

Supplementary material to:

Standardised comparison of limonene-derived monoterpenes identifies structural determinants of anti-inflammatory activity

Cátia Sousa^{a,b,c}, Alcino Jorge Leitão^{a,b,c}, Bruno Miguel Neves^d, Fernando Judas^{a,c,e},

Carlos Cavaleiro^{b,f}, Alexandrina Ferreira Mendes^{a,b,c}*

Affiliations:

^aCenter for Neuroscience and Cell Biology, University of Coimbra, Coimbra, Portugal

^bFaculty of Pharmacy, University of Coimbra, Coimbra, Portugal

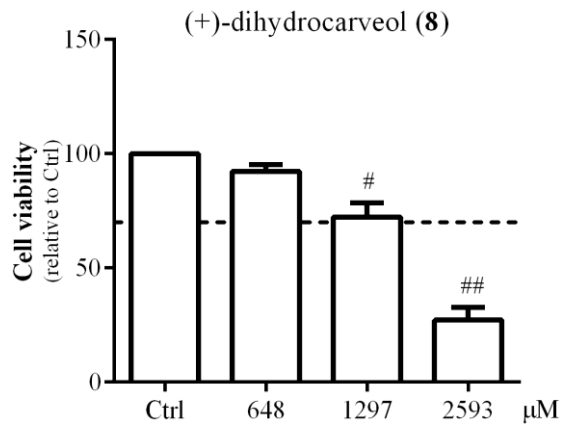
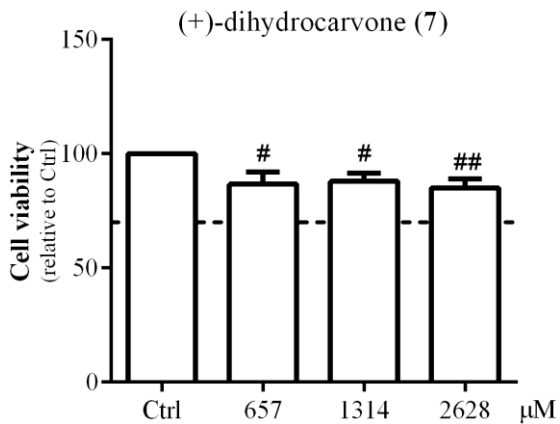
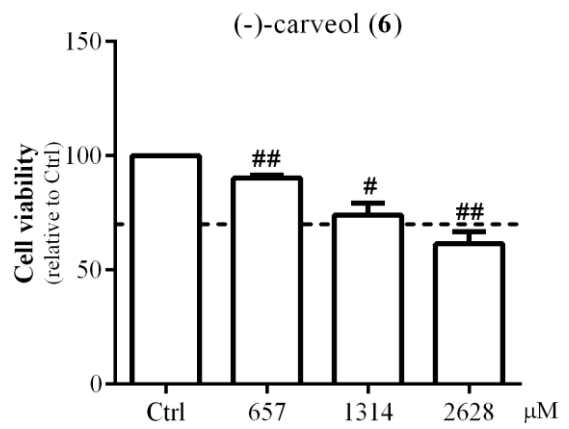
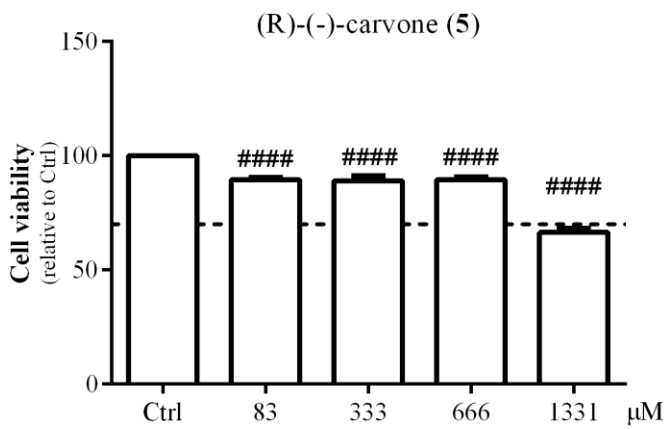
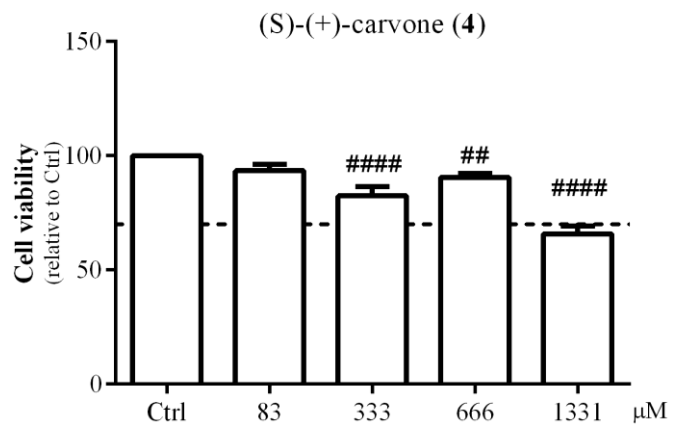
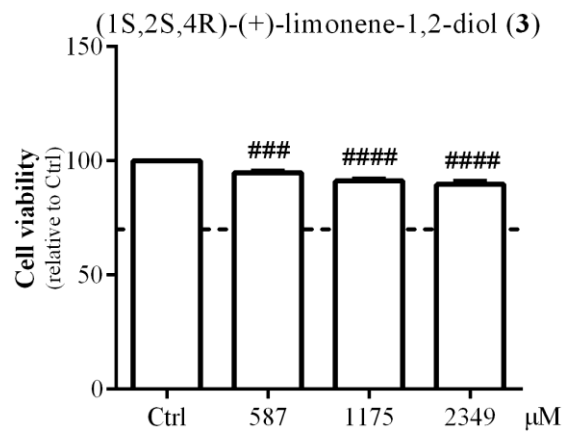
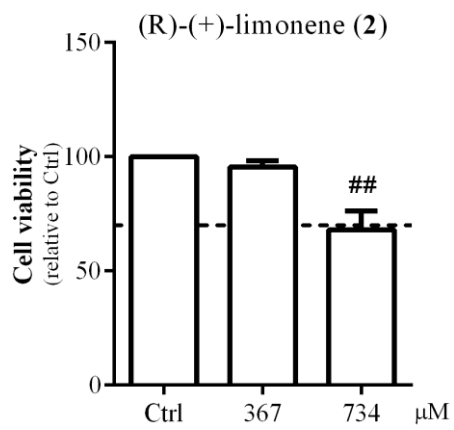
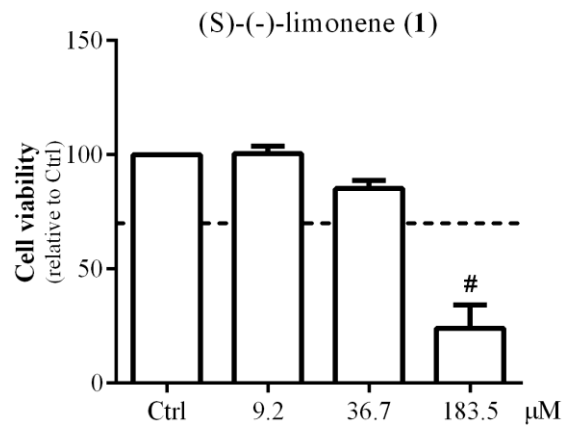
^cCentre for Innovative Biomedicine and Biotechnology, University of Coimbra, Coimbra, Portugal

