the presence of different classes of phytochemicals. The identification of each compound was possible thanks to the comparison of mass spectra from the NIST library spectral data bank. We found different compounds with significant values of probability of identification (> 95%): α-thujene, α-pinene, camphene, sabinene, β-myrcene, α-phellandrene, 3-carene, α-terpinene, p-cymene, γ-terpinene, linalool, α-terpineol, thymol methyl ether, carvacrol methyl ether, thymol and carvacrol; and four with lower percentage: limonene (94%), eucalyptol (94%), β-pinene (91%), α-terpinolene (91%) (Fig. 3S).

^{1}H-NMR analysis

According to ^{1}H-NMR analysis of the *M. didyma* essential oil at both 300 and 400 MHz, the main compounds identified were as follows: p-cymene, carvacrol, thymol, carvacrol methyl ether, 1-octen-3-ol, thymol methyl ether, linalool, γ-terpinene and α-terpinene (Fig. 2); α-pinene, α-terpineol, limonene, myrcene, and camphene were not detectable. ^{1}H-NMR spectra at 300 MHz in $CDCl_3$ of standard compounds were used for comparison.

Quantitative GC-FID analyses

The concentrations (Z) of the main compounds in M. didyma essential oil (mg/mL EO and %) are indicated in Table 3.

Essential oil antioxidant and anti-inflammatory activity

The essential oil showed a 50% DPPH scavenging activity with 263.4 µL of *M. didyma* essential oil ethanol solution (0.5 µL/mL), corresponding to the effect exerted by 0.4 µL of Ascorbic Acid ethanol solution (40 mg/mL) in a final volume of 400 µL. The analysis demonstrated a dose-dependent effect ($R^2 = 0.991$).

The anti-inflammatory effect of *M. didyma* essential oil was investigated treating U937 cells with an essential oil ethanol solution (0.5 µL/mL final concentration) during a pro-inflammatory stimulus (LPS, 1 µg/mL final concentration). We used this dose of essential oil since we found that it had a good antioxidant activity without creating any cellular toxicity (data not shown). LPS treated U937 cells showed the typical inflammatory condition: down regulation of miR-146a expression level and high amounts of the pro-inflammatory markers IL-6 and IRAK-1. As shown in figure 2, when treating the cells with the essential oil (MdEO), we observed that the anti-inflammatory effect was particularly evident, resulting in an overexpression of miR-146a and the consequent down regulation of IRAK-1 and IL-6. When considering U937 cells, pre-treated with *M. didyma* essential oil, under a pro-inflammatory stimulation (LPS + MdEO), the up-regulation of miR-146a was evident as well but, as expected, a little lower than that observed in MdEO experimental group, thus confirming that the essential oil phytochemical compounds can efficiently cope with the inflammatory cascade triggered by the LPS insult (Fig. 3). The ability of *M. didyma* essential oil to modulate the inflammatory response, through the inhibition of the TLR-4 signaling pathway and the reduced expression of IL-6, demonstrates that this is a good anti-inflammatory agent.

Discussion

The current study investigated the chemical composition of *M. didyma* essential oil from central Italy (Urbino, Marche region). The study of essential oils is extremely complex because of the great variability in their composition depending on various parameters such as the geographical origin of the plants, the cultivar/hybrid type, the plant growth conditions (soil, fertility, humidity, sunshine, length of the day), the state of development of the plant, and the plant material state (fresh, dry, whole, broken). Given the composition discrepancies reported in available literature about different *M. didyma* essential oils, much of our research was aimed at studying the chemical composition of the essential oil examined in this paper (i.e., the Urbino chemotype). Several compounds that we identified correspond to those found by (Fraternale et al., 2006), (Carnat, Lamaison & Remery, 1991) and (Ricci, Epifano & Fraternale, 2017): α-pinene, camphene, β-myrcene, p-cymene, limonene, linalool and α-terpineol, even if their concentrations differ according to each published result or from study to study. On the other hand, we report also carvacrol and carvacrol methyl ether which were not previously listed by these authors. Considering only the composition of essential oils extracted from plants cultivated in Italy (Fraternale et al., 2006; Ricci, Epifano & Fraternale, 2017), we found many differences despite the similar origin of the plants. In the essential oil investigated in the present study, carvacrol, p-cymene and thymol resulted to be the major compounds.

