

## Potential Health Benefits of *Origanum heracleoticum* Essential Oil: Phytochemical and Biological Variability among Different Calabrian Populations

Mariangela Marrelli<sup>a</sup>, Fabrizio Araniti<sup>b</sup>, Maria Rosa Abenavoli<sup>b</sup>, Giancarlo Statti<sup>a</sup> and Filomena Conforti<sup>a,\*</sup>

<sup>a</sup>Department of Pharmacy, Health and Nutritional Sciences, University of Calabria, I-87036 Rende, (CS), Italy

<sup>b</sup>Department of Agraria, University "Mediterranea" of Reggio Calabria, I-89100 Reggio Calabria, Italy

filomena.conforti@unical.it

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The potential health benefits of the essential oils of six different populations of *Origanum heracleoticum* L. from Calabria (Italy) were assessed, together with the evaluation of the influence of the site of collection on their metabolic profile and biological activity. Different terpenoids were identified, being thymol, carvacrol and *o*-cymene among the most abundant ones. Hierarchical cluster analysis allowed the identification of two main groups, which were discriminated by the altitude of collection. The antioxidant activity was tested by means of two *in vitro* assays: DPPH and  $\beta$ -carotene bleaching test. Samples EO-4 and EO-3 were the most active in protecting linoleic acid from peroxidation, with IC<sub>50</sub> values of 4.00 and 4.68  $\mu$ g/mL after 30 minutes of incubation. The anti-inflammatory potential was assessed through the evaluation of the capacity to inhibit NO production in lipopolysaccharide (LPS)-induced murine macrophage RAW 264.7 cell line. All essential oils induced a dose-depending inhibitory effect. An excellent activity was demonstrated for sample EO-4, followed by sample EO-3 (IC<sub>50</sub> values equal to 32.77 and 49.48  $\mu$ g/mL, respectively). Observed biological properties were correlated to the phytochemical content of analyzed samples.

**Keywords:** Oregano, Antioxidant activity, Anti-inflammatory activity, Nitric oxide.

Oregano is one of the most important spices in world trade. The name "oregano" refers to a number of species belonging to different families, whose essential oils (EOs) are characterized by a typical flavor originating from the high amounts of carvacrol [1]. *Origanum vulgare* L. is the species commonly known as oregano in most countries [2-4] and is present in the whole of Italy. *Origanum heracleoticum* L. (Lamiaceae) is instead typical of the South of the country [5].

Different studies dealt with the phytochemical variability of EOs from wild *Origanum heracleoticum* from different regions of Italy. A number of studies have shown that this species is a very variable taxon both in morphological and in chemical features, with an EO the main components of which are phenols, *p*-cymene, and  $\gamma$ -terpinene [6-8]. The antibacterial activity of the EO and the antioxidant potential of both extract and EO have also been explored [9-12]. We already tested the anti-inflammatory potential of the ethanolic extract of this plant through the evaluation of the inhibitory activity on nitric oxide (NO) production in LPS-stimulated RAW 264.7 macrophages [13].

The aim of this work was to evaluate the potential health benefits and applications of the EO of this species, particularly the antioxidant and anti-inflammatory potential. The influence of the site of collection on metabolic profile and biological activity of the EOs of six different populations of *O. heracleoticum* from Calabria (Italy) was also assessed.

The harvest times, the altitude and GPS coordinates of the location as well as the EO yield (percent of dry weight) of *O. heracleoticum* samples are reported in Table 1. The yield ranged from 3.47 to 5.43% and it was significantly higher in the samples collected at the highest altitudes (Table 1).