^dDepartment of Medical Sciences and Institute of Biomedicine – iBiMED, University of Aveiro, Aveiro, Portugal

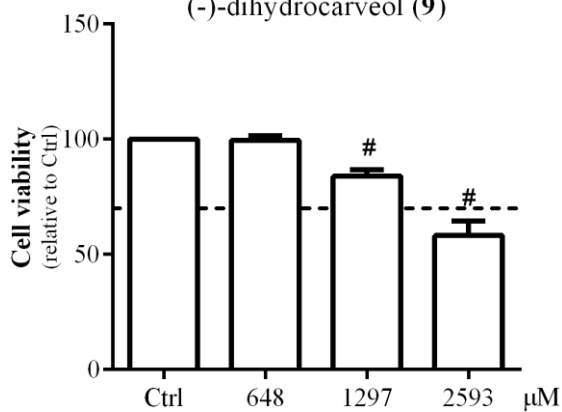
^eOrthopaedics Department and Bone Bank, University and Hospital Center of Coimbra, Coimbra, Portugal

^fChemical Process Engineering and Forest Products Research Centre, Chemical Engineering Department, Faculty of Sciences and Technology, University of Coimbra, Coimbra, Portugal

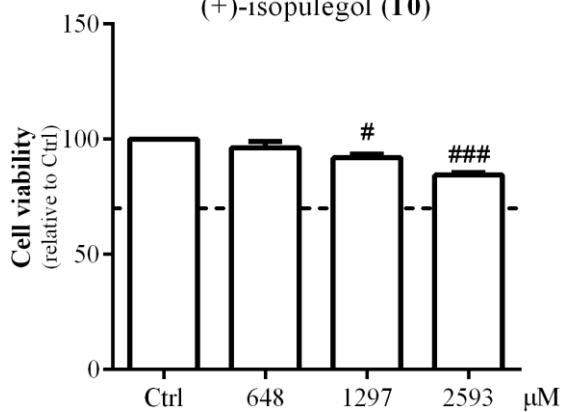
Figure S1



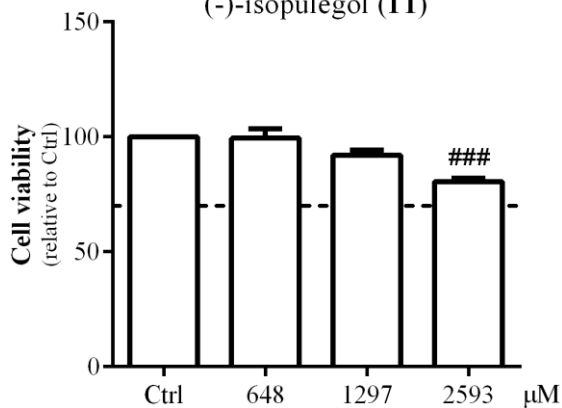
(-)-dihydrocarveol (9)



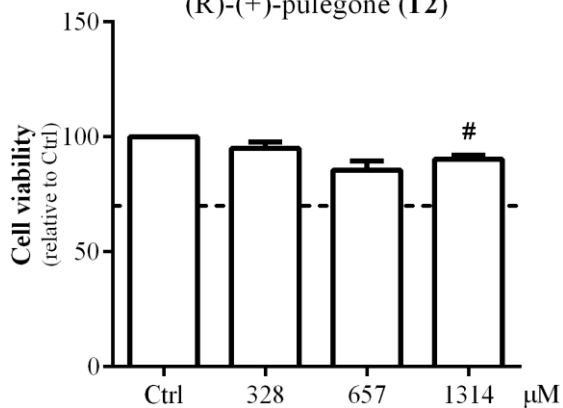
(+)-isopulegol (10)



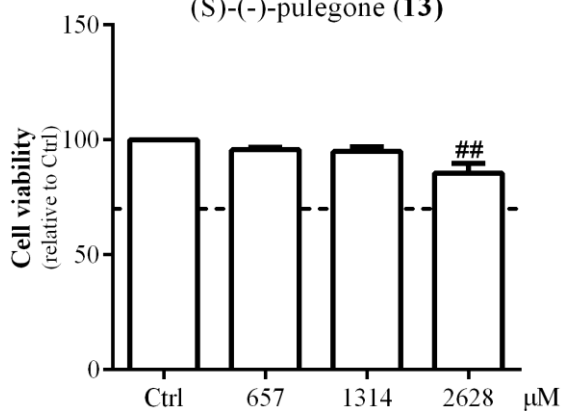
(-)-isopulegol (11)



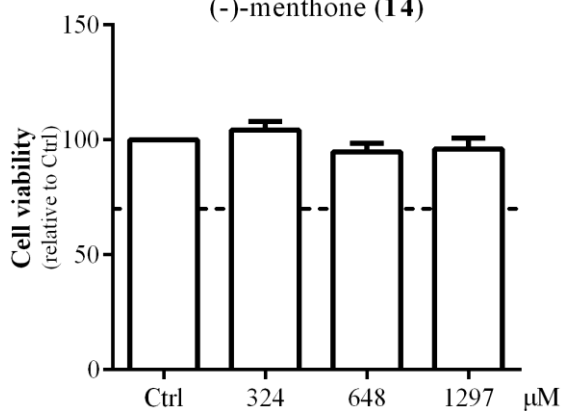
(R)-(+)-pulegone (12)



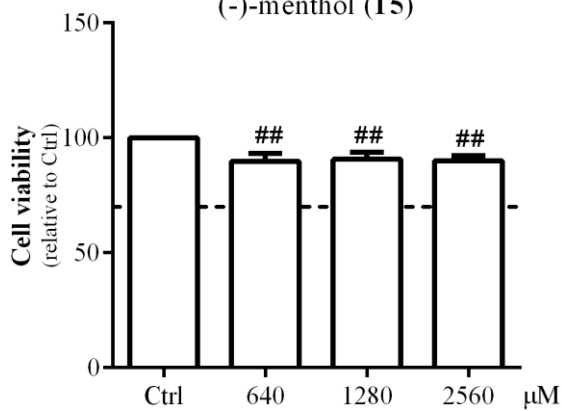
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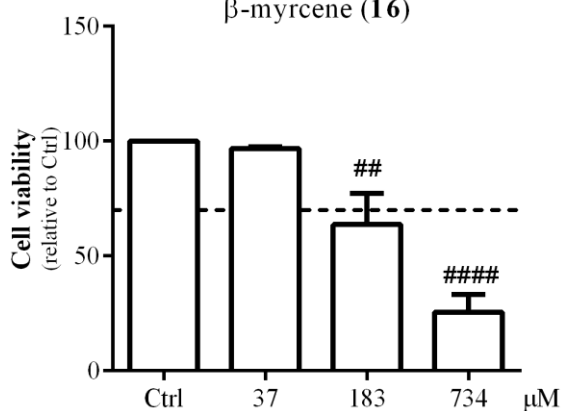
(-)-menthone (14)



