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Anti-Inflammatory and Analgesic Properties of Pulegone, a Major Component in *Calamintha nepeta*

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ABSTRACT

Monoterpenes are small molecules, composed of two isoprene units, able to pass through the blood brain barrier, allowing to target both peripheral and central pain pathways. They are the main components of essential oils, responsible for their diverse well-known biological activities. Menthol, the main monoterpene found in *Mentha piperita* (L.) is known to modulate nociceptive threshold and is present in different curative preparations that reduces sensory hypersensitivities in pain conditions. While pulegone is a menthol-like monoterpene, only a limited number of studies focuses on its putative analgesic effects. Pulegone is the most abundant monoterpene presents in *Calamintha nepeta* (L.), a Laminaceae plant used in traditional medicine to alleviate rheumatic disorders, a chronic inflammatory disease. Here, we compared the impacts of menthol and pulegone on pain and inflammation. First, we described that both monoterpenes are anti-inflammatory compounds. Secondly, we found that while menthol is highly cytotoxic at anti-inflammatory concentrations, the cytotoxic effects of pulegone are limited, if not absent. Finally, in a model of peripheral inflammatory-induced pain a pulegone treatment exerts a significantly higher anti-hyperalgesic effect than menthol in response to mechanical stimuli, heat and cold thermal stimulations than a menthol treatment. In conclusion we demonstrated that pulegone is an anti-inflammatory compound and it is acting a potent pain-killer in acute inflammatory pain condition.

INTRODUCTION

Due to the mighty side effects of available clinical drugs alike opioids prescribed for the treatment of chronic pain[1], the search for bioactive natural compounds from plants as an alternative treatment has skyrocketed.

Herbal medicine has been used for thousands of years and today the demand for treatments of various diseases based on plants and natural molecules is highly increasing[2]. Medicinal plants, among which the Lamiaceae family, including *Mentha piperita* (L.) and *Calamintha nepetha* (L.) savi, have been used in traditional medicine since the ancient Greeks and the Renaissance[3]. This plant was used to heal wounds and bruises and to cure jaundice and snakebites[4]. Lately, in the 20th century, a calamint decoction was recommended to break fevers[5].

The main bioactive compounds in Laminaceae plants are secondary metabolites including terpenoids, alkaloids and phenolic compounds[6]. With about 70,000 structures described today[7], terpenoids are considered the largest family of natural compounds[8]. Those natural compounds are classified by the number of isoprene units contained in their chemical structure. Monoterpenes, composed of two isoprene units, are the main components of essential oils and are responsible for their diverse well-known biological activities[9]. Less than 50 monoterpenes have been described as potential analgesic[10] and/or anti-inflammatory molecules[11]. In general, monoterpenes are derived from geranyl diphosphate (GPP) which is a product of the head-to-tail coupling of the primary metabolites isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP)[12]. Pulegone, a major monoterpene found in essential oil of *Calamintha nepetha* (L.) savi[13], is obtained from GPP through limonene and piperitone, isopulegone is its direct precursor[14]. Menthol, the major monoterpene found in *Mentha piperita* (L.) [15] is involved in the same biosynthetic pathway than pulegone, which is a precursor in the biosynthesis of menthol through menthone[13].

Interestingly, a recent review of Quintans et al. (2019)[16] lists several terpenes, among which menthol and pulegone, that modulate the activity of cytokines, including the tumor necrosis factor (TNF-α), a cytokine involved in the primary onset of inflammatory responses maintenance and finally chronicity[17]. Despite this potent anti-inflammatory action, only a few studies explore the potential analgesic actions of terpenes. Among them, menthol seems to have potent *in vivo* pain-killer effect on visceral[18], inflammatory[19] as well as neuropathic pain[20]. Despite the similarities between menthol and pulegone, the analgesic potential of pulegone was only studied twice in visceral pain models[21,22]. Despite these observations, little is known regarding the putative effect of a curative pulegone treatment on mechanical, heat and cold sensitivities in inflammatory pain condition.

Therefore, we hypothesized that pulegone might have potent analgesic action on peripheral inflammatory pain. Therefore, in the present study we characterized the *in vitro* anti-inflammatory effect and cytotoxicity, as well as the putative analgesic effects of both menthol and pulegone in an *in vivo* inflammatory pain models.

RESULTS

Mentha piperita (L.) and *Calamintha nepeta* (L.) extracts analysis.

