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

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Abstract

In the present study, *Monarda didyma* L. essential oil (isolated from the flowering aerial parts of the plant) was examined to characterize its chemotype and to evaluate, in addition to the quali-quantitative chemical analysis, the associated antioxidant and anti-inflammatory activities. The plants were grown in central Italy, Urbino (PU), Marche region. Different analyses (TLC, GC-FID, GC-MS and ^{1}H-NMR) allowed to identify twenty compounds among which carvacrol, p-cymene and thymol were the most abundant. On this basis, the chemotype examined in the present study was indicated as *Monarda didyma* ct. carvacrol. The antioxidant effect was assessed by DPPH assay. Moreover, this chemotype was investigated for the anti-inflammatory effect in an *in vitro* setting (i.e., LPS-stimulated U937 cells). The decreased expression of pro-inflammatory cytokine IL-6 and the increased expression of miR-146a are suggestive of the involvement of the Toll-like receptor-4 signaling pathway. Although further studies are needed to better investigate the action mechanism/s underlying the results observed in the experimental setting, our findings show that *M. didyma* essential oil is rich in bioactive compounds (mainly aromatic monoterpenes and phenolic monoterpenes) which are most likely responsible for its beneficial effect.

Introduction

40
41 The exploration of bioactive compounds from natural sources represents an important method for
42 the discovery of new potential therapeutic agents, alternative to chemically synthesized
43 compounds, which often require more complicated and wasteful manufacturing processes.
44 Aromatic plants play a fundamental role in this kind of investigations especially due to their
45 essential oils (EOs), simple to extract and rich in secondary metabolites, with some recognized
46 pharmacological properties. *Monarda didyma* L. (bergamot or beebalm) (Lamiaceae family) is a
47 perennial herbaceous aromatic plant native to North America, with verdant coarse leaves and
48 scarlet-red flowers in terminal tufts (Carnat, Lamaison & Rémery, 1991). Recently, *M. didyma*
49 flowers have been included in the group of edible flowers suitable for human consumption
50 (Grzeszczuk et al., 2018; Stefaniak & Grzeszczuk, 2019; Marchioni et al., 2020). Moreover, *M.*
51 *didyma* leaves and flowers are also used in the preparation of the "Oswego tea" a beverage by
52 the Oswego tribe of American Indians and imported in Great Britain for traditional English tea.
53 Native Americans didn't use Oswego tea only as a beverage (especially for digestive disorders)
54 but also for a wide range of medicinal purposes including treatment of fever, headache and
55 cough, heart ailments, bee stings, skin and mouth **(**Fraternale et al., 2006).
56 Thanks to its phenols content, this plant has been used for its medicinal properties: diuretic,
57 antipyretic, sudorific, carminative and antiseptic (Mattarelli et al., 2017). *Monarda didyma* is
58 also characterized by the presence of different bioactive compounds including carvacrol and
59 thymol that possess beneficial health properties (Nagoor Meeran et al., 2017; Mahmoodi et al.,
60 2019; Javed et al., 2021).
61 Thymol and carvacrol have an elevated antioxidant activity for the presence of phenolic
62 hydroxyls groups (Beena Kumar & Ravat 2013), which suggests that the presence of a group
63 donor of electrons in the function hydroxylates is crucial to obtain an effective antioxidant
64 activity.
65 The chemical composition of aromatic plants is complex and consists of two fractions: non-
66 volatile and volatile. The last one is composed of secondary metabolites which constitute the
67 essential oil (EO).
68 There are a number of studies dealing with isolation and characterization of *M. didyma* essential
69 oil from flowers or aerial parts (Mattarelli et al., 2017; Wróblewska et al., 2019; Marchioni et al.,
70 2020; Côté et al., 2021) (Fig. 1). First phytochemical studies reported the presence of flavonoids
71 such as didymin (Brieskorn & Meister, 1965; Scora, 1967) and linarin (Ch & Carron, 2007) from
72 leaves; a bis-malonylated anthocyanin: monardaein (Saito & Harborne, 1992) from flowers; and
73 interesting content in thymoquinone and thymohydroquinone from aerial parts, flowers and
74 leaves (Taborsky et al., 2012).
75 The chemical composition of an essential oil (also known as chemotype) may vary considerably
76 for the same species, as the biosynthesis of secondary metabolites is strongly affected by
77 environmental factors, depending either on the geographical origin of the plants or growth
78 conditions (soil, fertility, humidity, sunshine, length of the day) and state of development, which
79 leads to different chemotypes with a specific qualitative and quantitative chemical profiles.

80   Although the species is the same, many differences have been described in chemical composition
81   of *M. didyma* EOs (see for example, Carnat, Lamaison & Rémery, 1991; Fraternale et al., 2006;
82   Ricci, Epifano & Fraternale, 2017). The major compounds representing the chemotype could be
83   thymol (Shanaida et al., 2021), linalool (Carnat, Lamaison & Rémery, 1991, carvacrol (Di Vito
84   et al., 2021), geraniol (Mazza, Kiehn & Marshall, 1993 or borneol (Gwinn et al., 2010).
85   The phytochemical analyses on each *M. didyma* essential oil have been associated to *in vitro* or
86   *in vivo* biological and/or physical-chemical studies in order to evaluate a potential application in
87   various fields. In particular, most studies have focused on antifungal/antibacterial activities,
88   especially to target resistant strains (Shanaida et al., 2021) and to search safe alternatives to pure
89   chemical products, such as preservative for food (Wróblewska et al., 2019). Moreover, several
90   authors reported about the usage of *M. didyma* essential oil for human health as topical
91   application (Di Vito et al., 2021). The antioxidant, antibacterial and anti-inflammatory activities
92   of the essential oil were also reported by physical-chemical analysis or cell-based assay
93   (Fraternale et al., 2006; Côté et al., 2021).
94   Among the explored biological properties of *M. didyma* EO, the anti-inflammatory effect
95   deserves additional investigation. The anti-inflammatory activity of essential oils may be
96   attributed not only to their antioxidant properties but also to their interactions with signaling
97   pathway that involves the expression of pro-inflammatory genes and consequently the cytokine
98   production (Miguel, 2010). Some authors found that the essential oils studied suppressed the
99   protein and mRNA expression of the cytokines in different lipopolysaccharide (LPS) stimulated
100   cells, assuming that this inhibitory effect seems to be mediated mainly at a transcriptional level
101   (Gandhi et al., 2020; Yadav & Chandra, 2017; Yoon et al., 2010). The stimulation with LPS
102   modulating the Toll-like-receptor-4 (TLR-4) cell signaling pathway can activate inflammatory
103   responses (Kuzmich et al., 2017). There are two adapter molecules in this signaling cascade: IL-
104   1 receptor-associated kinase 1 (IRAK-1) and TNF receptor-associated factor 6 (TRAF6); these
105   molecules provoke the activation of NF-kB (Nuclear Factor-kB), which in turn leads to the
106   production of pro-inflammatory cytokine (IL-6) and miR-146a. Cytokine expression is
107   modulated by TLR-4 through a negative feedback regulation loop involving the down-regulation
108   of IRAK-1 protein level targeted by miR-146a (Olivieri et al., 2013). On these bases, as a
109   continuation of a previous study (Fraternale et al., 2006), we decided to investigate *M. didyma*
110   essential oil from plants collected in Urbino area (Central Italy, Marche Region) to establish, in
111   addition to its quali-quantitative chemical profile, the antioxidant activity and to assess the anti-
112   inflammatory effect in an *in vitro* setting. The experimental setting was carried out in order to
113   investigate whether *M. didyma* essential oil anti-inflammatory effect was mediated through TLR-
114   4 signaling pathway and miR-146a negative feedback loop using lipopolysaccharide-stimulated
115   monocyte human tumor cells (U937 cells).
116