(-)-menthol (15)



β-myrcene (16)



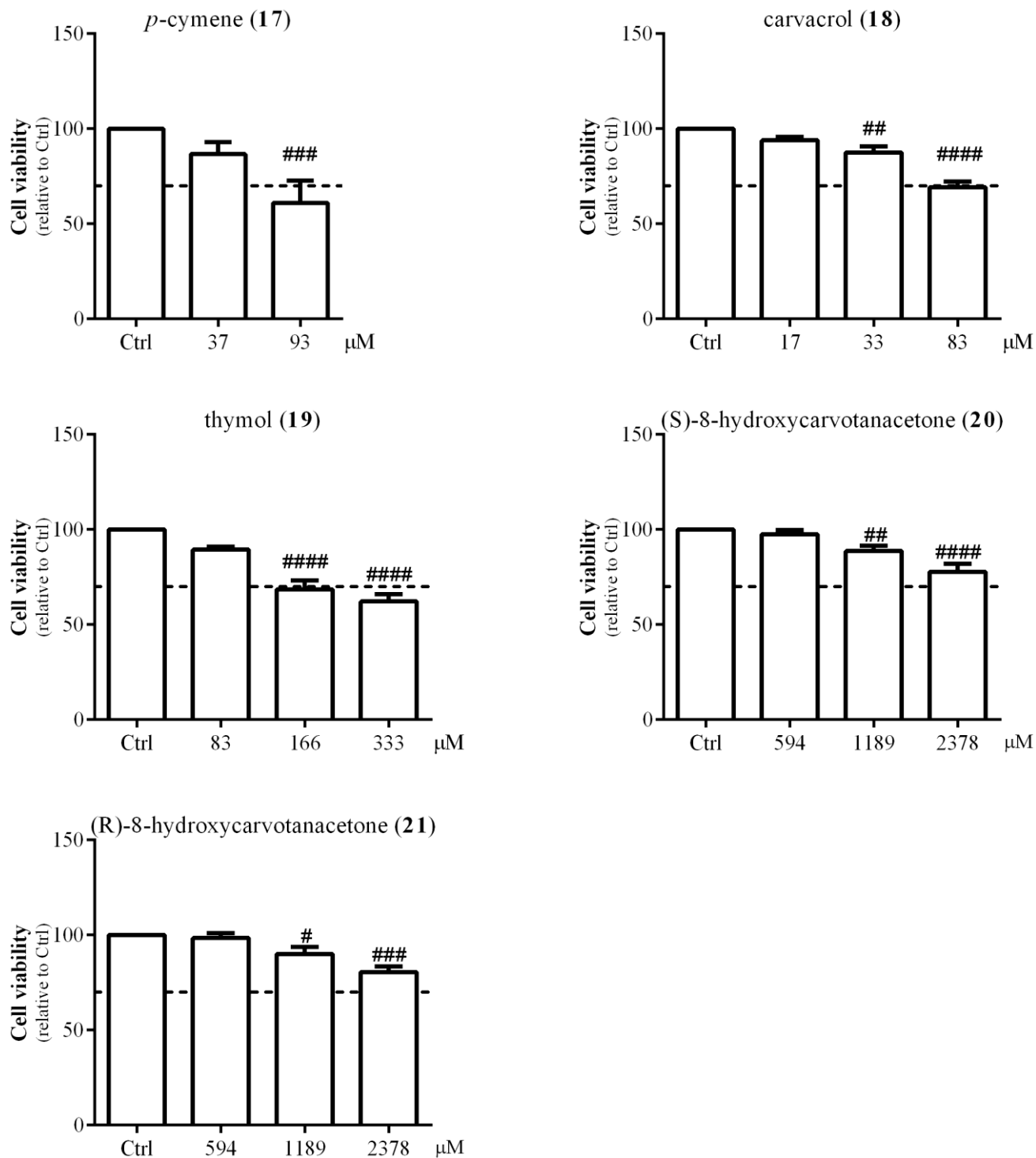
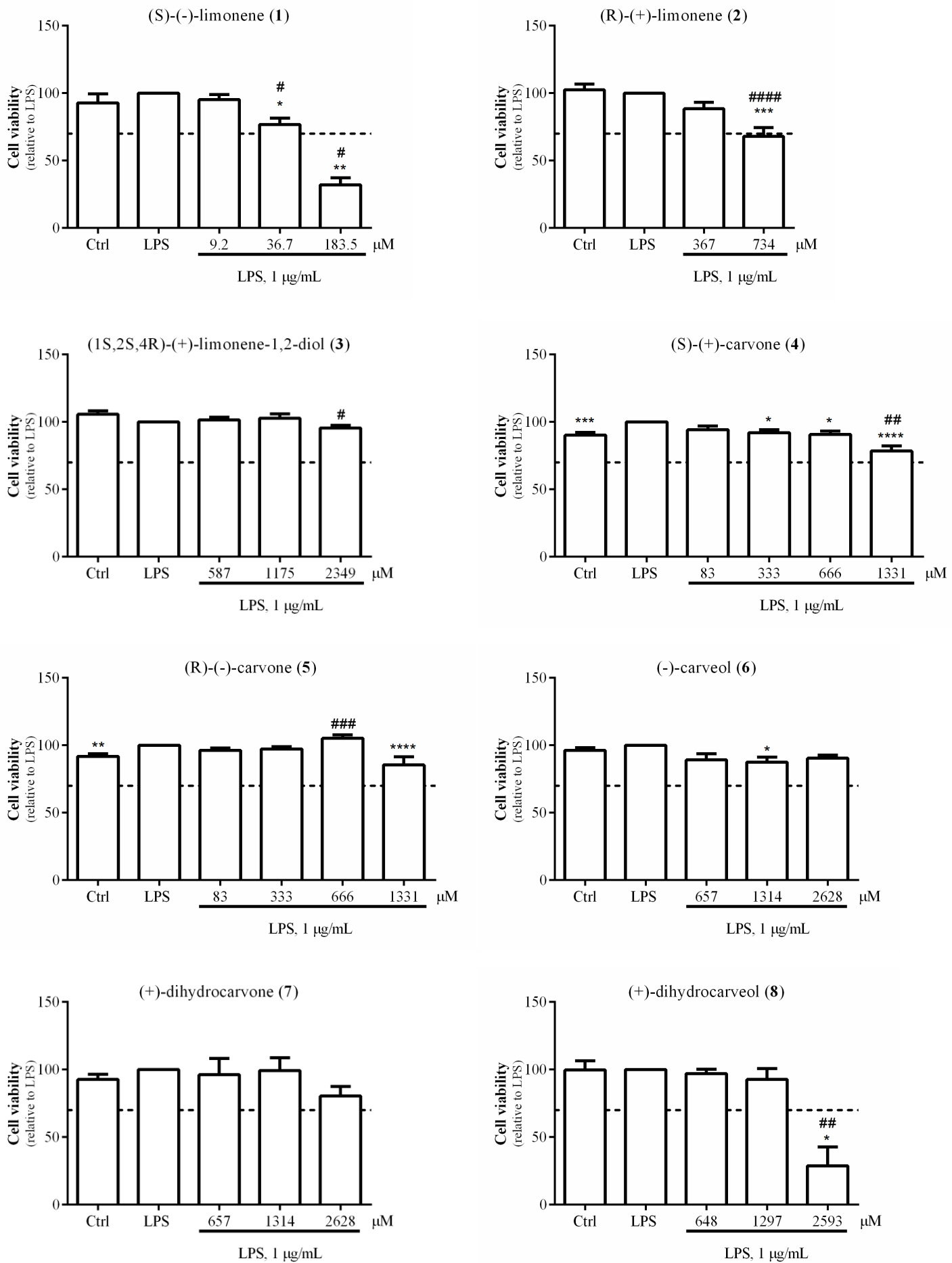
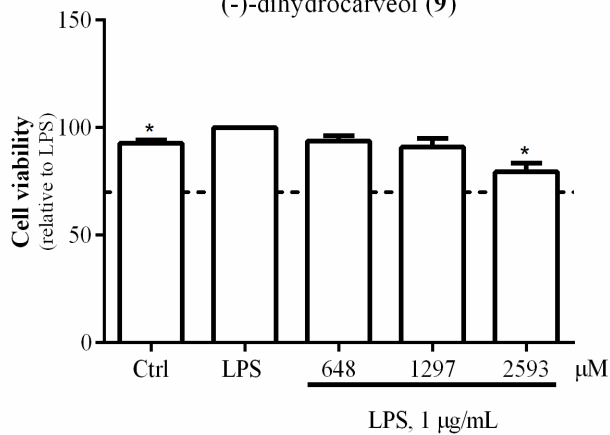