Mentha piperita (L.) and *Calamintha nepeta* (L.) savi extracts were obtained by pressurized liquid extraction (PLE) and analyzed by gas chromatography-mass spectrometry (GC-MS) (Fig. 1a; S1). Overall, 11 volatile compounds were identified in the *Mentha piperita* (L.) extract (Fig. 1b) and 10 in the *Calamintha nepeta* (L.) savi PLE extract. As expected, the main constituent of the *Calamintha nepeta* (L.) savi extract was pulegone (49.41 %) while the one of the *Mentha pieperita* (L.) extract was menthol (42.85 %). Isopulegone is obtained in the *Calamintha nepeta* (L.) savi extract (Fig. 1b), meaning that the isomerase conversion was not complete. In the same extract, menthol, isomenthone and menthone can be found at lower concentrations than pulegone. Given the absence of pulegone detection in *Mentha piperita* (L.) extracts, its analgesic effects in traditional medicine cannot be attributed to pulegone. In the other hand, it raises the question of pulegone putative analgesic action, as it is the principal monoterpene found in *Calamintha nepeta* (L.) savi extracts[3].

Considering these results, we then restricted our investigation to the potential anti-inflammatory and analgesic properties of these two major compounds, pulegone and menthol.

Menthol and pulegone display *in vitro* anti-inflammatory effects.

The *in vitro* anti-inflammatory activity of menthol and pulegone were evaluated by measuring the secretion of the tumor necrosis factor (TNF-α) from THP-1 cells after 4 h incubation with either menthol or pulegone (Fig. 2a-c; S2). We used LPS to induce production of TNF-α, an effect that is blocked by celastrol (500 mM), a triterpene used as positive control of the inhibition of the secretion of TNF-α[23]; Fig. 2a; ethanol 1%: 78.3±0.7 % vs. celastrol 500 mM: 1.1 ± 0.1 %, n = 3, $p < 0.001$). Interestingly, we observed a similar reduction of the secretion of TNF-α secretion after incubation with menthol 3 mM (reduced to 1.2 ± 0.05 % n = 3, $p < 0.001$) or pulegone 3mM (reduced to 12.5 ± 2.2 % n = 3, $p < 0.001$) (Fig. 2a). We then evaluated the *in vitro* cytotoxicity of those different compounds (Fig. 2d-f). Interestingly, pulegone 3 mM present a low cytotoxicity, comparable to the one of celastrol 500 mM, our positive control (5.6 ± 0.2 % n=3, and 1.4 ± 0.2 % n=3, respectively; Fig. 2d). However, menthol 3 mM displayed a significant cytotoxicity (32.5 ± 11.1 %, n=3) compared to vehicle (1 % ethanol; 8.4 ± 0.3 %, n=3, $p = 0.038$; Fig. 2d). Therefore, pulegone showed a promising anti-inflammatory activity conjugated with a low cytotoxicity.

To refine this gross characterization, we performed dose response curves (0.03 to 13 mM) of the anti-inflammatory activity (Fig. 2b) and the cytotoxicity (Fig. 2e) of menthol and pulegone. Interestingly, the anti-inflammatory activity curve of the two compounds presents a similar distribution and an EC_{50} value in the same range (menthol: 1.5 ± 0.1 mM, n=3; pulegone: 1.2 ± 0.2 mM, n= 3; Fig. 2c), suggesting equivalent anti-inflammatory activities of both menthol and pulegone. However, the cytotoxicity curves were significantly different, showing a 100 % cells death rate with a concentration of 12.8 mM of menthol (Fig. 2e), while similar pulegone concentration induced only 45.1 % cells death rate. In adequation with this observation, the EC_{50} of the cytotoxicity was 3.5 ± 0.2 mM for menthol and 6.6 ± 0.3 mM for pulegone ($p < 0.001$; Fig. 2f). Taken together, those results indicate that THP-1 cells, which are among the

first cells involved in the inflammation process, can withstand a higher concentration of pulegone than menthol.

To further characterize the effect on menthol and pulegone, we then investigated their potential pain-killer effects *in vivo* on nociceptive thresholds.

Menthol and pulegone induce *in vivo* anti-hyperalgesia.

We first performed a dose-response curve of the putative analgesic effects of menthol and pulegone, measured on mechanical, thermal heat and thermal cold sensitivities, 40 min after a single intra-peritoneal (i.p) injection that occured 24 h after intra-plantar injection of complete Freund adjuvant[24] (CFA; Fig. 3;). CFA-model, like others inflammatory-induced pain hypersensitivity models, induces mechanical, thermal heat and cold hyperalgesia, with a plateau in hyperalgesia observed between 24 h and 48 h following the injection[24].