**Table 1:** Locality, altitude, collection date and EOs yield of the 6 wild populations of *O. heracleoticum* collected in Calabria (Southern Italy).

| Sample | Locality                  | Altitude (m. a.s.l.) | GPS coordinates             | Collection date | Yield (%) |
|--------|---------------------------|----------------------|-----------------------------|-----------------|-----------|
| EO-1   | Sant'Eufemia d'Aspromonte | 420                  | 38°16'00.4"N - 15°51'07.8"E | July            | 3.65      |
| EO-2   | Podargoni                 | 500                  | 38°09'48.1"N - 15°46'57.7"E | July            | 3.47      |
| EO-3   | Bagaladi                  | 460                  | 38°01'21.4"N - 15°49'12.5"E | July            | 3.94      |
| EO-4   | Longobucco                | 784                  | 39°27'05.4"N - 16°36'56.6"E | August          | 5.23      |
| EO-5   | Rogliano                  | 660                  | 39°10'14.0"N - 16°19'31.5"E | August          | 5.43      |
| EO-6   | Acri                      | 720                  | 39°29'24.0"N - 16°22'28.2"E | August          | 4.85      |

The relative abundances of the chemicals identified in the EOs of the 6 populations of *O. heracleoticum* collected at two different altitudes are reported in Table 2.

A wide array of terpenoids were identified. In particular, 29 terpenes were found, among which the two monoterpenic phenols thymol and carvacrol were among the most abundant ones together with *o*-cymene. Fifteen sesquiterpenoids were also found, among which caryophyllene, muurolene and cadinene were the most abundant ones.

To verify how location influenced the metabolic profile, the EOs isolated from 6 populations of *O. heracleoticum* were evaluated through GC-MS and then data were analyzed through unsupervised Principal Component Analysis (Figure 1).

The PCA score plot, which allowed the separation of the 6 populations basing on their metabolite profiles, is reported in Figure 1-A, whereas the PCA loading plot where the metabolites contributing in score-plot samples separation were highlighted is reported in Fig 1-B. The separation of the EOs belonging to different populations was achieved using the principal components PC1 vs PC2, which explained a total variance of 51.2%. In particular, PC1 explained the 31.5% of the variance while PC2 the 19.7%. Principal component analysis pointed out a clear separation