The observed antioxidant property of *M. didyma* essential oil is probably due to different compounds, which may act by scavenging free radicals and prevent lipid peroxidation. Recent studies have demonstrated that flavonoids (Maleki, Crespo & Cabanillas, 2019) and phenolic compounds (Yahfoufi et al., 2018) can inhibit regulatory enzymes or transcription factors important for controlling mediators involved in inflammation. Since we found a significant antioxidant activity of *M. didym*a essential oil, we investigated whether it could influence the TLR4 signaling pathway, which regulates NF-kB and inflammatory genes activation, in pro-inflammatory stimulated human tumor monocyte U937 cells. TLR4 is a member of the pattern-recognition receptors family, which can be activated by LPS. Following the TLR4 stimulation, NF-kB is activated by IRAK-1 and TRAF-6 (TNF receptor-associated factor 6), thus allowing the expression of pro-inflammatory cytokines such as IL-6. In the same time NF-kB up-regulates miR-146a, which in turn inhibits IRAK-1 and TRAF-6 expression by a negative feedback loop (Olivieri et al., 2013). Our findings indicate that during an acute LPS stimulation in *M. didyma* essential oil pre-treated U937 cells, miR-146a increases and targets IRAK-1, decreasing its expression, with a consequent reduction of IL-6 amounts. Such a result strongly supports the hypothesis that the mechanism of action by which *M. didyma* essential oil exerts its anti-inflammatory activity involves the TLR4 signaling pathway.

Conclusions

Aiming at providing our contribute in investigating plant-derived phytochemical compounds as prototypes in the attempt to develop more effective and less toxic substances, the essential oil of *M. didyma* aerial parts from Urbino (Central Italy, Marche region) was examined for the first

time. According to the quali-quantitative analysis, twenty compounds were identified among which nine (carvacrol, p-cymene, thymol, γ-terpinene, carvacrol methyl ether, 1-octen-3-ol, thymol methyl ether, linalool and α-terpinene) reached 72% of the essential oil composition. Although further studies are needed for a better elucidation of the molecular mechanisms underlying the observed associated antioxidant and anti-inflammatory activities, they are most likely due to the bio-functional properties of the monoterpenes (mainly aromatic and phenolic monoterpenes) present in the essential oil. Noteworthy, such bioactive compounds may be considered, after normalization, in herbal medicinal products or food supplements and could be proposed as a natural anti-inflammatory agent in further applications.

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Additional information and declarations

Competing interests

Maria Cristina Albertini is an Academic Editor for PeerJ. The authors have no additional conflicts of interest to declare.

Author contribution

MCA, HD and MC realized the conceptualization of the experimental project. DF prepared and identified the plant. DF, RDA, CB and SC performed experiments. RDA and SC performed statistical analysis. MC and BDG performed data analysis; HD, MCA, and MC prepared the original draft; MCA and MC wrote and reviewed the final version. SC, DM and SR carried out graphical editing and figures preparation. All authors have read and agreed to the final version of the manuscript.

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Legends

Figure 1 Graphical representation of major biological properties of *Monarda didyma* essential oil. Many biological properties of *M. didyma* essential oil have been documented in available literature, the most relevant of which (antifungal/antibacterial, antioxidant and anti-inflammatory activities) are briefly reported.

Figure 2 ^{1}H-NMR spectra of *Monarda didyma* essential oil at 400 MHz in CDCl3. The main constituents detectable in the essential oil were the following: p-cymene, carvacrol, thymol, carvacrol methyl ether, 1-octen-3-ol, thymol methyl ether, linalool, γ-terpinene and α-terpinene.