117   **Materials & Methods**
118   **Chemicals and Reagents**

119 Thymol methyl ether, carvacrol methyl ether, (+)-2-carene, 1-octen-3-ol, myrcene, trans-
120 anethole, α-terpineol were purchased from Sigma-Aldrich (St. Louis, MO, USA). Carvacrol, γ-
121 terpinene, 3-carene, p-cymene, camphene, α-terpinene, terpinolene, (+/-)-α-pinene, (-/-)-β-
122 pinene, R-(+)-limonene, S-(-)-limonene, were purchased from Extrasynthese (Genay, France).
123 Thymol, linalool, α-phellandrene were purchased from Merck Millipore (Massachusetts, USA).
124 All reagents and solvents have European Pharmacopoeia quality.
125

126 **Plant material**
127 The plants were grown in the city of Urbino at 450 m a.s.l. (Marche Region, Central Italy, GPS
128 coordinates: 43° 42' 50.3" N, 12° 36' 40.7" E) and identified, by morphological analysis, as
129 *Monarda didyma* L. by Professor Daniele Fraternale from the Department of Biomolecular
130 Sciences (DISB) of the University of Urbino Carlo Bo. The plants were harvested until the
131 beginning of October 2017. The flowering period (September 2017) showed an average
132 maximum temperature of 20.4 °C and an average minimum temperature of 12.6 °C. Precipitation
133 was 162.2 mm and average relative humidity was 74%. These data are available at the
134 "Osservatorio Meteorologico Alessandro Serpieri – Università degli Studi di Urbino Carlo Bo"
135 (https://ossmeteo.uniurb.it/). A specimen of the plant has been preserved in the herbarium of the
136 Botanical Garden of University of Urbino Carlo Bo with accession number: Md 19-63.
137

138 **Essential Oil Isolation**
139 The flowering aerial parts (5.0 Kg fresh weight) of the plants were steam distilled by hydro-
140 distillation method with a yield (v/w) of 2.55 mL/Kg dry weight. The oil was dried and, after
141 filtration, stored at 4 °C until use.
142

143 **Essential Oil Analyses**
144 **Thin Layer Chromatography (TLC)**
145 TLC analyses were performed using the following indications of paragraph 2.2.27 'Thin-Layer
146 Chromatography' of the European Pharmacopoeia 7.0 ("European Pharmacopoeia 7.0 Thin-layer
147 chromatography"), and the guidelines of the Technical Guide for the Elaboration of monographs
148 ("EDQM 7 Edition 2015"). The TLC fingerprint profile was obtained using the following
149 conditions; stationary phase: TLC pre-coated silica gel 60 F254 and HIRSCHMANN® ring
150 caps® 1.2.3.4.5 μL with reproducibility ≤ 0.6% and accuracy ≤ ±0.30% (REF 960 01 05, LOT
151 861744); essential oil: 0.1 mL/mL in toluene; reference substances: 6-7 mg/mL; mobile phase:
152 toluene/ethyl acetate (93:7 v/v); spotted volume: 3 μL; start position: 15 mm from the plate edge.
153 The visualization of spots on TLC plates was performed under UV light at 254 nm (UV lamp
154 Benda, NU-8 KL, SN: 6001003): compounds containing at least two conjugated double bonds
155 quench fluorescence and appear as dark zones against the light-green fluorescent background of
156 the TLC plate. We used a vanillin-sulphuric acid solution equal volume of 1% vanillin in
157 anhydrous ethanol w/v and 1% sulphuric acid in anhydrous ethanol v/v as spray reagent and heat
158 for 1 min at 120 °C.

159

160 **GC-FID analysis**

161 GC-FID analysis was carried out in an Agilent GC-7820A (Agilent Technologies, USA)

162 equipped with a Flame Ionization Detector (FID) and coupled to an electronic integrator. The

163 column used was HP-5 column, ref. 19091J-413, lot n° USA563455H (5% Phenyl Methyl

164 Siloxane, 30 m x 0.32 mm i.d. x 0.25 µm); Temperature limits: from -60 °C to 325 °C. The

165 carrier gas was helium (1 mL/min); the injector and detector temperatures were 250 °C and 270

166 °C, respectively. The samples for the analysis were prepared by diluting 10 µL of essential oil in

167 1 mL of heptane.

168 We used two different methods for GC-FID analysis: Method A, the analysis of the *M. didyma*

169 essential oil samples and the reference substances was carried out in sequence for a preliminary

170 identification of the peaks of each compound and to observe their possible presence in the

171 sample by comparing their retention time in the spectra. Method B, the essential oil was co-

172 injected with the various reference substances to confirm their presence in the samples by

173 observing an increase in the intensity of the relative peaks.

174 In the method A the column temperature was programmed from 40 °C to 220°C (from 40 °C to

175 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

176 °C/min; then 220 °C for 5 min). Running Time: 32 min 54 sec, and 5 min (Post Run) at 40 °C.

177 In the method B the column temperature was programmed from 40 °C to 300 °C (from 40 °C to

178 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

179 °C/min; from 220 °C to 300 °C at 40 °C/min; then 300 °C for 3 min). Running Time: 34 min 54

180 sec, and 3 min (Post Run) at 40 °C. Compound identification was carried out by comparison of

181 calculated retention indices with those reported in the literature (Khan et al., 2018). Two

182 repetitions of essential oil samples at three concentration levels (50, 100 and 150%) were

183 analyzed on three consecutive days.

184 The quantification of the most abundant compounds was carried out by external calibration from

185 the areas of the chromatographic peaks obtained by GC-FID analysis method A, considering

186 their relative FID areas up to 90% of the total FID area. The choice was based on the verified

187 presence of these compounds by GC-MS analysis, their detection by ^{1}H-NMR in $CDCl_3$ solution

188 at 400 MHz, and on the availability of the standards. We did not consider some less abundant

189 compounds with relative peak areas under 2.5% (Table 1). A stock solution of α-terpinene, p-

190 cymene, γ-terpinene, linalool, 1-octen-3-ol, thymol methyl ether, carvacrol methyl ether, thymol

191 and carvacrol was serially diluted with the same solvent to prepare calibration curves ranging

192 from 20-170 mg/mL. The R^2 coefficients for the calibration curves were > 0.99. We performed

193 two repetitions for each sample at each concentration level (50, 100 and 150%). All analyses

194 were repeated three times; each time the samples were prepared by the same operator before

195 starting the analysis.

196

197 **GC-MS analysis**

198 GC-MS analysis was performed using a Shimadzu gas chromatograph, model GC-MS-
199 QP2010SE, equipped with a quadrupole analyzer ionization mode with electronic impact and
200 DB-5 capillary column (30 m x 0.25 mm i.d. x 0.25 µm, ref. 122-5032, lot n° USR146513H;
201 Agilent Technologies, USA). The oven temperature was programmed from 40 °C to 220 °C
202 (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
203 220 °C at 10° C/min; then 220 °C for 5 min). Running time: 39 min 54 sec. Helium was used as
204 carrier gas (constant flow rate 36.1 cm/sec). The temperature of ion source and interface were
205 maintained at 220 °C. The injection volume was 1 µL. Prior to injection, the essential oil was
206 diluted (10 µL /1 mL heptane). The acquisition data and instrument control were performed by
207 the GC-MS Solution software. The identity of each compound was assigned by comparison with
208 the mass spectra characteristic features obtained with the NIST library spectral data bank. For
209 semi-quantification purpose the normalized peak area abundances without correction factors
210 were used. Compounds can be identified by a comparison of their retention index, relative to a
211 standard mixture of n-alkanes (Adams, 2009).
212

213 **Proton Nuclear Magnetic Resonance (^{1}H-NMR)**
214 The ^{1}H-NMR spectra were recorded at 400 MHz for ^{1}H experiments on a Bruker Avance 400
215 MHz spectrometer. NMR Fourier transform, integration and peak picking were done with Bruker
216 Top Spin software. Chemical shifts (δ) are reported in ppm. The one-dimensional spectra were
217 performed in CDCl$_3$. The residual solvent was used as a reference (δ = 7.26 ppm).
218