Figure S1. Evaluation of the cytotoxicity of the test compounds. Raw 264.7 cells were treated with the indicated concentrations of the each compound or with the vehicle (0.1% DMSO, control, Ctrl) for 18 h. Each column represents the mean \pm SEM of, at least, three independent experiments. # $p \leq 0.05$, ## $p \leq 0.01$, ### $p \leq 0.001$ and #### $p \leq 0.0001$ relative to Ctrl. The dotted line represents the threshold (70% of maximal viability) below which cytotoxicity is recognized, in agreement with standard ISO 10993-5.

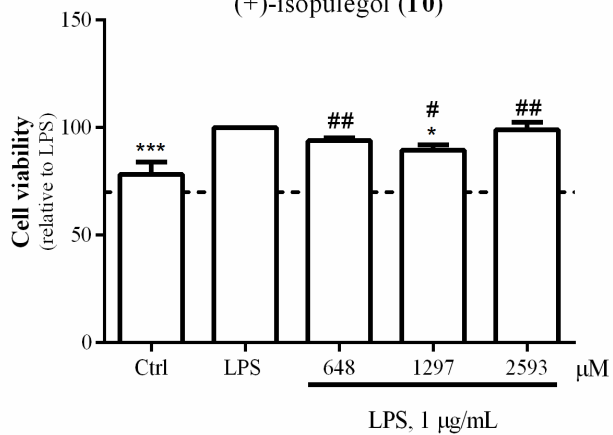
Figure S2



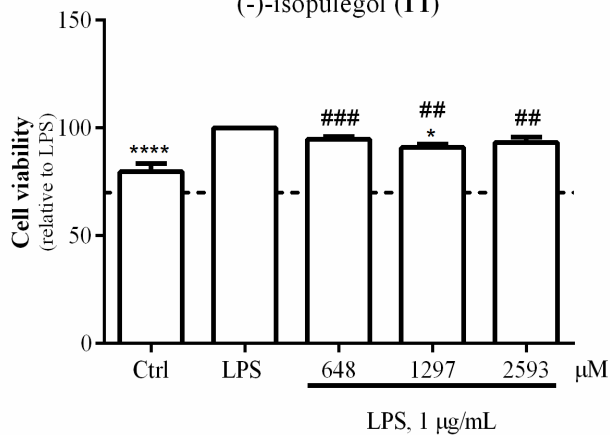
(-)-dihydrocarveol (9)



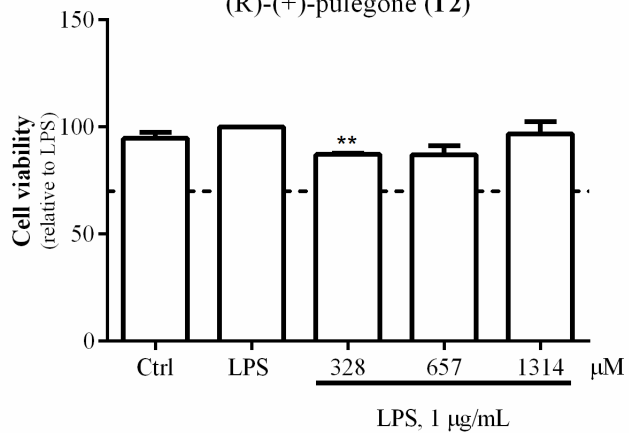
(+)-isopulegol (10)



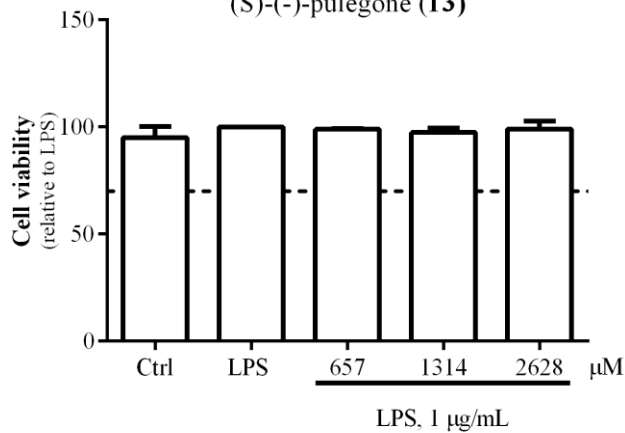
(-)-isopulegol (11)



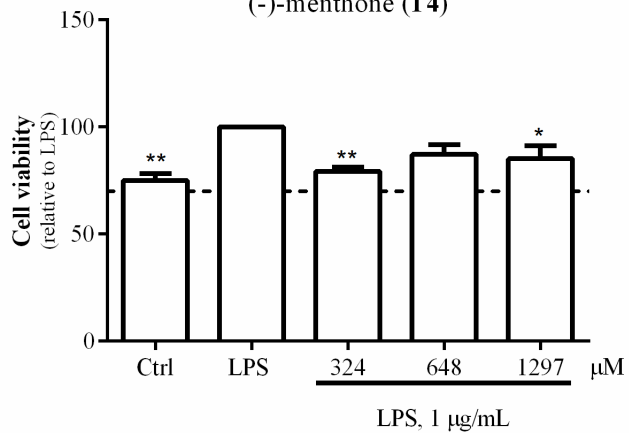
(R)-(+)-pulegone (12)