First, both menthol and pulegone induced dose-dependent increase of the mechanical threshold (Fig. 3a, b), up to a plateau value obtained after 50 mg/kg for menthol (84.83 ± 17.87 ∆g, n = 6; p = 0.0162) and 100 mg/kg for pulegone (150.2 ± 67.79 ∆g, n = 8; p = 0.0007). Interestingly, while anti-hyperalgesic effects of menthol start at lower concentration than the one of pulegone (50 mg/kg vs 100 mg/kg), the plateau effect induced by pulegone was slightly higher than the one induced by menthol. Similarly, both menthol and pulegone induced dose-dependent increase of thermal heat threshold latency (Fig. 3c, d), up to a plateau value obtained after 100 mg/kg (menthol: 1.713 ± 0.951 ∆s, n = 8; p = 0.0016; pulegone 3.688 ± 0.852 ∆s, n = 8; p = 0.0005). Again, the anti-hyperalgesic effect of pulegone was significantly higher than the one induced by menthol (p = 0.0019). Finally, pulegone but not menthol induced a significant decrease in thermal cold acetone score (Fig. 3e, f) following 100 mg/kg injection (menthol: -1.25 ± 2.19 ∆score, n = 8; p = 0.0546; pulegone -1,63 ± 1.6 ∆score, n = 8; p = 0.0256). Finally, the anti-hyperalgesic effects of both terpenes were compared through their respective dose-response distribution, and showed that pulegone induced a significantly higher anti-hyperalgesic effect than menthol on all tested modalities (p < 0.001; Fig. 3b, d, f). Interestingly, none of those observations were reproduced on contra-lateral paw (i.e. paw without inflammatory sensitization), suggesting an absence of potential deleterious anti-nociceptive effect (Fig. S3).

Along with this dose-response, that allowed us to determine the optimal dose of i.p. terpene injection in regards of their anti-hyperalgesia effect (100 mg/kg), we next aimed to evaluate its duration. We thus monitored the mechanical, thermal heat and thermal cold sensitivities every 20 min following i.p injection of either menthol or pulegone at 100 mg/kg, carboxymethyl cellulose (CMC, 1 %) in NaCl (0.9 %) as a control vehicle (Fig. 4; S4). The comparison of the AUC will be used to assess the global effect of each treatment.

Interestingly, pulegone induced an anti-hyperalgesic effect on all measured modalities as soon as 20 min after i.p. injection on mechanical threshold (pulegone: 274.25 ± 68.89 g, n = 8; vehicle: 160.88 ± 35.17 g, n = 8, p < 0.0001, Fig. 4a, b) ; as well on thermal heat threshold (pulegone: 4.09 ± 0.62 s, n = 8; vehicle: 2.25 ± 0.34 s, n = 8, p < 0.0001, Fig. 4c, d) ; and also on thermal cold threshold (pulegone: 2.25 ± 1.28 score, n = 8; vehicle: 4.75 ± 1.04 score, n = 8, p = 0.0003, Fig. 4e, f), while menthol effects only significantly start 40 min after i.p. injection (mechanical: menthol: 281.63 ± 45.52 g, n = 8; vehicle: 166.25 ± 35.4 g, n = 8, p < 0.0001, Fig.

4a; thermal heat: menthol: 3.65 ± 0.88 s, n = 8; vehicle: 2.19 ± 0.26 s, n = 8, < 0.0001, Fig. 4c). However, both terpenes effects fade 80 min after i.p. injection (mechanical: pulegone: 212 ± 42.46 g, n = 8; menthol: 209.38 ± 25.08 g, n = 8; vehicle: 179.92 ± 26.55 g, n = 8, p = 0.1979 (pulegone), p = 0.2579 (menthol), Fig. 4a; thermal heat: pulegone: 2.84 ± 0.64 s, n = 8; menthol: 2.76 ± 0.89 s, n = 8; vehicle: 2.19 ± 0.44 s, n = 8, p = 0.1155 (pulegone), p = 0.1951 (menthol), Fig. 4c; thermal cold: pulegone: 4.5 ± 1.6 score, n = 8; vehicle: 5.13 ± 0.64 score, n = 8, p = 0.6344, Fig. 4e). Of note, menthol only slightly alleviate thermal cold hyperalgesia at after i.p. injection (thermal cold: menthol: 91.39 ± 13.93 %, n = 8, p = 0.2696, Fig. 4e). Importantly, those effects were totally absent in non-hyperalgesic animals that received NaCl intraplantar injection (Fig. 4b, d, f) as well as in the CFA contra-lateral hindpaw (Fig. S4), illustrating an anti-hyperalgesic action of those terpenes in absence of anti-nociceptive action. In addition, it is interesting to note that the anti-hyperalgesic effects of pulegone seems to be higher than those of menthol, especially for thermal heat modality (Fig. 4d).