219 **Determination of *Monarda didyma* essential oil anti-oxidant activity by DPPH radical assay**
220 The anti-oxidant activity was assessed by DPPH (diphenyl picrylhydrazyl) radical-scavenging
221 method described by Cuendet et al., (1997) with slight modifications, as previously described
222 (Fraternale et al., 2016). We used 80 µL of 0.5 mM DPPH ethanol solution (Sigma-Aldrich,
223 Missouri, US) and 40 µL of *M. didyma* essential oil were diluted in ethanol at final
224 concentrations ranging from 0.10 to 0.50 µL/mL (to achieve a final volume of 400 µL). After
225 one hour in the dark, the absorbance was measured at 517 nm by UV/Vis spectrophotometer.
226 The control was prepared with 8 µL of 0.5 mM DPPH diluted in 400 µL of ethanol and ethanol
227 without DPPH was used as a blank. Lower absorbance is indicative of higher free radical
228 scavenging activity. The equation to calculate the Inhibitory activity (I) of DPPH radical was the
229 following:
230 I (%) = 100 x (A0-As)/A0
231 In this equation, A0 is the absorbance of the control and As is the absorbance of the tested
232 sample.
233 Different amounts of *M. didyma* essential oil ethanol solution (0.5 µL/mL) ranging from 20 to
234 160 µL were used. All the analyses were run in triplicate. EC50 L-ascorbic acid (Sigma-Aldrich,
235 Missouri, US) was used as positive control (40 µg/mL final concentration). *M. didyma* essential
236 oil EC50 (50% DPPH scavenging activity) was calculated by regression analysis.
237

238 **Biological Assays**
239 **Cell culture and treatments**
240 U937 cells (purchased at Euroclone, Milan, Italy) were maintained in RPMI 1640 culture
241 medium (Sigma Aldrich, Missouri, US) with 10% fetal bovine serum (FBS), penicillin (50
242 U/mL) and streptomycin (50 µg/mL) (Euroclone, Milan, Italy). U937 were incubated at 37 °C in
243 tissue culture (T-75 flasks; Corning, NY) with an atmosphere of 95% air to 5% CO_2. To
244 investigate the anti-inflammatory activity of the essential oil, U937 cells were differentiated into
245 macrophages with 100 nM Phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich, Missouri,
246 US) for 48 h. U937 were then incubated for 24 hours without PMA. To expose U937 cells to a
247 pro-inflammatory stimulus, 1 µg/mL lipopolysaccharide (LPS; Sigma-Aldrich, Missouri, US)
248 final concentration was added to the medium for 6 hours. The essential oil (0.5 µL/mL final
249 concentration) was added one hour before LPS stimulation. Three replicates were performed.
250
251 **Cell viability**
252 To analyze cell viability either Hoechst and Trypan blue have been used to identify apoptosis
253 and necrosis, respectively. Apoptosis was quantified by staining cells with Hoechst 33342
254 (Sigma-Aldrich). Cells with nuclear apoptotic morphology, detected using a fluorescence
255 microscope (Olympus, Milan, Italy), were counted (at least 100 cells in at least 3 independent
256 fields) and the fraction of apoptotic cells among total cells was evaluated as percentage (Radogna
257 et al., 2017). The percentage of necrotic cells was assessed by trypan blue exclusion test using
258 cell suspension diluted 1:2 (v/v) with 0.4% trypan blue.
259
260 **Quantitative real time PCR (RT-qPCR) of mature microRNAs**
261 The total RNA purification kit (Norgen Biotek Corp., Ontario, Canada) was used to isolate total
262 RNA from 1 x 10^6 U937 cells, as recommended in the manufacturer's protocol. Samples were
263 subsequently analyzed for nucleic acid quality and quantity using the Nano-Drop ND-1000
264 spectrophotometer (Nano-Drop Technologies, Wilmington, DE, USA) and stored at -80 °C until
265 use. Human miR-146a and human RNU44 (reference miRNA) expressions were quantified using
266 the TaqMan MicroRNA assay (Applied Biosystems) (see Olivieri et al., 2013). Experimental
267 protocol was as described in a previous paper by Fraternale et al. (2016). More specifically, the
268 TaqMan MicroRNA reverse transcription kit was used to reverse transcribe the total RNA; 5 µL
269 of RT mix contained 1 µL of each miR-specific has-miR-146a stem-loop primers, 1.67 mL of
270 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.
271 The mixture was incubated at 16 °C for 30 min, at 42 °C for 30 min, and at 85 °C for 5 min. RT-
272 qPCR was performed in 20 µL of PCR mix containing 1 µL of 20 x TaqMan MicroRNA assay –
273 which in turn contained PCR primers and probes (5'-FAM) – 10 µL of 2 x TaqMan Universal
274 PCR Master Mix No Amp Erase UNG (Applied Biosystems), and 5 µL of reverse-transcribed
275 product. The reaction was maintained at 95 °C for 10 min, then incubated at 40 cycles at 95 °C
276 for 15 s and at 60 °C for 1 min. The qRT-PCR was run on an ABIPRISM 7500 Real Time PCR
277 System (Applied Biosystems). Data were analyzed by a 7500-system software (1 1.4.0) with the

278   automatic comparative threshold (Ct) setting for adapting baseline. The relative amount of miR-
279   146a was calculated using the Ct method: $\Delta Ct = Ct\ (miR146a) - Ct\ (RNU44)$; 2-$\Delta Ct$
280   Results are expressed as fold change 2-$\Delta\Delta Ct$ related to control (CTRL).
281
282   **Quantitative real time PCR (RT-qPCR) of mature mRNAs**
283   Total RNA was extracted and analyzed for quality and quantity using the same techniques
284   described before. The RNA extracted was used to synthesize cDNA using a reverse transcription
285   kit (Applied Biosystems) according to the manufacturer's protocol. RT-qPCR was performed
286   with the SYBR Green PCR master mix (Applied Biosystems, Foster City, CA, USA) on an ABI
287   Prism 7500 Real Time PCR System (Applied Biosystems). The primers we used were the ones
288   designed by (Angel-Morales, Noratto & Mertens-Talcott, 2012). TATA binding protein (TBP)
289   was used as the endogenous reference and forward/reverse primers were purchased from Sigma-
290   Aldrich. Dissociation curve analysis was used to verify product specificity.
291   Primer sequences were: TBP, forward: TGCACAGGAGCCAAGAGTGAA, reverse:
292   CACATCACAGCTCCCCACCA; IL-6, forward: AGGGCTCTTCGGCAAATGTA, reverse:
293   GAAGGAATGCCCATTAACAACAA; and IRAK-1: forward
294   CAGACAGGGAAGGGAAACATTTT and reverse CATGAAACCTGACTTGCTTCTGAA).
295   The relative amounts of IL-6 and IRAK-1 were calculated using the Ct method:
296    $\Delta Ct = Ct\ (IL-6) - Ct\ (TBP)$; 2-$\Delta Ct$
297   Results are expressed as fold change 2-$\Delta\Delta Ct$ related to control (CTRL).
298
299   **Statistical analysis**
300   All measurements were expressed as mean values ± standard deviation (SD) from the mean of at
301   least three independent experiments. The two-tailed paired Student's t-test was used for the
302   analyses. The results were considered significant at the level of $p < 0.05$.
303

304   # Results
305   **TLC analysis**
306   TLC analysis showed the presence of thymol, carvacrol, thymol methyl ether, carvacrol methyl
307   ether, 1-octen-3-ol, and linalool. Results obtained for p-cymene, α-terpinene, γ-terpinene, α-
308   terpineol and eucalyptol were uncertain (Fig. 2).
309

310   **Qualitative GC-FID analysis**
311   Two different methods (Method A and Method B) were successfully used. GC-FID by method A
312   (analysis, in sequence, of the essential oil samples and the reference substances) resulted in
313   fifteen compounds identified, among which nine were selected as the most abundant ones,
314   whereas the presence of β-pinene, δ-2-carene, δ-3-carene, β-myrcene and α-phellandrene was
315   doubtful (Table 1, see also Fig. 1S).
316   To have further confirmation of the identified compounds, the essential oil was co-injected with
317   the standards (Method B). Compounds identified are listed in Table 2 (see also Fig. 2S). Using