Figure 3 Anti-inflammatory effect of *Monarda didyma* essential oil on miR-146a, IRAK-1 and IL-6 expression levels in U937 cells. Both experimental groups treated with the essential oil (LPS + MdEO and MdEO,) showed miR-146a up-regulation with a decreased expression of IRAK-1 and IL-6. Results are reported as fold change related to CTRL.
CTRL: controls; LPS: LPS-stimulated cells (1 μg/mL final concentration); LPS + MdEO: cells treated with MdEO (0.5 μL/mL final concentration) 1 h before LPS stimulation (1 μg/mL final concentration); MdEO: cells treated with the essential oil (0.5 μL/mL final concentration). Two-tailed paired Student's t-test: $*p < 0.05$ vs. CTRL; $°p < 0.05$ vs. LPS.

Figure 1

Graphical representation of major biological properties of *Monarda didyma* essential oil.

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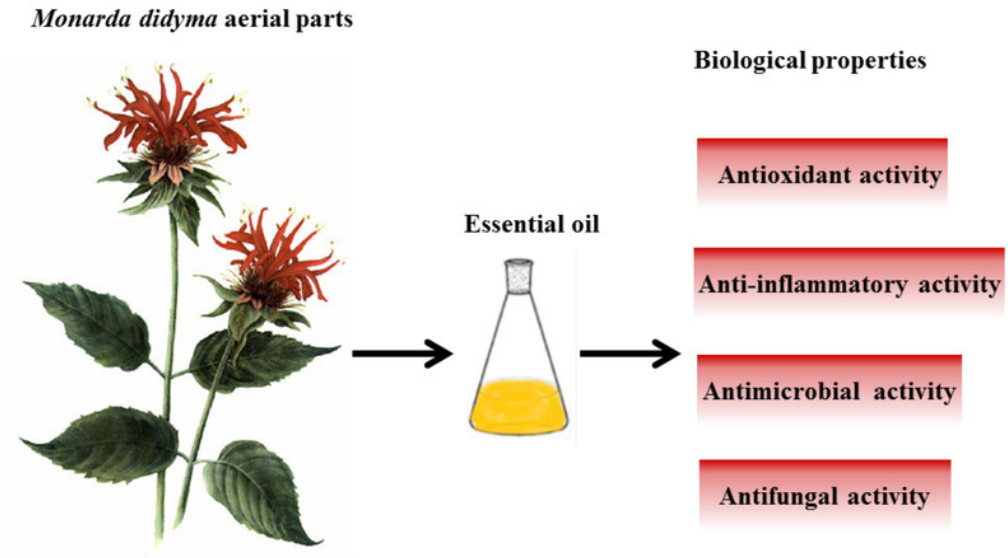

Figure 2

^{1}H-NMR spectra of *Monarda didyma* essential oil at 400 MHz in CDCl3.