Altogether, these results indicate that a single i.p. injection of menthol or pulegone exerts a significant and middle-lasting anti-hyperalgesic action on both mechanical, thermal heat and thermal cold sensitivities in an inflammatory-induced pain hypersensitivity rat model.

Menthol and pulegone are devoid of *in vivo* locomotor side effects.

Given that monoterpenes might have strong unwilling side-effects when injected at high doses (pulegone > 200 mg/kg)[22,25], we performed a number of controls to assess that the previous experiments are free of motor reflex bias. To do so, we quantified hindpaw diameter as well as the motor activity of the rats after the i.p injection of either pulegone or menthol, at the highest dose of 150 mg/kg (Fig. 3), using two different experimental paradigms.

In few of monoterpenes known to have anti-inflammatory action[11,26] the hindpaw edema diameter due to CFA was not reduced by neither pulegone nor menthol i.p. injection (vehicle, 10.55 ± 0.86 mm, n = 8; menthol, 10.18 ± 0.66 mm, n = 8; pulegone, 10.29 ± 0.66 mm, n = 8; Fig. 5a). This suggests that acute terpenes injection, at this stage of the CFA-induced inflammation, may have no or limited anti-inflammatory effect but rather acts directly on nociceptive thresholds through a mechanism that remains to be explain.

To control that menthol or pulegone i.p. injection did not affect the locomotor ability of the rats, and consequently alters the assessment of the nociceptive thresholds during the experimentation, we monitored their locomotor functions after i.p injection using the beam walk assay (Fig. 5b) as well as the rotarod assay (Fig. 5c). In those two tests, both menthol and pulegone failed to impair the locomotor abilities of the animals.

DISCUSSION

In the present study, we confirmed that the monoterpenes menthol and pulegone are the major monoterpenes present in *Mentha piperita* (L.) savi and *Calamintha nepeta* (L.) savi extracts, respectively (Fig. 1). We found that menthol and pulegone share very similar *in vitro* anti-inflammatory activities, while pulegone seems to present only limited cytotoxic effects (Fig. 2). Finally, we revealed anti-hyperalgesic properties of both menthol and pulegone, with a significant advantage for pulegone (Fig. 3 and 4), without negative locomotor side effects (Fig. 5).

Our results are in agreement with previous investigations, confirming that menthol and pulegone are the main monoterpenes present in *Mentha piperita* (L.) and *Calamintha nepeta* (L.) savi extracts, respectively[13,15].

An interesting result obtain here shed light on the high *in vitro* cytotoxicity effect of menthol, at doses known to display anti-inflammatory properties. Surprisingly, pulegone seems to only have a limited cytotoxicity action, allowing us to consider pulegone as a more potent anti-inflammatory (and pain-killer) candidate. However, limited hypothesis explains the difference of cytotoxicity between those two compounds. Indeed, both pulegone and menthol share common molecular targets, such as the transient receptor potential (TRP) melastatin 8 (TRPM8) and TRP ankyrin 1 (TRPA1) channels, all expressed by neurons and immune cells[27]. It has been reported a bidirectional action where the activation of immune cells TRPM8 leads to the inhibition of TNF-α release, while activation of TPH-1 macrophages TRPA1 leads to the release of pro-inflammatory cytokines[27]. Therefore, one can hypothesize that pulegone might have a lower affinity for the TRPA1, focusing its effect on TRPM8 and the subsequent an inhibition of the TNF-α release without the cytotoxicity consequences of the activation of TRPA1.

Investigating the analgesic properties of menthol and pulegone in an *in vivo* on CFA-induced inflammatory hypersalgesia, we found that both menthol and pulegone were able to alleviate the mechanical and thermal heat hyperalgesia. However, only pulegone was able to alleviate thermal cold hyperalgesia. In addition, we show that, at similar concentration, pulegone have a higher anti-hyperalgesic potency than menthol, both in intensity and duration (Fig. 3 and 4). While the underlying mechanisms are unknown, we propose a framework of hypothesis that remain to be explored. After peripheral inflammation, central sensitization leads to molecular and cellular changes in the central nervous systems where affinity and expression of channels and receptors like the gamma-aminobutyric acid receptor type A (GABA-A) increase and directly contribute to pain hypersensibility[28]. Thanks to their small molecular size and lipophilic properties, monoterpenes can cross the blood brain (BBB)[29,30]. Interestingly, has been shown that both menthol and pulegone can potentialize GABA-A receptors currents responsible of the inhibition of neuronal activity[31,32,33] and in this case contribute to analgesia.