**Table 2:** Chemical composition of *O. heracleoticum* EOs.

| Monoterpenes                            | RT    | EO-1     | EO-2     | EO-3     | EO-4     | EO-5     | EO-6     |
|---|-------|----------|----------|----------|----------|----------|----------|
| $\alpha$ -Thujene                       | 09.90 | 14.46    | -        | -        | 2368.91  | 53.03    | -        |
| $\alpha$ -Pinene                        | 10.07 | 375.79   | 524.34   | 106.45   | 3388.29  | 220.70   | 397.27   |
| Camphene                                | 10.50 | 47.54    | 50.06    | 13.68    | 404.69   | -        | 40.33    |
| $\beta$ -Pinene                         | 11.20 | 41.39    | 100.47   | 15.05    | 642.82   | 41.11    | 75.12    |
| $\beta$ -Myrcene                        | 11.54 | 472.25   | 1063.98  | 118.08   | 6199.70  | 445.71   | 804.92   |
| Sabinene                                | 11.77 | 12.41    | 79.01    | 18.82    | -        | -        | -        |
| $\Delta$ -3-Carene                      | 11.88 | 52.14    | 67.63    | 16.37    | 424.42   | -        | 47.89    |
| $\alpha$ -Terpinene                     | 11.99 | 181.62   | 477.50   | 116.65   | 251.29   | -        | -        |
| <i>o</i> -Cymene                        | 12.14 | 8119.62  | 7450.03  | 3304.13  | 48175.94 | 6348.5   | 6888.6   |
| Limonene                                | 12.20 | 434.74   | 470.30   | 111.03   | 2515.31  | 230.34   | 302.94   |
| Eucalyptol                              | 12.24 | 112.05   | 74.04    | 22.75    | 809.58   | 60.35    | 57.47    |
| $\beta$ -Ocimene                        | 12.34 | 6.54     | 177.80   | 16.25    | 783.18   | 152.88   | 164.14   |
| $\gamma$ -Terpinene                     | 12.65 | 23.65    | 4570.53  | 750.77   | 19132.72 | 1170.81  | 2199.87  |
| <i>trans</i> -4-Thujanol                | 12.78 | 43.73    | 196.66   | 91.31    | 1212.04  | 154.72   | 140.32   |
| Linalool                                | 13.18 | 1315.05  | 614.19   | 320.60   | -        | 725.91   | 355.83   |
| <i>trans</i> - <i>p</i> -2-Menthen-1-ol | 13.46 | 14.26    | 35.12    | 21.27    | -        | -        | -        |
| <i>cis</i> -2- <i>p</i> -Menthen-1-ol   | 13.67 | 13.48    | -        | 17.40    | -        | -        | -        |
| Myrtenol                                | 13.73 | 19.87    | 43.51    | 16.13    | 406.19   | 39.22    | 74.68    |
| Borneol                                 | 13.97 | 208.64   | 271.16   | 125.55   | 2435.39  | 297.50   | 551.85   |
| 4-Terpineol                             | 14.07 | 1099.06  | 805.82   | 901.76   | 3384.45  | 653.01   | 432.95   |
| <i>p</i> -Cymen-8-ol                    | 14.14 | 95.69    | -        | 39.92    | 236.68   | 36.50    | 34.55    |
| $\alpha$ -Terpineol                     | 14.20 | 413.19   | 212.38   | 334.61   | 1150.75  | 201.73   | 246.77   |
| Carvone                                 | 14.25 | 37.32    | -        | 86.09    | 463.43   | -        | -        |
| 3-methoxy- <i>p</i> -Cymene             | 14.56 | 737.8    | 671.48   | 69.38    | 2732.95  | 1519.11  | 400.93   |
| Carvacrol-methyl-ether                  | 14.64 | 3275.21  | 2657.99  | 2908.22  | 19843.72 | 2839.54  | 2900.29  |
| Carvenone                               | 14.87 | 38.55    | -        | -        | 507.28   | 57.34    | 155.03   |
| 3- <i>p</i> -Cymenol                    | 14.99 | 413.08   | 265.27   | 113.94   | 1728.28  | 289.79   | 252.28   |
| Thymol                                  | 15.06 | 10781.78 | 11145.27 | 6814.88  | 54459.34 | 13146.99 | 9104.01  |
| Carvacrol                               | 15.14 | 4568.63  | 3311.01  | 15117.63 | 68379.03 | 6374.46  | 12494.85 |
| <b>Sesquiterpenes</b>                   |       |          |          |          |          |          |          |
| Ylangene                                | 15.72 | 59.31    | 53.96    | 51.76    | 443.07   | 57.21    | 34.41    |
| $\alpha$ -Copaene                       | 15.75 | 138.73   | 93.87    | 139.30   | 964.29   | 115.55   | 79.94    |
| $\beta$ -Caryophyllene                  | 16.10 | 1192.22  | 1696.70  | 1182.44  | 16107.17 | 2500.99  | 1625.29  |
| $\alpha$ -Bergamotene                   | 16.13 | 43.92    | 82.763   | 40.06    | 633.74   | 63.99    | 75.34    |
| Aromadendrene                           | 16.23 | 45.52    | 52.10    | 45.20    | 362.70   | 96.99    | 57.49    |
| $\alpha$ -Humulene                      | 16.33 | 157.39   | 223.63   | 153.76   | 2518.07  | 442.70   | 268.93   |
| $\gamma$ -Muurolole                     | 16.45 | 2028.02  | 2488.83  | 2716.3   | 10824.34 | 1893.07  | 1364.28  |
| $\alpha$ -Muurolole                     | 16.57 | 157.08   | 79.54    | 164.15   | 1079.27  | 164.43   | 80.91    |
| $\beta$ -Bisabolene                     | 16.71 | 472.80   | 448.56   | 517.23   | 3853.02  | 525.29   | 395.92   |
| $\Delta$ -Cadinene                      | 16.74 | 1137.75  | 1108.69  | 1235.82  | 7096.58  | 1049.77  | 675.96   |
| $\alpha$ -Calacorene                    | 16.92 | 71.85    | 39.476   | 99.57    | 386.20   | -        | -        |
| Spathulenol                             | 17.20 | 49.87    | 40.90    | 703.89   | 5005.14  | 350.74   | 205.10   |
| Cadinol                                 | 17.64 | 66.23    | 92.56    | 136.55   | 1075.13  | 168.15   | 111.18   |
| $\alpha$ -Cadinol                       | 17.76 | 33.68    | 117.97   | 83.74    | 568.03   | 90.86    | 95.71    |
| Guaiazulene                             | 17.94 | 98.72    | -        | -        | -        | -        | -        |
| <b>Other compounds</b>                  |       |          |          |          |          |          |          |
| Methyl-2-methylbutanoate                | 04.43 | 54.29    | 562.53   | -        | -        | -        | 218.57   |
| Octan-3-one                             | 11.45 | 31.71    | -        | 5.15     | -        | -        | -        |
| 3-methyl-6-hydroxybenzo-c- dihydrofuran | 15.51 | 127.02   | 272.54   | 45.36    | 946.11   | 136.82   | 67.67    |