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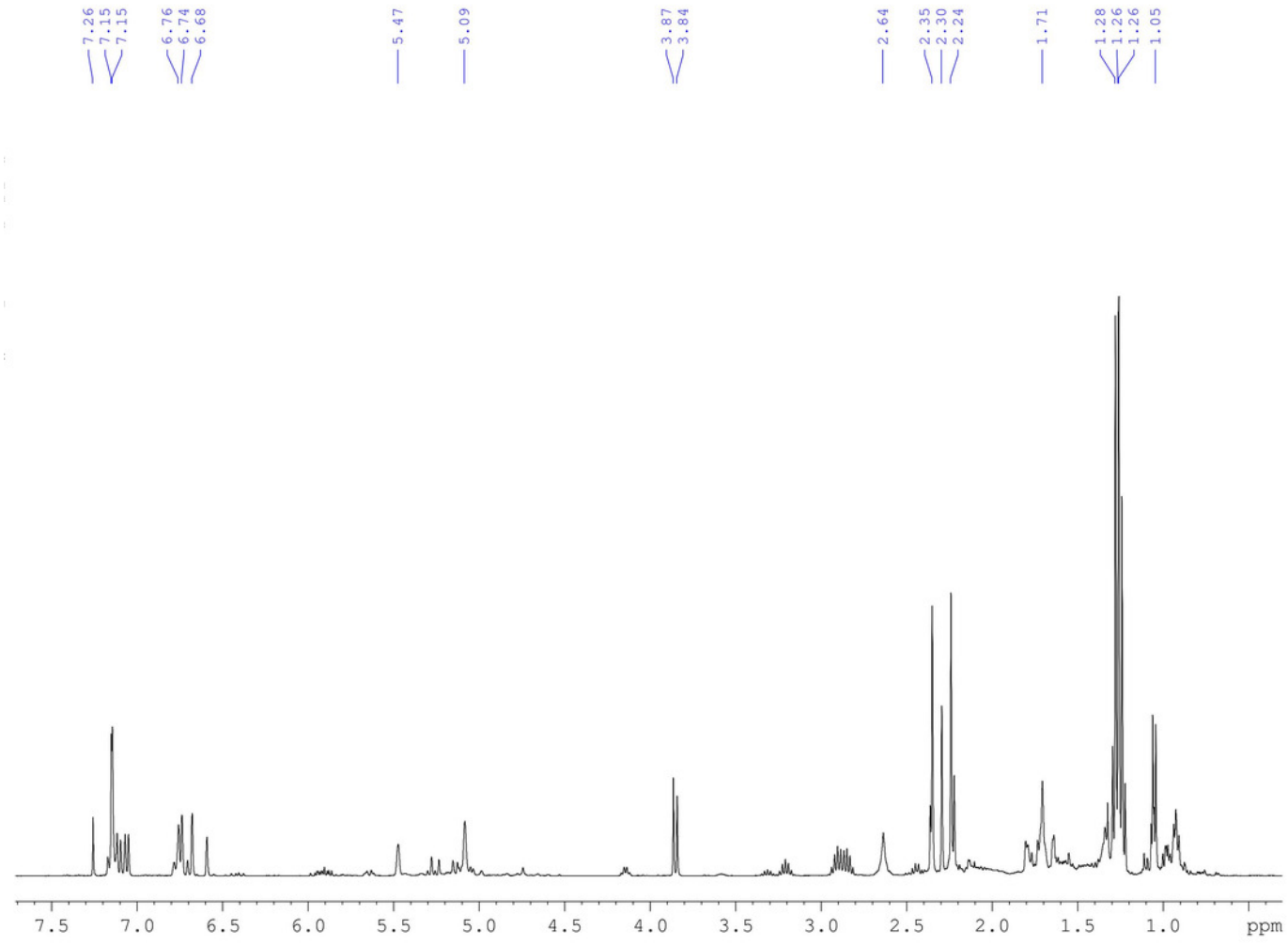