Another possible pathway for menthol and pulegone to act as pain-killer might be to directly modulate the electrical activity of nociceptors though the desensitization of their receptors targets involved in the maintenance maintains of hypersensitivities. Indeed, TRPM8 and TRPA1 are particularly involved in detection and transduction of the cold temperature and noxious stimulations into neuronal activity[17,34]. TRPA1, a known target of menthol and pulegone, is a cation channel contributing to transduce noxious temperatures above 42 °C[35]

and involved in the propagation of nociceptive mechanical stimulation after inflammation[35]. In addition, it has been demonstrated that TRPA1 upregulation contributes to peripheral sensitization during inflammation and is responsible for both mechanical pain and thermal hypersensitivity[36]. Furthermore, high concentration of agonists, such as monoterpenes, in the vicinity of sensory neurons can induce desensitization of both TRPM8[37,38] and TRPA1[39,40]. Therefore, one can hypothesize that monoterpene will induce a desensitization of TRPM8 and TRPA1, leading to a decrease of mechanical and cold thresholds, eventually leading to analgesia. Comparable mechanism exists for the TRP vanilloid 1 (TRPV1), a channel involved in the detection of nociceptive hot temperatures and activated by the red chili pepper active compound, capsaicin[17]: plantar capsaicin injection induces in naïve animals nocifensives reactions and pain hypersensitivities[41,42], while long exposure of capsaicin induces a long-term desensitization of the TRPV1 contributing to capsaicin-induced analgesia.

If these concepts are true and transposable to human and due to their capability to cross the BBB and interact with many different molecular targets during inflammation situations with no or limited locomotor side effect, monoterpenes might be key candidates for clinical use. This might lead to the development of drugs easy to take every few hours to limit pain symptoms.

In conclusion we showed that pulegone has a significantly higher effect than the menthol on nociceptive sensory sensitivities in an inflammatory-induced pain hypersensitivity rat model. It opens the door to consider using pulegone in the composition of anti-inflammatory and analgesic medication to alleviate mechanical and thermal hypersensitivities in inflammatory pain disease. Finally, given that pulegone is an approved molecule by the US Food and Drug Administration (FDA) for food use and was included by the Council of Europe in 1975 on the list of artificial flavoring substances, pulegone-based treatment might represent an interesting route for further clinical investigations.

FIGURES LEGENDS

Figure 1: (**a**) Chromatograms of *Calamintha nepeta* (L.) savi and *Menta piperita* (L.) PLE extracts 10-fold diluted were analyzed by GC-MS. On both chromatograms, the main components of each extract are identified: pulegone (T_R = 19.04 min) for *Calamintha nepeta* (L.) savi and menthol (T_R = 18.75 min) for *Mentha piperita* (L.). (**b**) Extract composition (in %) together with retention index (RI) of each found compound.

Figure 2: (**a**) Anti-inflammatory activities and (**d**) cytotoxicities of *Calamintha nepeta* (L.) savi and *Mentha piperita* (L.) extracts diluted by 5 and their main constituents pulegone and menthol at 3 mM in 1 % ethanol after 4 hours LPS-stimulation of THP-1 cells. Experiments (n = 3) were conducted on the same 96-wells plate to compare to the same negative control (1 % ethanol) (*** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$). Celastrol at 500 mM was used as the positive control. (**b**, **e**) Dose-response curves of (**b**) anti-inflammatory activity and (**e**) cytotoxicity of pulegone and menthol after 4 hours LPS-stimulation of THP-1 cells. Concentration range: 0.03 to 13 mM. Each concentration was tested in triplicates on the same 96-wells plate. (**c**, **f**) EC_{50} values calculated by the dose-response curves of (**c**) the anti-inflammatory activity and (**f**) cytotoxicity of menthol and pulegone. Ratios of the EC_{50} value of the anti-inflammatory activity over the EC_{50} value of the cytotoxicity for menthol (in blue) and pulegone (in green) (*** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$).

Figure 3. Dose-response of the analgesic properties of menthol and pulegone on CFA-induced inflammatory pain model. Effect of menthol 10 (n = 6), 50 (n = 6), 100 (n = 8) and 150 mg/kg (n = 7) and pulegone 10 (n = 6), 50 (n = 6), 100 (n = 8) and 150 mg/kg (n = 8) or their vehicle (n = 8) measured 40 min after i.p. injection on mechanical (**a**), thermal heat (**c**) and thermal cold (**e**) CFA-induced hyperalgesia. Dose-response distribution fits for menthol and pulegone on mechanical (**b**), thermal heat (**d**) and thermal cold (**f**). Data are expressed as mean ± SD. Asterisks indicate statistical significance (*** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$) using Kurskal-Wallis test followed by Dunn's multiple comparisons post-hoc test to compare each delta with the delta of the vehicle, to compare each treatment depending on the time post i.p. (0 vs 40 min) it is a paired t test or a Wilcoxon test, depending on the data's normal distribution.