318   Method B conditions, the absence of β-pinene and δ-2-carene was confirmed; whereas
319   camphene, δ-3-carene and α-terpinolene showed too small peaks to be identified.
320
321   **GC-MS analysis**
322   GC-MS analysis pointed to the presence of different classes of phytochemicals. The
323   identification of each compound was possible thanks to the comparison of mass spectra from the
324   NIST library spectral data bank. We found different compounds with significant values of
325   probability of identification (> 95%): α-thujene, α-pinene, camphene, sabinene, β-myrcene, α-
326   phellandrene, δ-3-carene, α-terpinene, p-cymene, γ-terpinene, linalool, α-terpineol, thymol
327   methyl ether, carvacrol methyl ether, thymol and carvacrol; and four with lower percentage:
328   limonene (94%), eucalyptol (94%), β-pinene (91%), α-terpinolene (91%) (Fig. 3S).
329
330   **^{1}H-NMR analysis**
331   *M. didyma* essential oil was also analyzed by ^{1}H-NMR at 400 MHz in CDCl$_3$. The main
332   compounds identified were as follows: p-cymene (Ribeiro, Serra & d' A. Rocha Gonsalves,
333   2010), carvacrol (Han & Armstrong, 2005), thymol (Chung et al., 2007), carvacrol methyl ether
334   (Narkhede et al., 2008), 1-octen-3-ol (Felluga et al., 2007), thymol methyl ether (Maraš, Polanc
335   & Kočevar, 2008), linalool (Shibuya, Tomizawa & Iwabuchi, 2008), γ-terpinene (Ishifune et al.,
336   2003) and α-terpinene (Utenkova et al., 2017) (Fig. 3); due to lower concentration in the mixture,
337   and to signal overlapping, α-pinene, α-terpineol, limonene, myrcene, and camphene were
338   difficult to detect.
339   In particular, despite the expected overlap of most of the resonance signals, some portions of the
340   spectra can be decoded, and some set of signals can be assigned to single compounds, already
341   identified by GC-FID/GC-MS analysis. Most significant resonances in the ^{1}H-NMR spectrum
342   reported in Fig. 3, were as follows: a multiplet at 7.15 ppm for the aromatic protons of p-cymene;
343   a series of overlapped doublets in the 6.7–7.1 ppm range for the aromatic H5 and H6 of
344   carvacrol, thymol, carvacrol methyl ether and thymol methyl ether; two broad singlets at 6.68
345   and about 6.6 ppm for the aromatic H3 of carvacrol and carvacrol methyl ether, and the aromatic
346   H2 of thymol and thymol methyl ether; two overlapped multiplets at 5.9 ppm for the olefinic H2
347   (proximal to the -OH substituent) of both 1-octen-3-ol and linalool; an isolated broad multiplet in
348   the 5.6-5.7 ppm range for the olefinic H2 and H3 of α-terpinene; a broad singlet at 5.47 ppm for
349   the olefinic H2 and H5 of γ-terpinene; a set of overlapped doublets from 5 to 5.3 ppm for the
350   olefinic H1 of 1-octen-3-ol and olefinic H1 and H6 of linalool; a broad singlet for both the OH
351   protons of thymol and carvacrol; a broad isolated quartet at approximately 4.1-4.2 ppm for the
352   allylic H3 of 1-octen-3-ol; two isolated and distinctive singlets for the methoxyl substituent of
353   carvacrol methyl ether (OMe2) and thymol methyl ether (OMe3), at 3.87 and 3.84 ppm
354   respectively; a set of multiplets typical for the isopropyl C-H (H7), at 3.3 ppm for thymol methyl
355   ether, at 3.2 ppm for thymol, and from 2.8 to 3 ppm for carvacrol, carvacrol methyl ether, p-
356   cymene and α-terpinene; a broad signal at approximately 2.6-2.7 ppm, for methylene protons H3
357   and H6 of γ-terpinene; a set of five distinct singlets in the 2.2-2.4 ppm range, for the methyl

358 substituent on the aromatic ring (Me1) of carvacrol methyl ether, carvacrol, thymol, p-cymene
359 and thymol methyl ether; a series of overlapped doublets at 1.2-1.3 ppm, for the geminal methyl
360 protons of the isopropyl substituent (Me7) of thymol methyl ether, p-cymene, thymol, carvacrol,
361 carvacrol methyl ether, and two overlapped doublets at 1.0-1.1 ppm, for the same type of protons
362 (Me7) of γ-terpinene and α-terpinene.
363
364 **Quantitative GC-FID analyses**
365 The concentrations ± standard deviation (SD) of the most abundant compounds in *M. didyma*
366 essential oil are indicated in Table 3.
367
368 **Essential oil antioxidant and anti-inflammatory activity**
369 The essential oil showed a 50% DPPH free radical scavenging activity with 160.214 μL of *M.*
370 *didyma* essential oil ethanol solution (0.5 μL/mL), corresponding to the effect exerted by 0.4 μL
371 of Ascorbic Acid ethanol solution (40 mg/mL) in a final volume of 400 μL. The analysis
372 demonstrated a dose-dependent effect (R^2 = 0.9803) (Fig. 4a).
373 The anti-inflammatory effect of *M. didyma* essential oil was investigated treating U937 cells with
374 an essential oil ethanol solution (0.5 μL/mL final concentration) during a pro-inflammatory
375 stimulus (LPS, 1 μg/mL final concentration). We used this dose of essential oil since we found
376 that it had a good antioxidant activity without creating any cellular toxicity (Fig. 4b). LPS treated
377 U937 cells showed the typical inflammatory condition: down regulation of miR-146a expression
378 level (Fig. 5a) and high amounts of the pro-inflammatory markers IRAK-1 and IL-6 (Figs. 5b, 5c).
379 When treating the cells with the essential oil (MdEO), we observed that the anti-inflammatory
380 effect was particularly evident, resulting in an overexpression of miR-146a (Fig. 5a) and the
381 consequent down regulation of IRAK-1 and IL-6 (Fig. 5b, 5c). When considering MdEO-
382 pretreated U937 cells, under a pro-inflammatory stimulation (LPS + MdEO), the up-regulation of
383 miR-146a was evident as well but, as expected, a little lower than that observed in MdEO
384 experimental group (Fig. 5a), thus confirming that the essential oil phytochemical compounds can
385 efficiently cope with the inflammatory cascade triggered by the LPS insult. The ability of *M.*
386 *didyma* essential oil to modulate the inflammatory response, through the inhibition of the TLR-4
387 signaling pathway and the reduced expression of IL-6 (Fig. 5d), demonstrates that it is a down
388 modulator of transcriptional regulation of pro-inflammatory molecules, at least in an *in vitro*
389 setting. This result is suggestive of a potential good *in vivo* anti-inflammatory activity.
390

Discussion

392 Essential oils (EOs) from aromatic and medicinal plants are known to possess biological
393 activity. In order to provide our contribute in investigating plant-derived phytochemical
394 compounds we studied the chemical composition of the essential oil of *M. didyma* aerial parts
395 from Urbino (Central Italy, Marche region) and evaluated the antioxidant activity and its
396 potential as an anti-inflammatory agent in an *in vitro* setting. According to the quali-
397 quantitative chemical analysis, twenty compounds were identified. Among these, we quantified