among EO-1 – EO-6. Although well separated, populations EO-1 – EO3, which were collected at the same altitude and in closer locations, tended to group together (Figure 1-A) and a similar trend was observed also on population EO-5 - EO-6. Only the population EO-4 was highly separated by the other populations.

On the other hand, using hierarchical cluster analysis, two main groups were observed (Figure 1-C), which can be classified as populations collected at an altitude of ~ 450 above s.l (EO-1 - EO-3) and populations collected at an altitude of ~ 720 above s.l (EO-4 - EO-6) (Figure 1-C).

The PCA loading plot reported in Figure 1-B allowed identifying the chemical compounds that contributed to samples separation. In particular, the PC1 was largely affected by  $\gamma$ -terpinene,  $\beta$ -ocimene,  $\alpha$ -terpinene and  $\alpha$ -calacorene, whereas the PC2 was mainly affected by camphene,  $\beta$ -pinene, linalool and spathulenol.

The radical scavenging activity of the different EOs was evaluated using DPPH test. Samples showed a modest radical scavenging activity, being EO-4 the most effective one, with an IC<sub>50</sub> value of 320.9  $\mu$ g/mL (Table 3). The antioxidant activity was also evaluated by means of a second assay, the  $\beta$ -carotene bleaching test. Sample EO-4, together with sample EO-3, was the most active one in

protecting linoleic acid from peroxidation, with IC<sub>50</sub> values of 4 and 4.68  $\mu$ g/mL after 30 minutes of incubation (Figure 2) Also after 60 minutes of incubation the biological activity was significantly high (IC<sub>50</sub> values equal to 6.91  $\mu$ g/mL for sample EO-4 and 15.39  $\mu$ g/mL for sample EO-3).

Essential oil EO-4 was the most interesting one. Its antioxidant activity could be related to the highest content of the two monoterpenoid phenols thymol and carvacrol [14,15].

The anti-inflammatory potential was tested through the evaluation of the capacity to inhibit NO production in lipopolysaccharide (LPS)-induced murine macrophage RAW 264.7 cell line. The presence of nitrite, a stable oxidized product of NO, was determined in cell culture media after 24 h by the Griess reagent. Dinitrogen trioxide (N<sub>2</sub>O<sub>3</sub>) generated from acidified nitrite (or from the autooxidation of NO) reacts with sulfanilamide to yield a diazonium derivative. This reactive intermediate interacts with N-1-naphthylethelene diamine to yield a colored diazo product that absorbs strongly at 540 nm [16]. The inhibitory activity observed at the concentration of 100  $\mu$ g/mL is reported in Figure 3.