Figure 4. Time-course of the analgesic properties of menthol and pulegone on CFA-induced inflammatory pain model. Baseline, time-course and relative-to-baseline AUC (%) of the effects of i.p. menthol 100 mg/kg (n = 8), pulegone 100 mg/kg (n = 8) or the vehicle (n = 8) on CFA-induced mechanical (**a**), thermal heat (**c**) and thermal cold (**e**) hyperalgesia. Relative-to-baseline AUC (%) of the effects of i.p. menthol 100 mg/kg (n = 8), pulegone 100 mg/kg (n = 8) on mechanical (**b**), thermal heat (**d**) and thermal cold (**f**) sensitivities of NaCl-injected hindpaw. Data are expressed as mean ± SD. Asterisks indicate statistical significance (*** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$) using two-way ANOVA followed by Dunnett or Sidak multiple comparisons test for the time course, for the AUC is a one-way ANOVA followed by a Holm-Sidak multiple comparisons test or T-test, depending on the data's normal distribution.

Figure 5. Control of terpenes effect on edema and locomotor activity. (**a**) Time-course of the effects of i.p. menthol 100 mg/kg (n = 8), pulegone 100 mg/kg (n = 8) or the vehicle (n = 8), on CFA-induced edema size. Time-course of the locomotor activity after an i.p. injection (Vehicle, n = 6; Menthol (150 mg/kg), n = 6; Pulegone (150 mg/kg), n = 6) tested with the (**b**)

Rotarod and the (**c**) beam walk. Data are expressed as mean ± SD. No statistical significance where observed, using two-way ANOVA followed by Dunnett multiple comparisons post-hoc test.

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AUTHOR CONTRIBUTIONS

Conceptualization, AC, HP; Methodology, AC, CM, EM, HP, LH, ZT; Chemical analysis, CM, FE, ZT; Behavior, LH; Writing, AC, HP, PD; Funding acquisition, AC, HP; Supervision, AC, HP; Project administration, AC, HP.

CONFLICT OF INTERESTS

Dr. Hugues Petitjean, Louis Hilfiger and Zélie Triaux are or were salaries of the Benephyt company. All other authors declare no potential conflict of interest.

MATERIALS AND METHODS

All the protocols, test and use of living animals were performed in accordance with European committee council Direction, from the regional ethic committee (Comité Régional d'Ethique en Matière d'Expérimentation Animale de Strasbourg, CREMEAS) and with authorization from French Department of Agriculture (APAFIS# 19006-2019020714109922 v3).

Reagents and chemicals. Ethanol was purchased from Sigma-Aldrich (Steinheim, Germany). Milli-Q water (18.2 MΩ) was generated by Millipore synergy system (Molsheim, France). Nitrogen was of 4.5 grade and helium of 6.0 grade (Sol France, Saint-Ouen l'Aumone, France). Pulegone (97 %) and menthol (99 %) were analytical standards obtained from Sigma-Aldrich (Steinheim, Germany). RPMI 1640 cell culture media was obtained from ATCC (LGC Standards, Molsheim, France). Penicilin and streptomycin were purchased from Cambrex Bio Science (St Beauzire, France). Fetal bovine serum (FBS) was obtained from Lonza BioWhittaker (Fisher Scientific, Illkirch, France). Lipopolysaccharide (LPS) from *Salmonella abortus equi* and celastrol were purchased from Sigma-Aldrich (Steinheim, Germany). Propidium iodide was obtained from Miltenyi Biotec Inc. (Auburn, USA). For *in vivo* biological assays, terpenes were emulsified in warmed (37 °C) carboxymethyl cellulose (CMC, 1 %) - NaCl (0.9 %) and administered at the temperature of 37 °C. Pulegone, menthol or vehicle were injected intraperitoneally (i.p.).

Plant extracts. *Plants. Calamintha nepeta* (L.) savi and *Mentha piperita* (L.) were obtained from the Ledermann-Mutschler nursery (Krautergersheim, France). Upon reception, leaves and stems of the plants were chopped and dried at room temperature and out of the light until constant weigh was obtained, indicating that the drying process was done. Dry plants were then cryogenically grounded (6870 Freeze/Mill, Spex CertiPrep, Stanmore, Royaume-Uni) into fine powders which were stored at 4 °C and protected from the light before extraction.