398   the most abundant ones including carvacrol, p-cymene, thymol, γ-terpinene, carvacrol methyl
399   ether, 1-octen-3-ol, thymol methyl ether, linalool and α-terpinene.
400   Taking into account that the chemical profile of the essential oils can be greatly variable,
401   especially due to genetic or climatic causes but also to geographic origin, we compared the
402   chemical composition of the *M. didyma* essential oil examined in this experimental set (i.e., *M.*
403   *didyma* ct. carvacrol) with those provided for two Italian chemotypes (Fraternale et al., 2006 and
404   Ricci, Epifano & Fraternale, 2017) and one French chemotype (Carnat, Lamaison & Rémery,
405   1991).
406   Several compounds that we identified correspond to those previously reported by the authors
407   cited above, such as α-pinene, camphene, β-myrcene, p-cymene, limonene, linalool and α-
408   terpineol, but, as expected, they show different concentration profiles, according to the
409   chemotype studied. On the other hand, compounds such as carvacrol and carvacrol methyl ether,
410   that we found in concentration of 16.74% and 5.67% respectively, have not been mentioned in
411   those previous studies.
412   We found remarkable differences just comparing the composition of the essential oils from two
413   *M. didyma* plants of related origin, from Italy: one cultivated in Urbino (same cultivation area of
414   our plant) (Fraternale et al., 2006) and the other one in Imola (Ricci, Epifano & Fraternale,
415   2017).
416   In particular, in these other Italian *M. didyma* EOs, the most abundant compounds were thymol
417   (51.7%-59.3%) and p-cymene (10.5%-9.7%), while in our essential oil the most abundant
418   compounds are carvacrol (16.74%), p-cymene (16.00%) and thymol (10.51%).
419   Considering the composition of the two essential oils obtained from plants grown in
420   Urbino (Fraternale et al., 2006 and present paper), the observed differences could be ascribed to
421   the different season (solar inclination, luminosity, temperature) and different climatic conditions
422   that occurred in the two distinct flowering periods; July 2006 and September 2017. In July 2006,
423   temperatures on average were higher (maximum temperature: 27.1 °C vs. 20.4 °C, minimum
424   temperature: 18.2 °C vs. 12.6 °C); it was less rainy (precipitation: 35 mm vs. 162.2 mm) and a
425   little less humid (relative humidity: 55% vs. 74%) (https://ossmeteo.uniurb.it/).
426   As concerns the most abundant compounds that are most biologically interesting in our essential
427   oil, there is an extensive literature reporting on the pharmacological activities and therapeutic
428   potential of thymol in view of its antioxidant, anti-inflammatory and anti-tumoral properties
429   (Nagoor Meeran et al., 2017; and references therein). In particular, the antioxidant and anti-
430   inflammatory properties of thymol have been well documented in various preclinical studies
431   including cell lines and animal models (see for example, Vigo et al., 2004; Marsik et al., 2005;
432   Braga et al., 2006; Undeger et al., 2009; Archana, Nageshwar Rao & Satish Rao, 2011; Nagoor
433   Meeran & Prince, 2012; Chauhan et al., 2014; Cabello et al., 2015; Nagoor Meeran, Jagadeesh &
434   Salvaraj, 2015; Nagoor Meeran, Jagadeesh & Salvaraj, 2016; Perez Roses et al., 2016; Zidan et
435   al., 2016; Wei et al., 2017).
436   As far as concerns carvacrol, a number of research studies have shown biological actions of
437   carvacrol as an immunomodulator agent (Mahmoodi et al., 2019) with a great therapeutic

438   potential. The *in vitro* and *in vivo* studies have shown multiple pharmacological properties such
439   as anticancer, antifungal, antibacterial, antioxidant, anti-inflammatory, vasorelaxant,
440   hepatoprotective, spasmolytic, immunomodulating and anti-viral (Javed et al., 2021).
441   p-cymene is an aromatic monoterpene with a widespread range of therapeutic properties
442   including antioxidant and anti-inflammatory activity (see de Oliveira Formiga et al., 2020; Sani
443   et al., 2022).
444   Finally, carvacrol methyl ether has been shown to have antibacterial activity with a high
445   potential in the food industry and agriculture (Simirgiotis et al., 2020).
446   Recent studies have demonstrated that flavonoids (Maleki, Crespo & Cabanillas, 2019) and
447   phenolic compounds (Yahfoufi et al., 2018) can inhibit regulatory enzymes or transcription
448   factors important for controlling mediators involved in inflammation.
449   Our findings indicate that during an acute LPS stimulation in *M. didyma* essential oil pretreated
450   U937 cells (MdEO + LPS), miR-146a increases (Fig. 5a) and targets IRAK-1, decreasing its
451   expression (Fig. 5b), with a consequent reduction of IL-6 amounts (Fig. 5c). Such a result
452   strongly supports the hypothesis that the mechanism of action by which *M. didyma* essential oil
453   exerts its anti-inflammatory activity involves the TLR-4 signaling pathway (Fig. 5d).
454   Based on the wide range of experimental studies reporting the potential of thymol, carvacrol and
455   p-cymene as antioxidant and anti-inflammatory drugs (see the above-mentioned literature), we
456   hypothesize that the antioxidant property of the essential oil of *M. didyma* ct. carvacrol we
457   observed in this study is probably due to one or more of these phytochemicals which may act by
458   scavenging free radicals and prevent lipid peroxidation. Moreover, one of the three most
459   abundant compounds alone, or a synergistic interaction of a particular combination of the
460   secondary EO metabolites, may be primarily responsible for the observed anti-inflammatory
461   effect, thus making *M. didyma* ct. carvacrol suitable candidate for further investigation.
462

463   ## Conclusions
464   Our study highlights an aspect still unexplored by the scientific literature regarding the real anti-
465   inflammatory activity of *M. didyma* essential oil. Although further studies are needed for a better
466   elucidation of the molecular mechanisms underlying the observed associated antioxidant and
467   anti-inflammatory bioactivities of *M. didyma* essential oil examined in the present study, they are
468   most likely due to the bio-functional properties of the monoterpenes (mainly aromatic and
469   phenolic monoterpenes) present in the essential oil. As far as concerns the anti-inflammatory
470   activity, in our experimental set, LPS-stimulated U937 cells showed a decreased expression of
471   IRAK-1 and IL-6 (a pro-inflammatory cytokine) and an increased expression of miR-146a,
472   which are suggestive of the involvement of the Toll-like receptor-4 signaling pathway.
473   In summary, this study characterizes a new special chemotype of *M. didyma* essential oil and
474   expands the knowledge about its biological activity confirming that it may be considered, after
475   normalization, in herbal medicinal products or food supplements and could be proposed as a
476   natural source of bioactive compounds.
477

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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 the final version; MCA, DF, BDG and MC revised 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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770 **Legends**

771 **Figure 1 Graphical representation of major biological properties of *Monarda didyma***

772 **essential oil**. Many biological properties of *M. didyma* essential oil have been documented in

773 available literature, the most relevant of which (antifungal/antibacterial, antioxidant and anti-

774 inflammatory activities) are briefly reported.

775 **Figure 2 TLC of *Monarda didyma* essential oil (EO).** *M. didyma* essential oil (MdEO 2, 3 and

776 4 µL) analysis with eight standards: 1-octen-3-ol (25 µL), α-terpineol (10 µL), thymol (2 µL),

777 carvacrol (20 µL), thymol methyl ether (30 µL), carvacrol methyl ether (30 µL), p-cymene (50

778 µL), linalool (2 µL), α-terpinene (50 µL), γ-terpinene (50 µL) and eucalyptol (4 µL).

779 **Figure 3 ^{1}H-NMR spectra of *Monarda didyma* essential oil at 400 MHz in CDCl$_3$.** a. ^{1}H-

780 NMR spectra of *Monarda didyma* essential oil at 400 MHz in CDCl$_3$ (δ 7.26 ppm) and structures

781 with suitable numbering of the detectable compounds; b. enlarged ^{1}H-NMR fragments with

782 annotation for the assignment of the most significant chemical shift ranges or peak resonances.

783 The main constituents detectable in the essential oil were the following: p-cymene (pCy),

784 carvacrol (Cr), thymol (Ty), carvacrol methyl ether (CrM), 1-octen-3-ol (Oct), thymol methyl

785 ether (TyM), linalool (Lin), γ-terpinen (γTer) and α-terpinen (αTer).

786 **Figure 4 DPPH antioxidant activity and cell viability.** a. The essential oil showed a 50%

787 DPPH scavenging activity with 160.214 µL of *M. didyma* essential oil (MdEO) and the analysis

788 demonstrated a dose-dependent effect (R^2 = 0.9803). b. Cell viability (Apoptosis and Necrosis)

789 has been evaluated in U937 cells treated at different concentrations of *M. didyma* essential oil

790 (MdEO): 0, 0.5, 5 and 50 µL. The values are expressed as mean values ± standard deviation (SD)

791 from the mean of at least three independent experiments. The two-tailed paired Student's t-test

792 was used to compare the results vs 0 µL of MdEO (* $p < 0.05$).