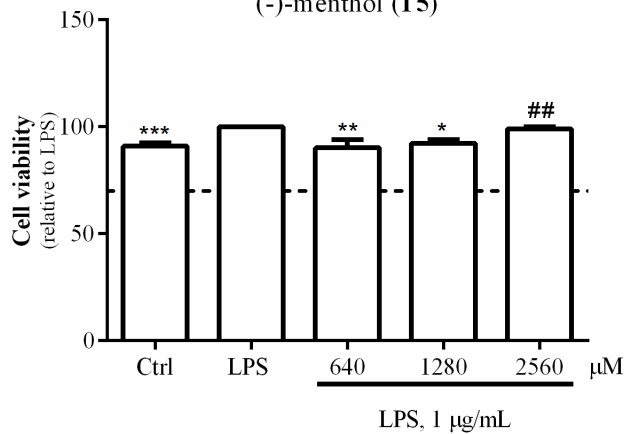
(S)-(-)-pulegone (13)



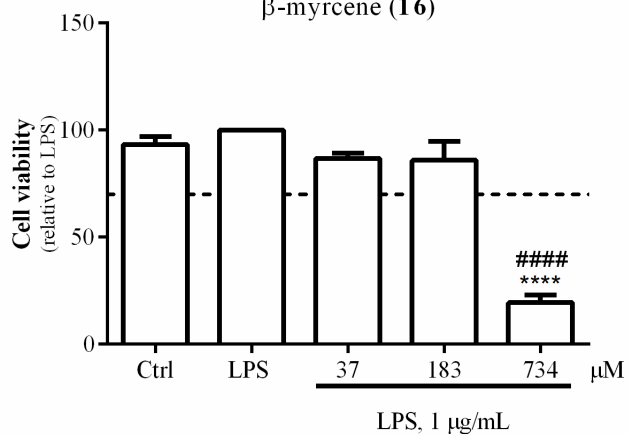
(-)-menthone (14)



(-)-menthol (15)



β-myrcene (16)



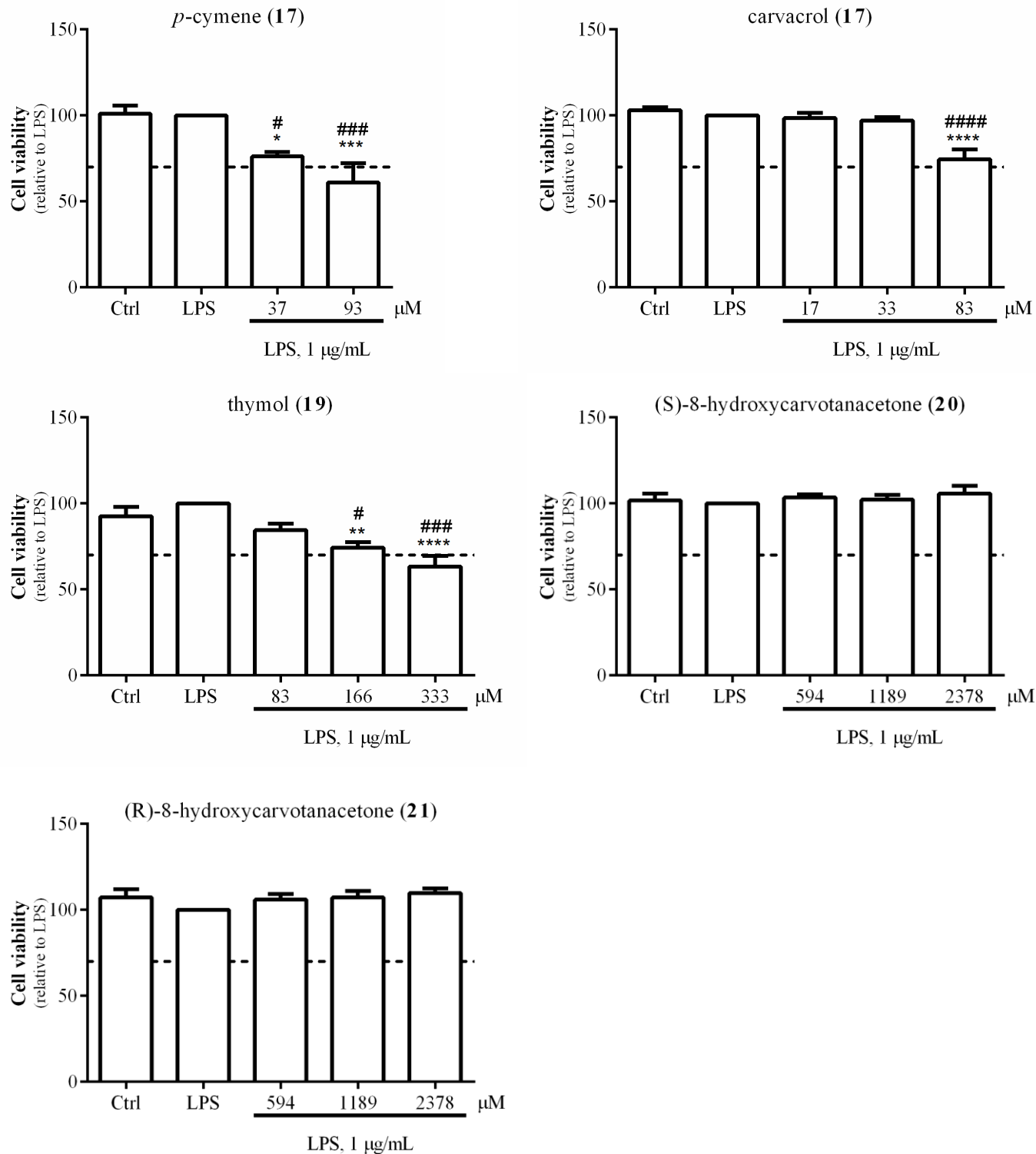
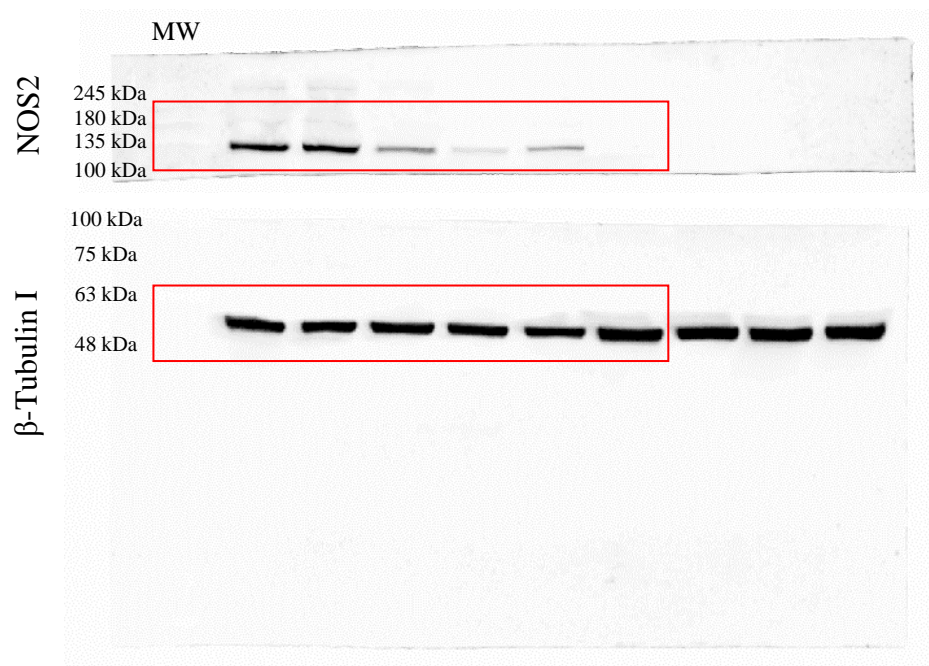
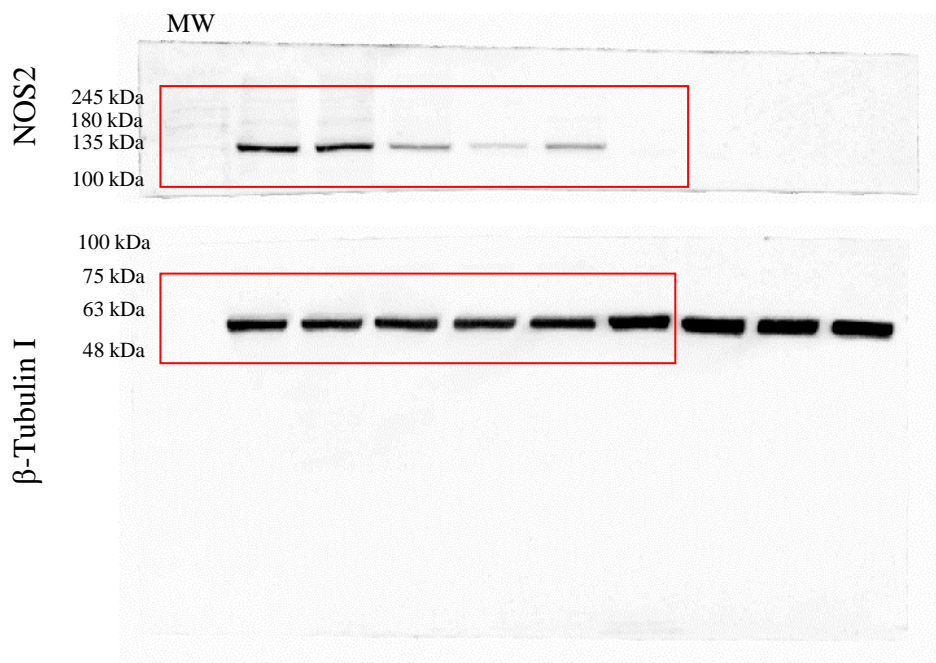