Pressurized liquid extraction. Plant extracts were obtained by solid/liquid extraction under pressure using an ASE-350 system (Dionex, Sunnyvale, CA, USA). For the extraction, 3 g of finely grounded plant powder mixed with chemically inert Fontainebleau sand (previously heated at 600 °C for 4 hours and stored at room temperature) were placed into a 10 mL stainless steel extraction cell. Two 27 mm cellulose filters (Dionex, Sunnyvale, CA, USA) were placed one at the bottom and one at the top of the extraction cell. The extraction cell was then subjected to one cycle of extraction at 125 °C under 100 bars for 7 min in the static extraction mode. The extraction solvent was a 50/50 % (v/v) mixture of water and ethanol. The volume of the collected extract was about 15 mL. Between runs, the system was washed with 20 mL of the extraction solvent.

Gas chromatography-mass spectrometer (GC-MS) conditions. Plant extracts were analyzed on a 450-GC/240-MS system (Varian, Les Ulis, France) equipped with a DB-WAX capillary column (60 m x 0.25 mm x 0.15 µm) (Agilent Technologies, Les Ulis, France). Two microliters of the extract were injected in a split/splitless injector at 210°C and carried through the column by helium carrier gas (99.9999 %) at 1 mL/min. The following temperature program was applied to the column oven to allow the separation of the different compounds: hold for 1 min at 40 °C, increased to 100 °C at 10 °C/min, increased to 130 °C at 5 °C/min, increased to 150 °C at 10 °C/min, increased to 180 °C at 5 °C/min, heated to 230 °C at 10 °C/min and finally held isothermal at 230 °C for 5 min. For the MS parameters, the transfer line temperature was

set to 200 °C and the ion source at 150 °C. The mass spectrometer was operated in electron impact (EI) mode and the ionizing electron energy was set to 70 eV. The mass spectra were registered in a full scan acquisition mode in the range of 50-200 *m/z*. Peaks were identified by referring the mass spectra to the NIST (National Institute of Standards and Technology) mass spectral database and by comparing their retention time to the one of the analytical standard.

Cell culture. THP-1 cell line, acute monocytic leukemia cells, was purchased from the American Type Culture Collection (ATCC TIB-202, LGC Standard, Molsheim, France). The cells were maintained in RPMI-1640 (ATCC) medium supplemented with 10 % (v/v) of fetal bovine serum and 1 % (v/v) of a mixture of penicillin (1000 UI/mL, Gibco™) and streptomycin (1000 µg/mL, Gibco ™). Cells were grown in 75 cm^2 flasks in a humidified atmosphere with 5 % CO_2 at 37 °C and were replicated every 2-3 days before reaching a concentration of 1 x 10^6 cells/mL. Cells were sub-cultured at a concentration of approximatively 2-3 x 10^5 cells/mL by adding fresh media to the flask.

TNF-α secretion assay. The TNF-α secreted by the THP-1 cells was measured in the culture medium using an assay kit (TNF-α secretion assay, Miltenyi Biotech, United States). TNF-α secretion was obtained by LPS activation of the THP-1 cells. 180 µL of THP-1 in suspension in the media at a concentration of 3 x 10^5 cells/mL were seeded in a 96-well plate. 20 µL of LPS were added in each well to reach a final concentration of 1 µg/mL. In appropriate wells, 20 µL of plant extract dissolved in 90/10 water/ethanol were added to the cell media. For the evaluation of the activities of the standards, 2 µL of a standard solution in ethanol were added to reach specified final concentrations (from 0.03 to 13 mM). Positive and negative controls were included in the TNF-α secretion assay. Celastrol, a triterpenoid known for its TNF-α inhibitor capacities in *in vitro* tests[21], was used as a positive control at a final concentration of 0.5 µM. LPS-stimulated cells with 1 % of ethanol were used as a negative control. The 96-well plate was then incubated at 37 °C in 5 % CO_2 incubator for 2 hours. After incubation, 2 µL of each antibody of the assay kit (catch reagent and detection reagent) were added in each well. The 96-well plate is once more incubated at 37 °C in 5 % CO_2 incubator for 2 hours. 2 µL of propidium iodide is then added to each well to reach a final concentration of 1 µg/mL. The 96-well plate is incubated one last time for 10 min at 37 °C in 5 % CO_2 incubator. Each terpene concentration was tested in triplicates on the same 96-well plate.

Flow cytometry parameters. Microcapillary flow cytometry system Guava® EasyCyte™ 12HT (Merck Millipore, Darmstadt, Germany) with blue laser (488 nm) was used for the acquisition of the *in vitro* inflammation assay. The detection antibody of the assay contains *R*-phycoerythrin, thus yellow fluorescence allows to tract the concentration of TNF-α secreted by the cells (F cell %, Fig. 2a). Dead cells were detected by the red fluorescence of propidium iodide (F cell %, Fig. 2d). The mean flow velocity used was 35 µL/min. Each well was agitated for 10 s before each analysis using a rotatory agitator. Between each sample, the microcapillary and the agitator were washed. Data were treated using GuavaSoft (InCyte 3.1.1.) software.