All samples induced a dose-dependent inhibition of NO production. An excellent activity was observed for essential oil EO- 4, collected



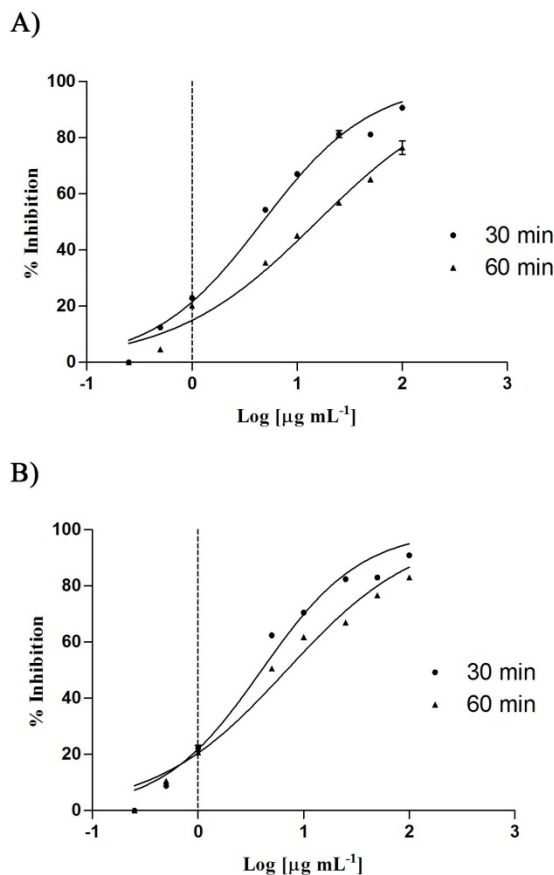
**Figure 1:** PCA and hierarchical cluster analysis carried on the metabolite identified and quantified in the EOs of 6 different populations of *O. heracleoticum* collected at two different altitudes in Calabria. A) Score plot of the Partial Least Square Discriminant Analysis (PLS-DA); B) loading plots; C) Hierarchical cluster analysis. N=3.

at about 720 m. above s.l., with an IC<sub>50</sub> value of 32.77 µg/mL, followed by sample EO-3, with an IC<sub>50</sub> value equal to 49.48 µg/mL (Table 4). Also at the lowest concentration tested, 25 µg/mL, these two last samples were effective, inducing more than 30% of inhibition.

**Table 3:** Antioxidant activity of *O. heracleoticum* EOs.

| Sample          | IC <sub>50</sub> (µg/mL) |                           |                           |
|-----------------|--------------------------|---------------------------|---------------------------|
|                 | DPPH test                | β-carotene bleaching test |                           |
|                 |                          | 30 min                    | 60 min                    |
| EO-1            | 430.6 ± 6.2 <sup>d</sup> | > 100                     | > 100                     |
| EO-2            | 466.4 ± 0.6 <sup>c</sup> | 55.12 ± 3.08 <sup>b</sup> | > 100                     |
| EO-3            | 390.3 ± 0.5 <sup>c</sup> | 4.68 ± 0.03 <sup>ab</sup> | 15.39 ± 0.49 <sup>c</sup> |
| EO-4            | 320.9 ± 3.6 <sup>b</sup> | 4.00 ± 0.03 <sup>ab</sup> | 6.91 ± 0.12 <sup>b</sup>  |
| EO-5            | 454.3 ± 1.1 <sup>c</sup> | 25.86 ± 1.00 <sup>c</sup> | 33.88 ± 0.93 <sup>f</sup> |
| EO-6            | 403.3 ± 5.9 <sup>c</sup> | 32.88 ± 0.92 <sup>f</sup> | 40.91 ± 1.17 <sup>g</sup> |
| Ascorbic acid*  | 2.00 ± 0.01 <sup>a</sup> | -                         | -                         |
| Propyl gallate* | -                        | 1.00 ± 0.02 <sup>a</sup>  | 1.00 ± 0.02 <sup>a</sup>  |

Data are expressed as mean ± SE (n = 3). Different letters along column (DPPH test) or between columns (β-carotene bleaching test) indicate statistically significant differences at P < 0.05 (Bonferroni test). \* Positive controls.