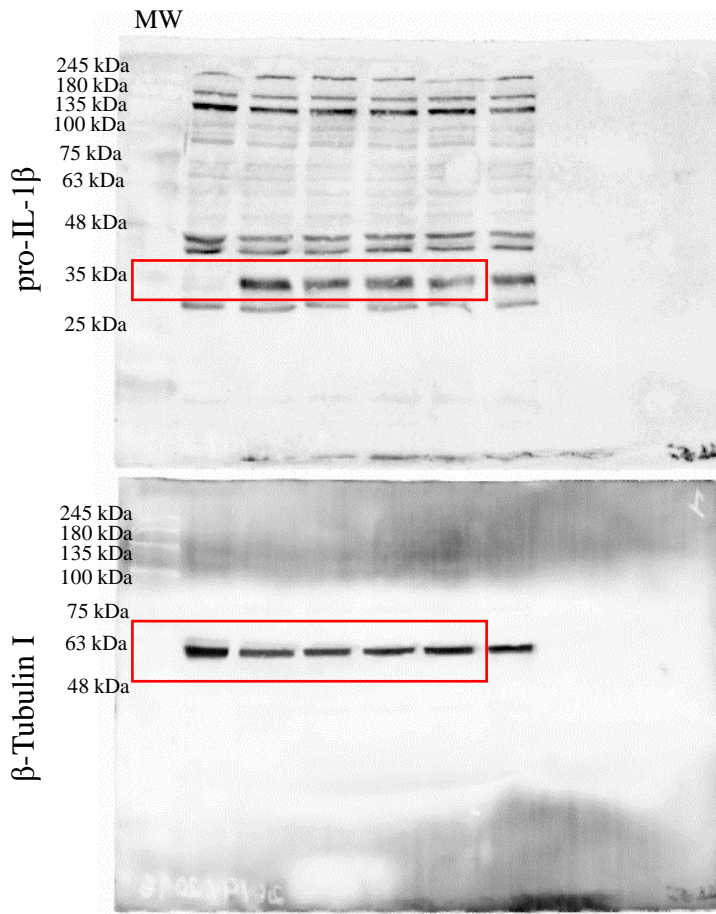
Figure S2. Evaluation of the cytotoxicity of the test compounds in the presence of bacterial lipopolysaccharide (LPS). Raw 264.7 cells were treated with LPS, 1 μg/mL, for 18 h, following pre-treatment for 1 h with the indicated concentrations of the test compounds or with vehicle (0.1% DMSO, Ctrl). Each column represents the mean ± SEM of ,at least, three independent experiments. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ and **** $p \leq 0.0001$ relative to LPS-treated cells. # $p \leq 0.05$, ## $p \leq 0.01$, ### $p \leq 0.001$ and #### $p \leq 0.0001$ relative to Ctrl. The dotted line represents the threshold (70% of maximal viability) below which cytotoxicity is recognized, in agreement with standard ISO 10993-5.