793 **Figure 5 Anti-inflammatory effect of *Monarda didyma* essential oil on miR-146a, IRAK-1**

794 **and IL-6 expression levels in U937 cells.** Both experimental groups treated with the essential

795 oil (LPS + MdEO and MdEO,) showed miR-146a up-regulation (5a) with a decreased expression

796 of IRAK-1 and IL-6 (5b and 5c). Results are reported as fold change related to CTRL.

797 CTRL: controls; LPS: LPS-stimulated cells (1 µg/mL final concentration); LPS + MdEO: cells

798 treated with MdEO (0.5 µL/mL final concentration) 1 h before LPS stimulation (1 µg/mL final

799 concentration); MdEO: cells treated with the essential oil (0.5 µL/mL final concentration).

800 Two-tailed paired Student's t-test: *$p < 0.05$ vs. CTRL; °$p < 0.05$ vs. LPS. TLR-4 signaling

801 pathway is illustrated in panel 5d.

803 **Supplementary material legends**
804 **Figure 1S GC-FID**. Chromatogram of *Monarda didyma* essential oil by GC-FID. Compounds
805 identified by comparison of their Retention Time with that obtained with standards (Method A),
806 are listed in Table 1.
807 **Figure 2S GC-FID.** Chromatogram of *Monarda didyma* essential oil co-injected with the
808 standards. Compounds identified by GC-FID in spiked essential oil (EO) with standards (Method
809 B) and their Retention Time, are listed in Table 2.
810 **Figure 3S GC-MS.** Mass chromatogram of *Monarda didyma* essential oil.
811

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.

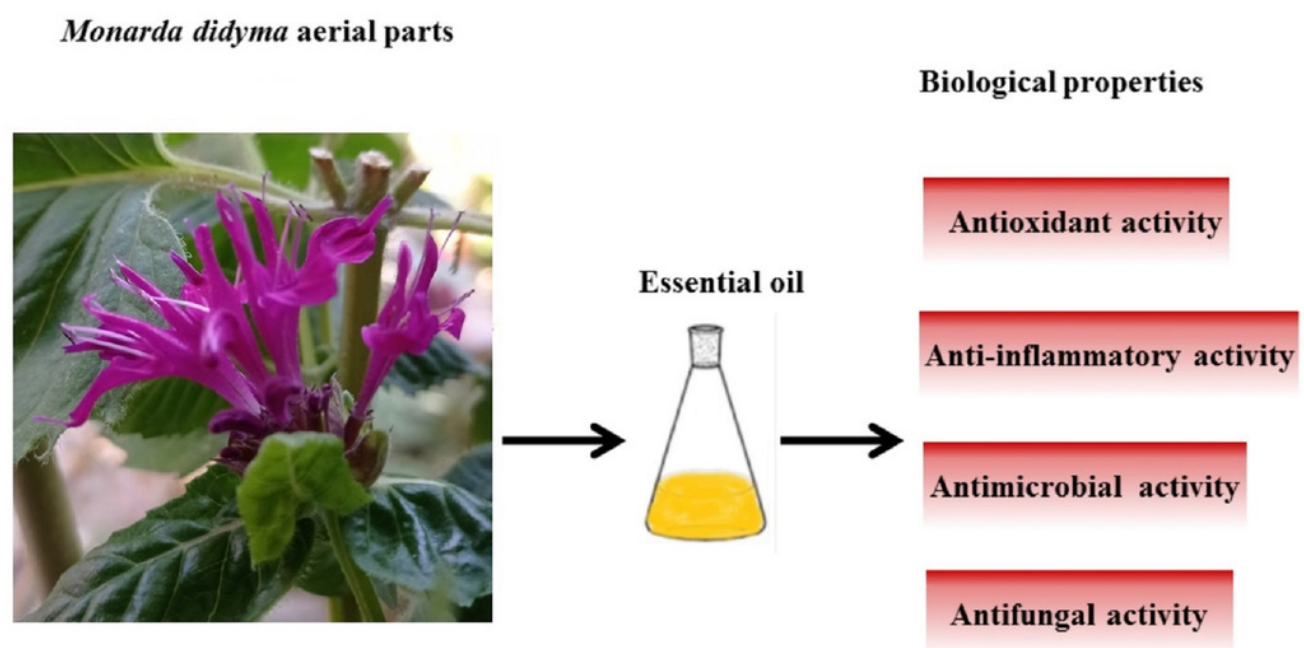



Figure 2

TLC of *Monarda didyma* essential oil (EO).

M. didyma essential oil (MdEO 2, 3 and 4 μL) analysis with eight standards: 1-octen-3-ol (25 μL), α-terpineol (10 μL), thymol (2 μL), carvacrol (20 μL), thymol methyl ether (30 μL), carvacrol methyl ether (30 μL), p-cymene (50 μL), linalool (2 μL), α-terpinene (50 μL), γ-terpinene (50 μL) and eucalyptol (4 μL).

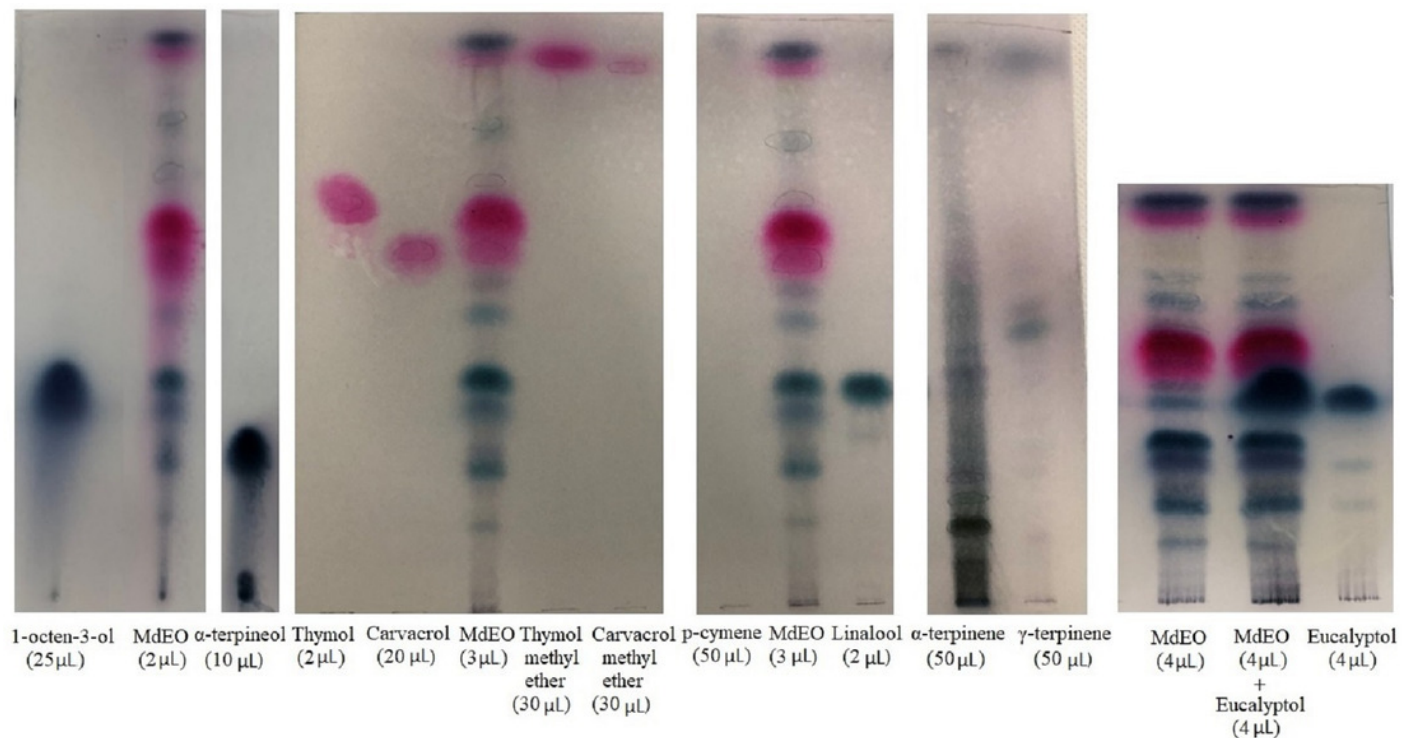



Figure 3

^{1}H-NMR spectra of *Monarda didyma* essential oil at 400 MHz in CDCl$_3$.