**Figure 2:** Inhibition of lipid peroxidation induced by *O. heracleoticum* essential oils EO-3 (A) and EO-4 (B).

**Table 4:** Inhibitory effects of *O. heracleoticum* EOs on the LPS-induced NO production in RAW 264.7 cells.

| Sample        | Locality                  | IC <sub>50</sub> (µg/mL)  |
|---------------|---------------------------|---------------------------|
| EO-1          | Sant' Eufemia d'Aspromnte | 128.7 ± 5.26 <sup>d</sup> |
| EO-2          | Podargoni                 | 78.62 ± 3.12 <sup>c</sup> |
| EO-3          | Bagaladi                  | 49.48 ± 0.62 <sup>b</sup> |
| EO-4          | Longobucco                | 32.77 ± 0.40 <sup>a</sup> |
| EO-5          | Rogliano                  | 118.0 ± 2.82 <sup>d</sup> |
| EO-6          | Acri                      | 170.9 ± 2.90 <sup>e</sup> |
| Indomethacin* |                           | 58.00 ± 0.90 <sup>b</sup> |
| L-NAME*       |                           | 45.86 ± 0.46 <sup>b</sup> |

Data are expressed as mean ± SE (n = 4). Different letters indicate statistically significant differences at P < 0.05 (Bonferroni test). \* Positive controls.

Among the other EOs an IC<sub>50</sub> value of 78.62 µg/mL was obtained for sample EO-2, while the other samples showed a lower activity (IC<sub>50</sub> values ranging from 118.0 to 170.9 µg/mL).

Besides their antioxidant activity, carvacrol and thymol could be responsible, at least in part, also for the *in vitro* anti-inflammatory activity observed, as it has been demonstrated a modest inhibition of NO production [17]. However, the effects observed on RAW cells could be more likely related to the major content of γ-terpinene and β-pinene found in essential oil EO-4. These compounds, as a matter of fact, were demonstrated to be effective inhibitors of NO production [18]. In conclusion, *O. heracleoticum* EO could be an interesting source of antioxidant and anti-inflammatory compounds. Therefore, due to these antioxidant and anti-inflammatory activities, this EO could be used in many applications in the future, including the use as a functional ingredient in health foods or as a drug for treating inflammatory related diseases.

### Experimental

**Chemicals:** 1,1-diphenyl-2-picrylhydrazyl radical (DPPH), ascorbic acid, β-carotene, linoleic acid, Tween 20, Dulbecco's modified Eagle's medium (DMEM), phosphate-buffered saline (PBS), fetal bovine serum (FBS), L-glutamine, penicillin/streptomycin, trypan blue and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma-Aldrich S.p.A. (Milan, Italy). Murine monocytic macrophage cell line RAW 264.7 was purchased from ATCC no. CRL-2278, UK. Ethanol, methanol and dimethyl sulfoxide (DMSO) were purchased from VWR International s.r.l. (Milan, Italy).

**Plant collection and EO extraction:** The study was carried out on the EOs isolated from six different Calabrian populations of *O. heracleoticum*. Samples were collected between July and August (depending by the altitude) at the beginning of the flowering stage (balsamic period) in Calabria (southern Italy) (Table 1). Fresh plant material was immediately processed after the harvest. EOs were extracted using a semi-industrial stainless steel distilling apparatus. Samples were distilled for four hours and the obtained oils were separated from the hydrolates and dried with anhydrous sodium sulfate, and stored in dark vials at -20°C.

**GC-MS analysis:** The EOs were chemically characterized using a Thermo Fisher gas chromatograph apparatus (Trace 1310) equipped with a single quadrupole mass spectrometer (ISQ LT). The capillary column was a TG-5MS 30 m×0.25 mm×0.25µm the gas carrier was helium with a flow of 1 mL/min. Injector and source were settled at the temperature of 200°C and 260°C, respectively. Samples were solubilized in ethanol at the concentration of 1000 ppm and fenchone was added as internal standard (2000 ppm). Samples were injected in a split mode with a split ratio of 60. The programmed temperature was as follow: isocratic for 7 minutes 45°C, from 45°C to 80°C with a rate of 10°C×min, from 80°C to 200°C with a rate of 20°C×min then isocratic for 3 minutes 200°C. Mass spectra were recorded in electronic impact (EI) mode at 70 eV, scanning the 45–500 *m/z* range. Compounds identification was carried out comparing the relative retention time and mass spectra of the molecules with those of the libraries (NIST).