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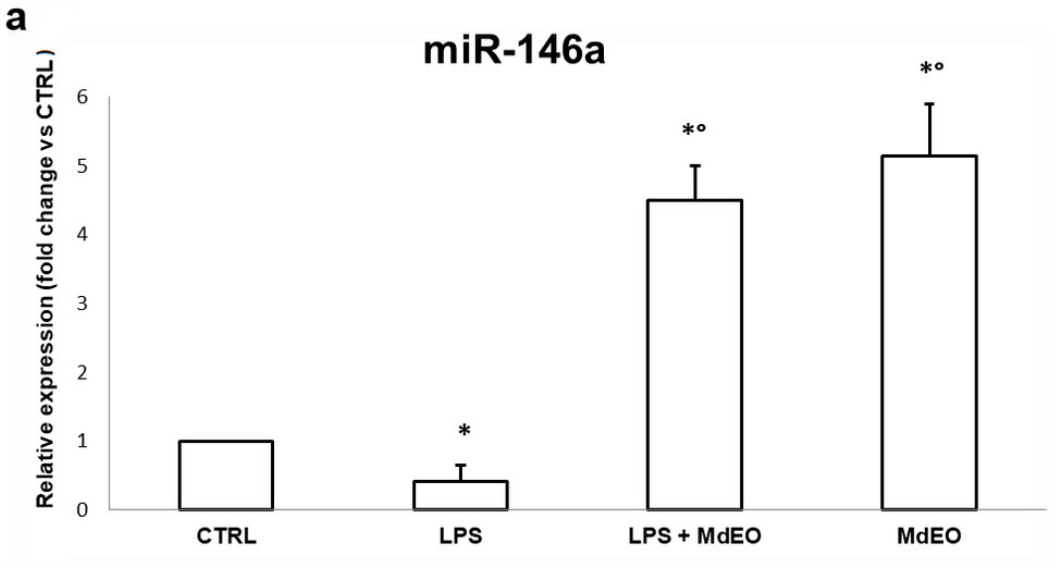
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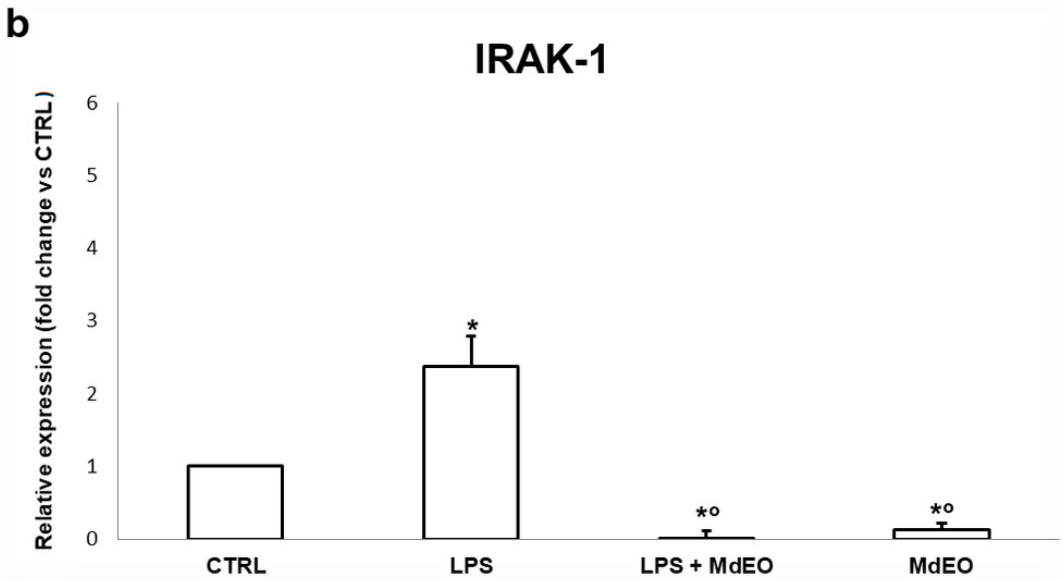
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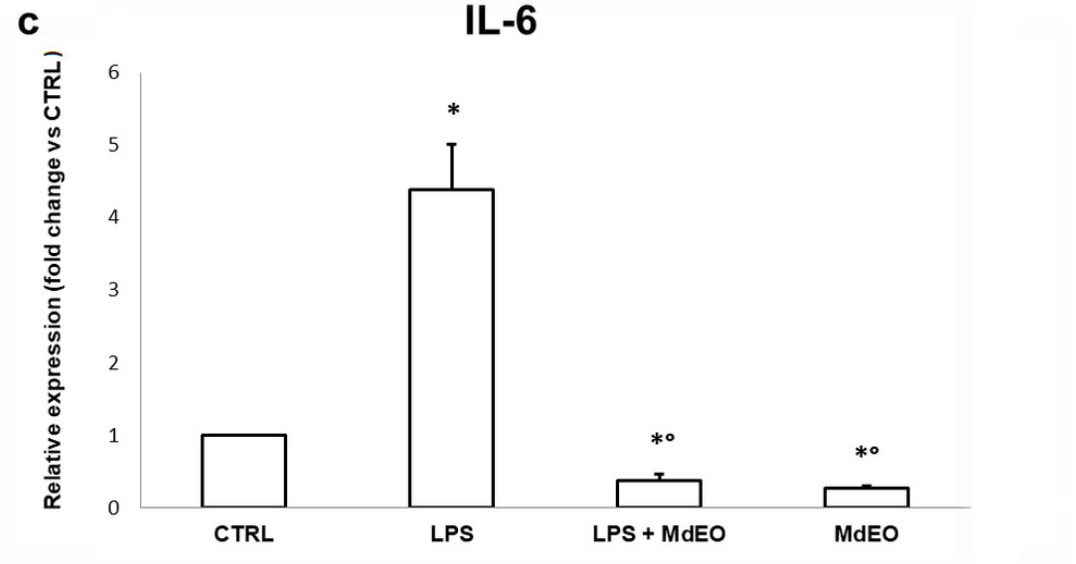
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Table 1(on next page)

Chemical composition of *Monarda didyma* essential oil by GC-FID (Method A).