a. ^{1}H-NMR spectra of *Monarda didyma* essential oil at 400 MHz in CDCl$_3$ (δ 7.26 ppm) and

structures with suitable numbering of the detectable compounds; b. enlarged ^{1}H-NMR

fragments with annotation for the assignment of the most significant chemical shift ranges or

peak resonances. The main constituents detectable in the essential oil were the following: p-

cymene (pCy), carvacrol (Cr), thymol (Ty), carvacrol methyl ether (CrM), 1-octen-3-ol (Oct),

thymol methyl ether (TyM), linalool (Lin), γ-terpinen (γTer) and α-terpinen (αTer).

a

p-cymene (pCy)   carvacrol (Cr)   thymol (Ty)   carvacrol methyl ether (CrM)   thymol methyl ether (TyM)

1-octen-3-ol (Oct)   linalool (Lin)   γ–terpinene (γTer)   α–terpinene (αTer)

b

TyM pCy Ty Cr CrM-Me1

TyM pCy Ty Cr CrM-Me7
Lin-Me3

γTer αTer-Me7

αTer γTer-Me1

Oct-Me7

γTer
-2H3
-2H6

Cr CrM pCy
αTer-H7

TyM-H7   Ty-H7

pCy-H2-H3
-H5-H6

Cr e CrM-H3
Ty e TyM-H2

CHCl₃   Cr CrM Ty e
TyM-H5-H6

Ty –OH3 Cr –OH2

γTer
-H2-H5   Oct-2H1
Lin-2H1-H6

Oct-H2
Lin-H2   αTer
-H2-H3

Oct-H3

CrM-OMe2
TyM-OMe3

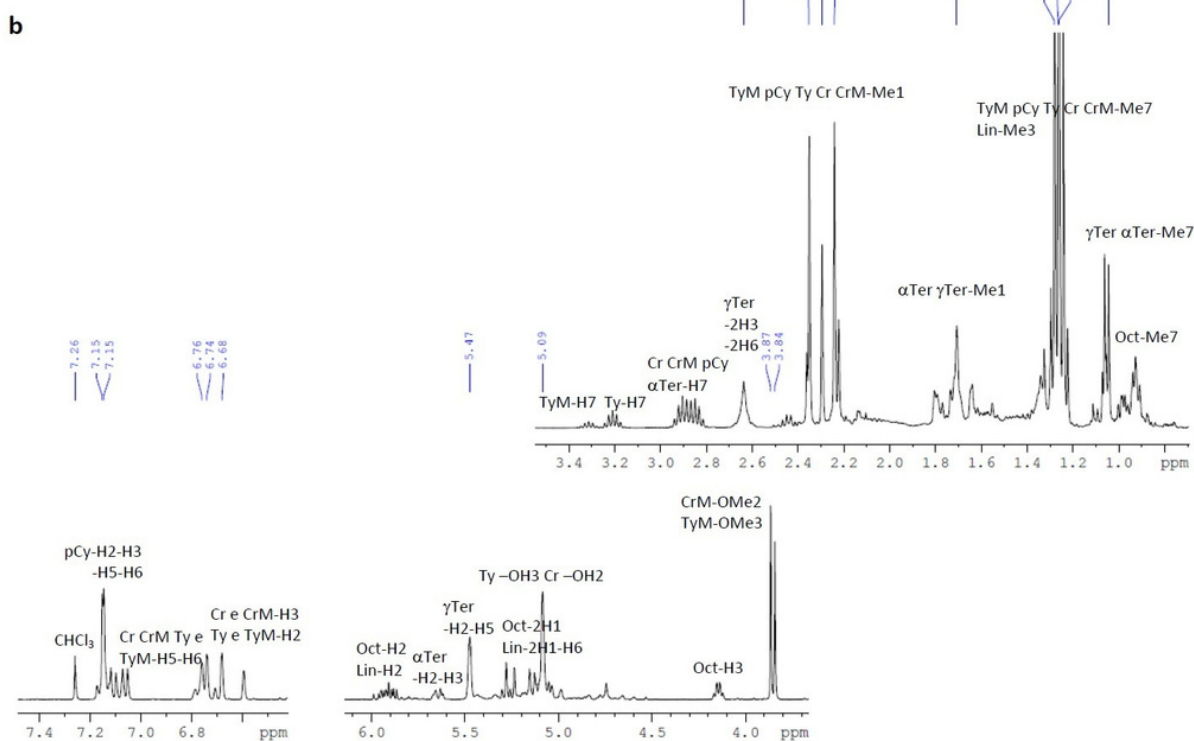
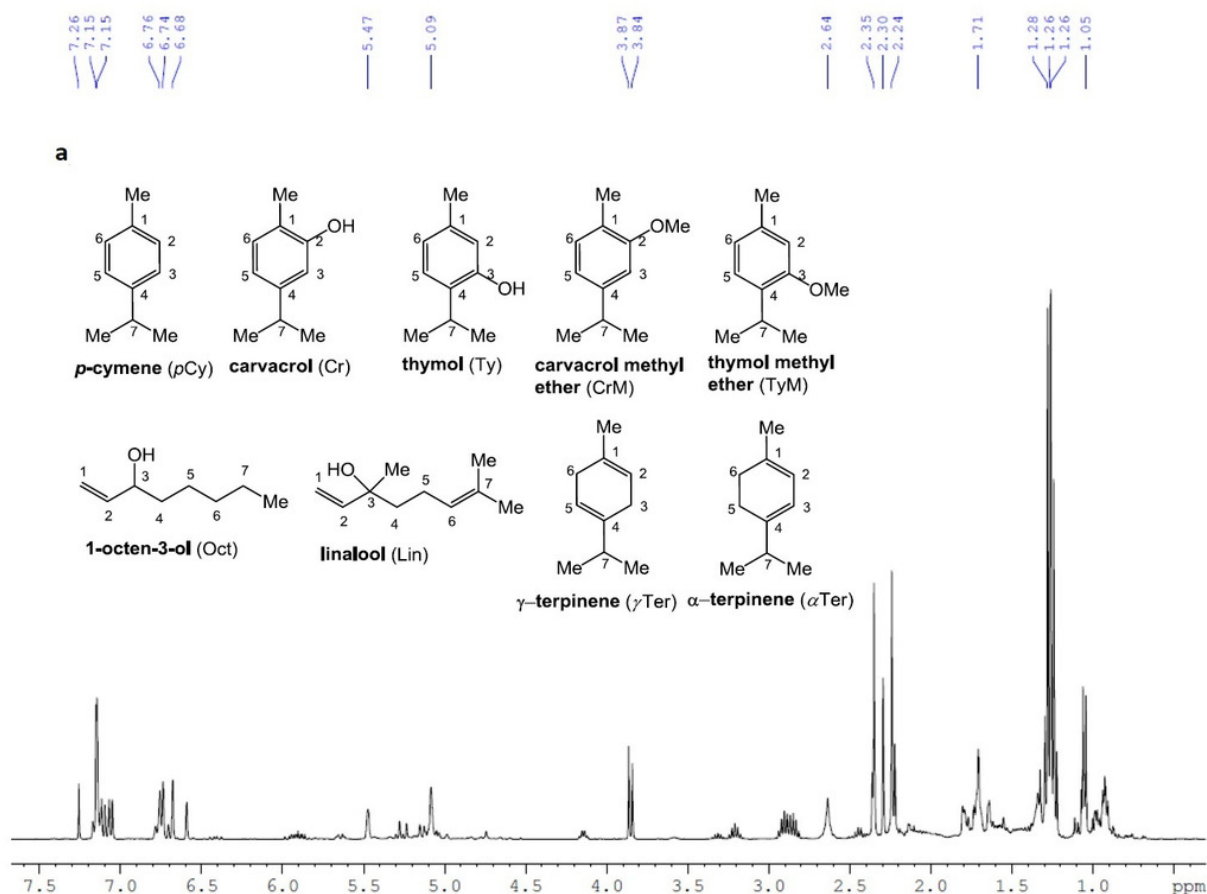



Figure 4

DPPH antioxidant activity and cell viability.

a. The essential oil showed a 50% DPPH scavenging activity with 160.214 µL of *M. didyma* essential oil (MdEO) and the analysis demonstrated a dose-dependent effect (R^2 = 0.9803). b. Cell viability (Apoptosis and Necrosis) has been evaluated in U937 cells treated at different concentrations of *M. didyma* essential oil (MdEO): 0, 0.5, 5 and 50 µL. The values are 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 to compare the results vs 0 µL of MdEO (* $p < 0.05$).