Uncropped blots shown in **Figure 2b****(S)-(+)-carvone (4)****(R)-(-)-carvone (5)**

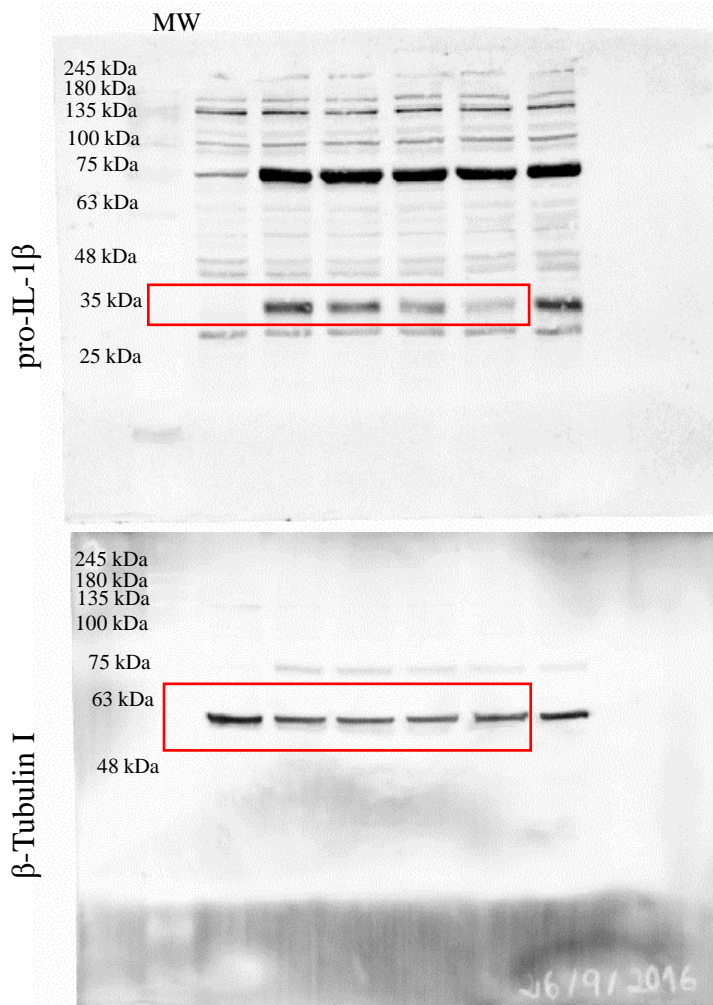
The membranes were cut at ≈ 100 kDa so that the upper piece was incubated with anti-NOS2 antibody and the lower one with the anti- β -Tubulin I antibody.

Uncropped blots shown in **Figure 3b**

(S)-(+)-carvone (4)

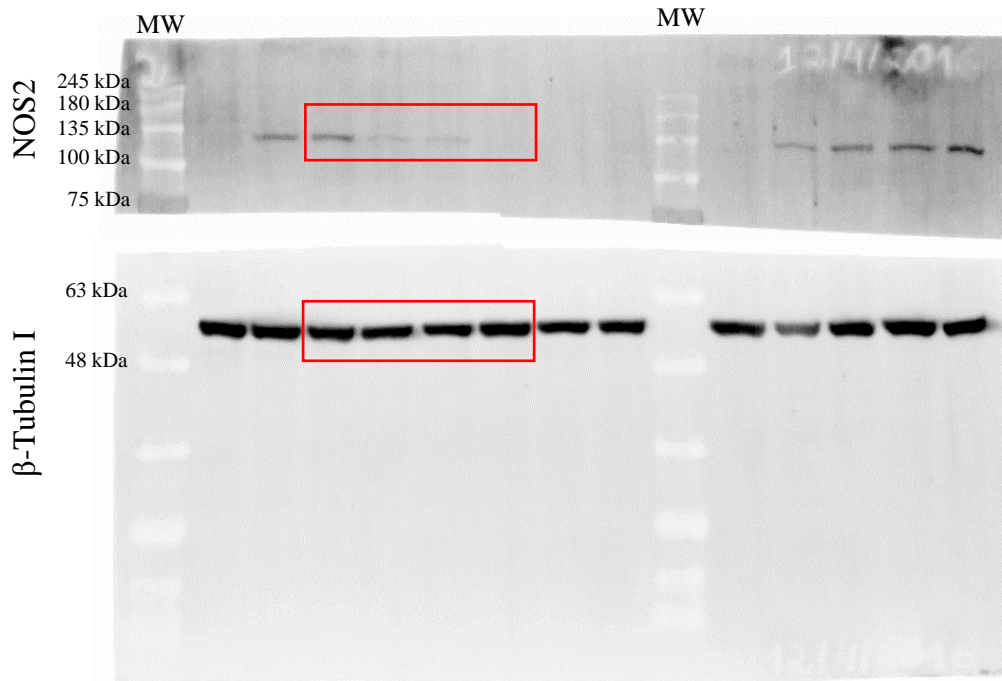


(R)-(-)-carvone (5)

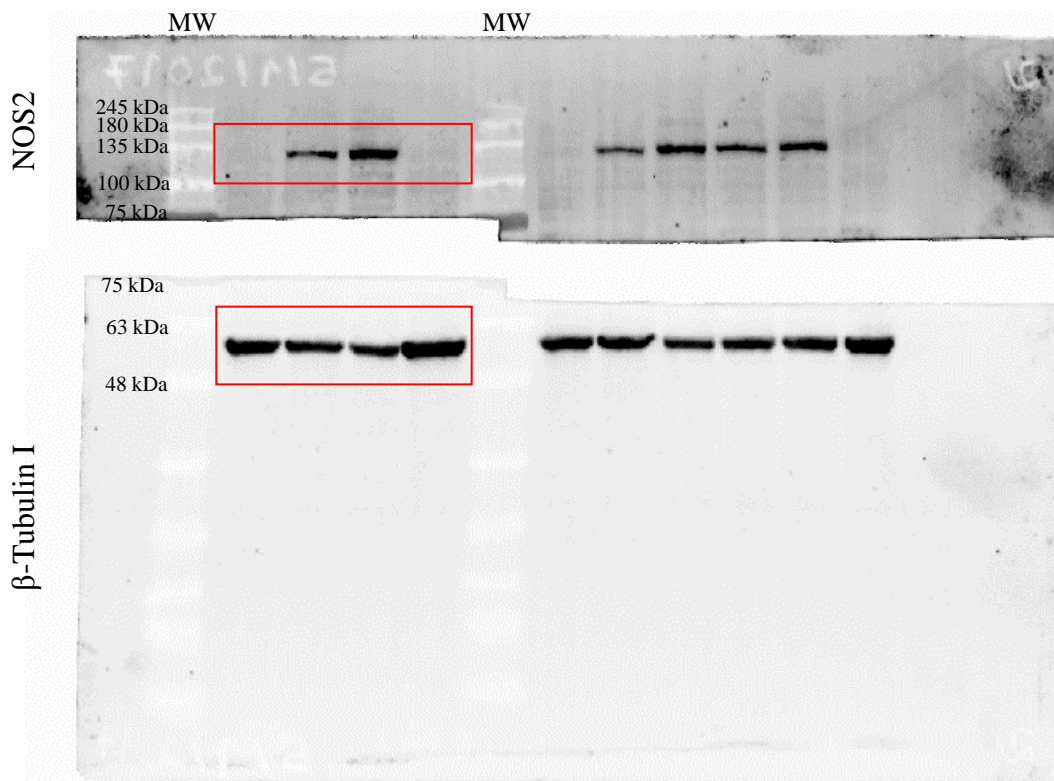


This membrane was also incubated with anti-COX2 antibody and the corresponding band appears near the 75 kDa molecular weight marker.