**Free radical scavenging activity assay:** Radical scavenging activity was determined using the free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) as previously described [19]. Briefly, about 200 µl of different concentrations of test samples solutions (ranging from 5 to 1000 µg/mL) were added to 800 µL of a 10<sup>-4</sup> M methanolic solution

of DPPH. Absorbance was measured at 517 nm after 30 minutes in the dark using a Perkin Elmer Lambda 40 UV/VIS spectrophotometer. Experiments were run in triplicate and ascorbic acid was used as positive control.

**β-carotene bleaching test:** The ability of *O. heracleoticum* EOs to inhibit lipid peroxidation was assessed by the β-carotene bleaching test [20]. A β-carotene–linoleic acid emulsion was prepared by adding a 0.2 mg/mL β-carotene chloroformic solution (1 mL) to linoleic acid (0.02 mL) and 100% Tween 20 (0.2 mL). After the evaporation of chloroform 100 mL of water were added. Different concentrations (ranging from 0.25 to 100 µg/mL) of each samples (0.2 mL) were combined with prepared emulsion (5 mL). Mixtures were then placed in a water bath at 45°C for 60 min. Propyl gallate was used as positive control and absorbance values were measured at 470 nm using the Perkin Elmer Lambda 40 UV/VIS spectrophotometer. Measurements were carried out at initial time (t = 0) and after 30 and 60 min and antioxidant activity was evaluated by measuring the prevention of β-carotene bleaching.

**Inhibition of NO production:** The *in vitro* anti-inflammatory activity of *O. heracleoticum* EOs was verified by evaluating the ability to inhibit NO production in LPS-stimulated murine macrophage RAW 264.7 cells. Cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine and 1% antibiotic solution (penicillin/streptomycin) under 5% CO<sub>2</sub> at 37 °C. Cells were removed from culture flask by scraping. Cells counts and viability were performed using a standard trypan blue cell counting technique and cells were subcultured onto 96 well culture plates (1 × 10<sup>5</sup> cells/well) used for experiments 24 h later. RAW 264.7 cells were then incubated with different concentrations of each EO (25–1000 µg/mL) in the presence of 1 µg/mL LPS for 24 h.

The Griess reagent (1% sulfanamide and 0.1% N-(1-naphtyl) ethylenediamine dihydrochloride in 2.5% H<sub>3</sub>PO<sub>4</sub>) was used to determine the presence in cell culture media of nitrite, a stable oxidized product of NO, as previously described [21]. Briefly, cell culture supernatant (100 µL) was combined with Griess reagent (100 µL) in a 96-well plates and absorbance was measured at 550 nm using a microplate reader (GDV DV 990 B/V, Roma, Italy). The absence of cytotoxic effects was assessed by means of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, as previously reported [22]. MTT was solubilized in phosphate buffered saline (0.5% w/v), and 100 µL were added to each well. After 4 h of incubation, 100 µL of dimethyl sulfoxide were added to each well to dissolve the formazan crystals and absorbance values at 550 nm were measured.

**Experimental design and statistical analysis:** EOs extraction and chemical characterization were carried out in triplicate. After chemical characterization and quantitation raw data were analyzed through unsupervised principal component analysis (PCA). Moreover, *Oregano* populations were clusterized through hierarchical cluster analysis where the distance was measured with the Euclidean method using the Ward's algorithm for clustering. Both multivariate and cluster analyses were performed using MetaboAnalyst 3.0 [23].

Biological activity assays were carried out in triplicate, with the exception of NO inhibition assay, that was conducted in quadruplicate. Data were expressed as means ± S.E.M. Raw data were fitted through non-linear regression in order to calculate the IC<sub>50</sub> values (concentration providing 50% inhibition). Data were checked for normality (D'Agostino-Pearson test) and tested for

homogeneity of variances (Levene's test). Statistical significance was assessed through one-way analysis of variance (ANOVA).

Significant differences among means were analyzed using Bonferroni post hoc test with  $P \leq 0.05$ .

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