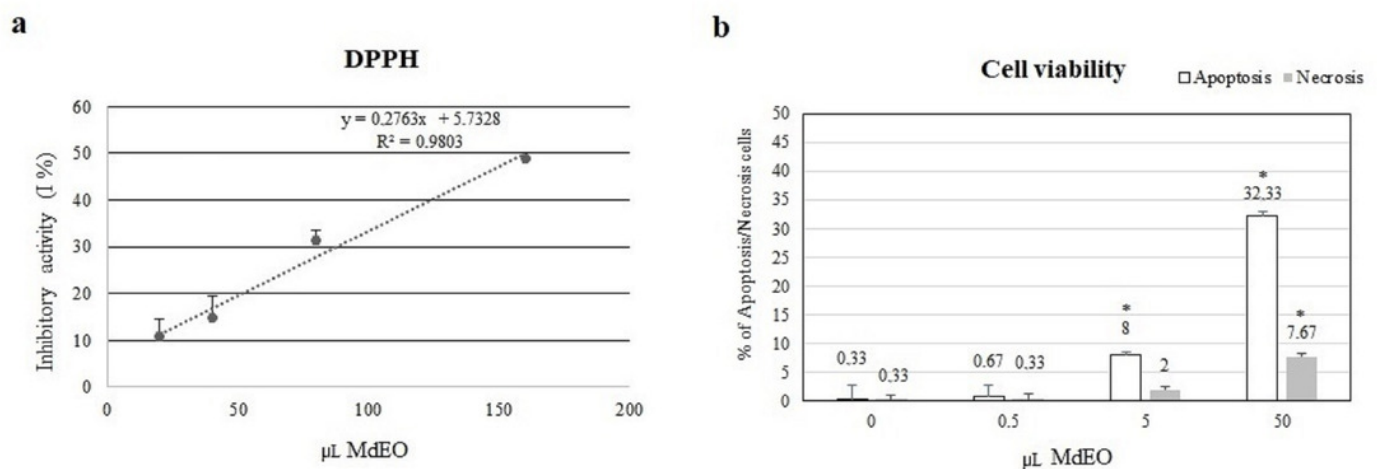
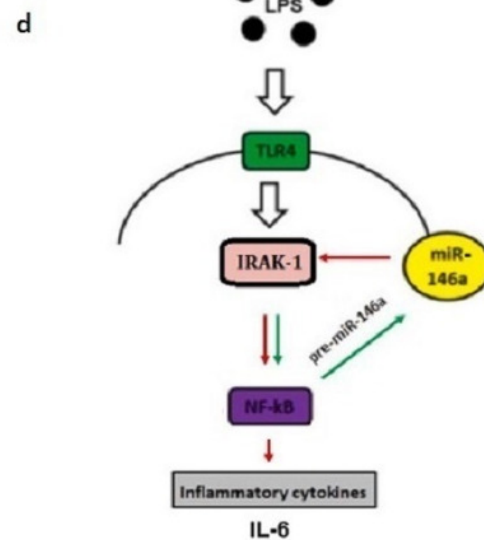
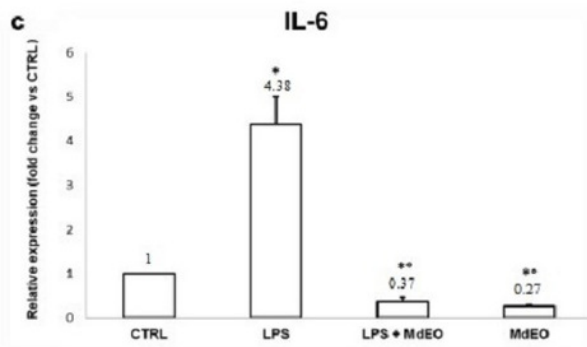
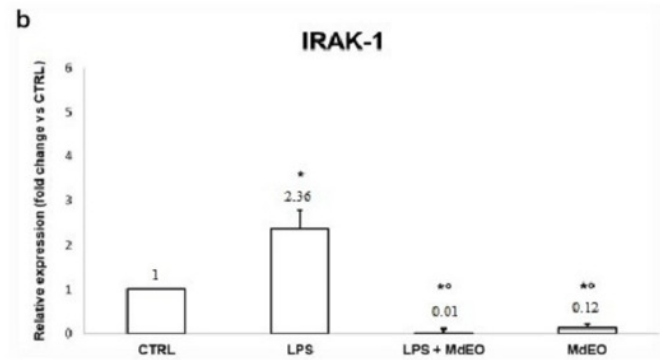
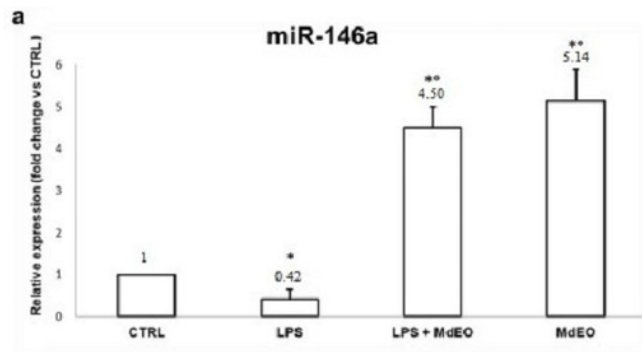



Figure 5

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 (5a) with a decreased expression of IRAK-1 and IL-6 (5b and 5c). 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.

Chemical composition of *Monarda didyma* essential oil (EO) 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 the most abundant components and their relative areas (> 2.5%). The 'Ret. Time EO' represents the mean retention time of 2 repetitions of essential oil samples (concentration level 100%) analyzed on 3 consecutive days.

1   **Table 1 Chemical composition of *Monarda didyma* essential oil (EO) by GC-FID (Method**

2   **A).** The Retention Time of the peaks of the different standards analysed (Ret. Time Standard)

3   and those of the corresponding peaks present in the essential oil spectrum (Ret. Time EO) are

4   indicated. The relative areas (%) of the GC-FID chromatogram peaks are also shown. In bold

5   the most abundant components and their relative areas (> 2.5%). The 'Ret. Time EO'

6   represents the mean retention time of 2 repetitions of essential oil samples (concentration level

7   100%) analyzed on 3 consecutive days.

Compounds	Relative 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

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.

1 **Table 2 Chemical composition of *Monarda didyma* essential oil by GC-FID (Method B).**

2 Compounds identified by GC-FID in the essential oil co-injected with the standards. The

3 Retention Time of the peaks of the essential oil (Ret. Time EO) and that of the essential oil co-

4 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

Quantitative GC-FID analysis of *Monarda didyma* essential oil (EO).

Quantities of the most abundant compounds (ca. 72% of the entire essential oil composition), expressed as mg/mL and percentage ± standard deviation, are listed below.

1   **Table 3 Quantitative GC-FID analysis of *Monarda didyma* essential oil (EO).** Quantities of

2   the most abundant compounds (ca. 72% of the entire essential oil composition), expressed as

3   mg/mL and percentage ± standard deviation, are listed below.

Compounds	mg/mL EO	% (g/100 mL) ± SD
Carvacrol	167.439	16.744±0.318
p-Cymene	159.999	16.000±0.972
Thymol	105.135	10.513±0.195
γ-Terpinene	78.095	7.810±0.384
Carvacrol methyl ether	56.725	5.673±0.204
1-Octen-3-ol	53.700	5.370±0.001
Thymol methyl ether	46.270	4.627±0.138
Linalool	25.212	2.521±0.068
α-Terpinene	24.336	2.434±0.078
	TOTAL %	71.691