Uncropped blots shown in **Figure 4b**

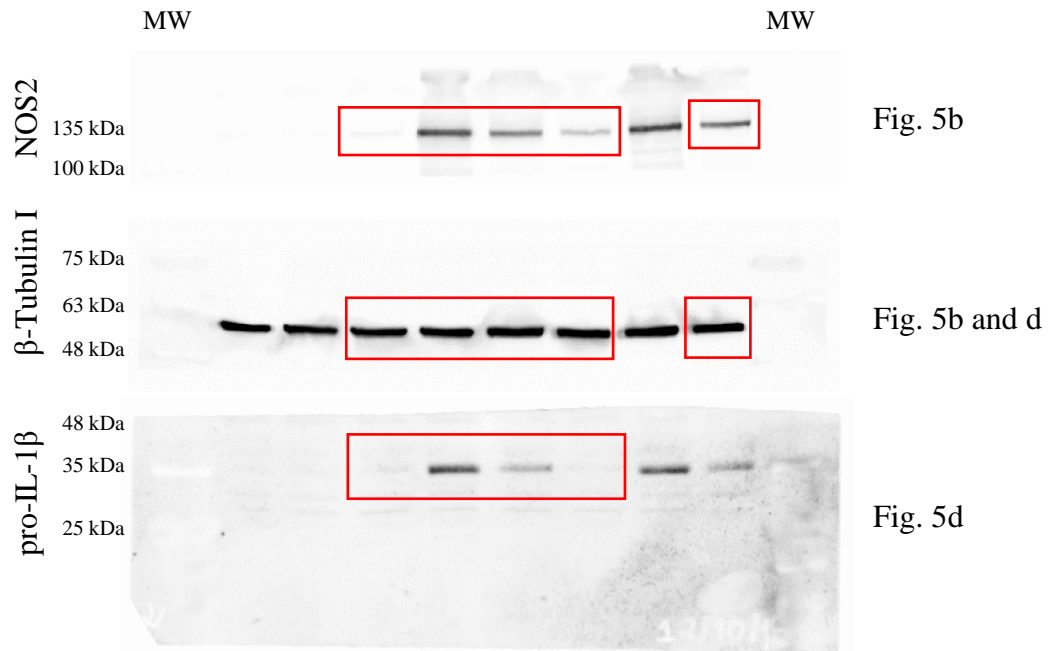


Uncropped blots shown in **Figure 4c**



The membranes were cut at ≈ 75 kDa so that the upper piece was incubated with anti-NOS2 antibody and the lower one with the anti- β -Tubulin I antibody.

Uncropped blots shown in **Figures 5b and 5d**



The membrane was cut between 100-75 kDa and 48 kDa so that the upper piece was incubated with anti-NOS2 antibody, the middle piece with anti- β -Tubulin I antibody and the lower one with the anti-IL-1 β antibody.

Figure S3: Uncropped images of the blots shown in figures 2-5.

Table S1. Purity and isomer composition of the test compounds

N°	Trivial name	IUPAC name of major isomer	Purity (%)*	Isomer composition	Catalog #, Vendor
1	(S)-(-)-limonene	(4S)-1-methyl-4-prop-1-en-2-ylcyclohexene	≥ 95%**	Purum	#62130, Sigma-Aldrich Co.
2	(R)-(+)-limonene	(4R)-1-methyl-4-prop-1-en-2-yl-cyclohexene	≥ 99%	Purum	#62118, Sigma-Aldrich Co.
3	(1S,2S,4R)-(+)-limonene-1,2-diol	(1S,2S,4R)-1-methyl-4-prop-1-en-2-ylcyclohexane-1,2-diol	≥ 97%	Purum	#669768, Sigma-Aldrich Co.
4	(S)-(+)-carvone	(5S)-2-methyl-4-prop-1-en-2-ylcyclohex-2-en-1-one	≥ 96%**	Purum	#435759, Sigma-Aldrich Co.
5	(R)-(-)-carvone	(5R)-2-methyl-4-prop-1-en-2-ylcyclohex-2-en-1-one	98%	Purum	#124931, Sigma-Aldrich Co.
6	(-)-carveol	(1R,5R)-2-methyl-5-prop-1-en-2-ylcyclohex-2-en-1-ol (1S,5R)-2-methyl-5-prop-1-en-2-ylcyclohex-2-en-1-ol	≥ 98%	Mixture of isomers	#61370, Sigma-Aldrich Co.
7	(+)-dihydrocarvone	(2R,5R)-2-methyl-5-prop-1-en-2-ylcyclohexan-1-one (2S,5R)-2-methyl-5-prop-1-en-2-ylcyclohexan-1-one	≥ 98%	Mixture of isomers	#09164, Sigma-Aldrich Co.
8	(+)-dihydrocarveol	(1S,2S,5S)- 2-methyl-5-prop-1-en-2-ylcyclohexan-1-ol	≥ 95%	Mixture of isomers – composition: n, ~ 75% iso, ~ 6% neo, ~ 3% neoiso, ~ 1.3%	#37277, Sigma-Aldrich Co.
9	(-)-dihydrocarveol	(1R,2R,5R)- 2-methyl-5-prop-1-en-2-ylcyclohexan-1-ol	≥ 95%	Mixture of isomers – composition: n, ~ 75% iso, ~ 6% neo, ~ 3% neoiso, ~ 1.3%	#37278, Sigma-Aldrich Co.

10	(+)-isopulegol	(1S,2R,5S)-5-methyl-2-prop-1-en-2-ylcyclohexan-1-ol	≥ 99%	Purum	#59765, Sigma-Aldrich Co.
11	(-)-isopulegol	(1R,2S,5R)-5-methyl-2-prop-1-en-2-ylcyclohexan-1-ol	≥ 99%	Purum	#59770, Sigma-Aldrich Co.
12	(R)-(+)-pulegone	(5R)-5-methyl-2-propan-2-ylidienecyclohexan-1-one	≥ 98.5%	Purum	#82569, Sigma-Aldrich Co.
13	(S)-(-)-pulegone	(5S)-5-methyl-2-propan-2-ylidienecyclohexan-1-one	98%	Purum	#328847, Sigma-Aldrich Co.
14	(-)-menthone	(2S,5R)-5-methyl-2-propan-2-ylcyclohexan-1-one	≥ 99%	Purum	#63677, Sigma-Aldrich Co.
15	(-)-menthol	(1R,2S,5R)-5-methyl-2-propan-2-ylcyclohexan-1-ol	≥ 99%	Purum	#63660, Sigma-Aldrich Co.
16	β-myrcene	7-methyl-3-methylideneocta-1,6-diene	≥ 90%	Purum	#64643, Sigma-Aldrich Co.
17	<i>p</i> -cymene	1-methyl-4-propan-2ylbenzene	≥ 99.5%	Purum	#30039, Sigma-Aldrich Co.
18	carvacrol	2-methyl-5-propan-2ylphenol	≥ 97%	Purum	#22051, Sigma-Aldrich Co.
19	thymol	5-methyl-2-propan-2ylphenol	98%	Purum	#30433, BDH

*Purity relative to sum of enantiomers determined by gas chromatography

The purities of **(1) (96.9%) and **(4)** (96.8%), which were in stock at our lab for over a year, were confirmed by GC-MS prior to starting the experiments and found to be within the limits defined by the manufacturer.