The Retention Time of the peaks of the different standards analysed (Ret. Time Standard) and those of the corresponding peaks present in the essential oil spectrum (Ret. Time EO) are indicated. The relative areas (%) of the GC-FID chromatogram peaks are also shown. In bold major components and their relative areas.

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Compounds	Area %	Ret. Time EO [min]	Ret. Time Standard [min]
α-Pinene	0.7	10.097	10.143
Camphene	0.3	10.607	10.621
β-Pinene	---	---	11.684
1-Octen-3-ol	**5.5**	11.690	11.810
β-Myrcene	2.1	12.132	12.169
δ-2-Carene	---	---	12.573
α-Phellandrene	0.3	12.673	12.739
δ-3-Carene	0.1	12.916	---
α-Terpinene	**2.7**	13.177	13.281
p-Cymene	**19.3**	13.521	13.616
D-Limonene	1.5	13.689	13.766
Eucalyptol	0.8	13.830	13.809

γ-Terpinene	**8.8**	15.026	15.071
α-Terpinolene	0.3	16.413	16.528
Linalool	**2.6**	16.976	17.141
α-Terpineol	0.5	21.773	22.006
Thymol Methyl Ether	**5.2**	24.092	24.108
Carvacrol Methyl Ether	**6.4**	24.411	24.436
Thymol	**12.3**	25.576	25.633
Carvacrol	**19.5**	25.753	25.754

Table 2(on next page)

Chemical composition of *Monarda didyma* essential oil by GC-FID (Method B).

Compounds identified by GC-FID in the essential oil co-injected with the standards. The Retention Time of the peaks of the essential oil (Ret. Time EO) and that of the essential oil co-injected with the standards (Ret. Time Standard co-injected with the EO) are indicated.

Table 2 Chemical composition of *Monarda didyma* essential oil by GC-FID (Method B). Compounds identified by GC-FID in the essential oil co-injected with the standards. The Retention Time of the peaks of the essential oil (Ret. Time EO) and that of the essential oil co-injected with the standards (Ret. Time Standard co-injected with the EO) are indicated.

Compounds	**Ret. Time EO** [min]	**Ret. Time Standard co-injected with the EO** [min]
α-Pinene	10.142	10.131
Camphene	---	10.640
β-Pinene	---	11.658
1-Octen-3-ol	11.746	11.723
β-Myrcene	12.024	12.169
δ-2-Carene	---	12.577
δ-3-Carene	---	12.954
α-Terpinene	13.230	13.211
p-Cymene	13.571	13.563
D-Limonene	13.743	13.739
γ-Terpinene	15.083	15.066
α-Terpinolene	---	16.447
Linalool	17.048	17.037
Thymol Methyl Ether	24.140	24.141
Carvacrol Methyl Ether	24.453	24.446
Thymol	25.604	25.595
Carvacrol	25.777	25.791

Table 3(on next page)

Quantitative GC-FID analysis.

Content (m/v and %) of the main compounds (ca 72%) found in *Monarda didyma* essential oil.

Table 3 Quantitative GC-FID analysis. Content (m/v and %) of the main compounds (ca 72%) found in *Monarda didyma* essential oil.

Compounds	mg/mL EO	%
Carvacrol	167.44	16.74
p-Cymene	160.00	16.00
Thymol	105.13	10.51
γ-Terpinene	78.10	7.81
Carvacrol methyl ether	56.73	5.67
1-Octen-3-ol	53.53	5.35
Thymol methyl ether	46.27	4.63
Linalool	25.21	2.52
α-Terpinene	24.34	2.43