Animals. Male Wistar rats (300 g; JANVIER LABS, Le Genest St. Isle, France) were used for this study. They were housed by groups of 3 or 4 under standard conditions (room temperature, 22 °C; 12 / 12 h light / dark cycle) with *ad libitum* access to food and water and behavioral enrichment. All animals were manipulated and habituated to the tests and to the room for at

least 2 weeks. All behavioral tests were done during the light period (i.e., between 7:00 and 19:00).

CFA model of inflammatory-induced hypersensitivities. In order to induce a peripheral inflammation, 100 µL of complete Freund adjuvant (CFA; Sigma, St. Louis, MO), was injected in the right hindpaw of the rat. All CFA injections were performed under light isoflurane anesthesia (3 %). Edema was quantified by measuring the width of the dorsoplantar aspect of the hind paw before and after the injection of CFA with a caliper.

Behavioral testing

Mechanical hyperalgesia. In all experimentations, to test the animal mechanical sensitivity, we used a calibrated forceps (Bioseb, Chaville, France). Briefly, the habituated rat is loosely restrained with a towel masking the eyes in order to limit stress by environmental stimulations. The tips of the forceps are placed at each side of the paw and a graduate force is applied. The pressure producing a withdrawal of the paw corresponded to the nociceptive threshold value. This manipulation was performed three times for each hindpaw and the values were averaged.

Thermal hot hyperalgesia. To test the animal heat sensitivity, we used the Plantar test with Hargreaves method (Ugo Basile, Comerio, Italy) to compare the response of each hindpaw when we tested healthy animals (unilateral intraplantar NaCl injection) and animals having received unilateral intraplantar CFA (Freund's Complete Adjuvant) injection. The habituated rat is placed in a small box and we wait until the animal is calmed then we exposed the hindpaw to a radiant heat, the latency time of paw withdrawal was measured. This manipulation was performed three times for each hindpaw and the values were averaged.

Thermal cold hyperalgesia. To test the animal cold sensitivity, we used the acetone test to compare the response of each hindpaw when we tested healthy animals (unilateral intraplantar NaCl injection) and animals having received unilateral intraplantar CFA (Freund's Complete Adjuvant) injection. The habituated rat is placed in a small box and we wait until the animal is calmed then we put a drop of acetone (≥ 99 %, Fisher Chemical) (between 50 and 100 µL) on the top of the hindpaw through a filed and curved needle without touching the hindpaw, then we scored the response of the animal during 20 s. 0 no response, 1 a short response or fast movement of the hindpaw (< 2 s), 2 a longer response (> 2 s), 3 licking of the hindpaw. This manipulation was performed three times for each hindpaw and the values were summed.

Rotarod test. We used the Rotarod (IITC Life Science, Woodland Hills, CA). The speed of the roll increased progressively from 5 rpm to 20 rpm in 4 min. The time spend by the animal on the rotarod before falling was measured. This test was reproduced two times for each animal and the values were averaged.

Beam walk test. The time need by the rats to cross the beam (PVC bar of length: 130 cm; width: 4 cm, placed at 80 cm from the floor) was recorded. This test was reproduced three times for each animal and the values were averaged.

Statistical analysis. Data are expressed as mean ± standard error of mean (SEM). Statistical tests were performed with GraphPad Prism 7.05 (GraphPas Software, San Diego, California, USA). Sigmoid dose-response curves and EC_{50} values were calculated with the following equation:

$$Y = Bottom + (Top - Bottom)/(1 + 10\text{^}((LogEC_{50} - X) * HillSlope$$

With *Y* the response, *X* the concentration, *Top* (resp. *Bottom*) the plateaus at the top (resp. bottom) of the sigmoid in the units of the y axis, *Hillslope* the steepness of the curve and EC_{50} the concentration that gives a concentration halfway between bottom and top.

Statistical analysis was performed using Student's t-test with a significance level of $\alpha=0.05$ meaning that differences between the negative control and the sample were considered significant when $p<0.05$. Results were considered to be statistically significant if p-values were below 0.05 (*), 0.01 (**), and 0.001 (***).

For behavioral tests, data were analyzed using repeated-measures two-way ANOVA for the time course experiments, with the following factors: treatment (between), and time (within); when 4 groups (treatment) were compared, the Dunnett's test was used for *post-hoc* multiple comparisons between individual groups and when 2 groups (treatment) were compared, the Sidak's test was used for *post-hoc* multiple comparisons between individual groups. To compare dose-response results, a Shapiro-Wilk normality test was performed to evaluate the hypothesis of normality, then we performed a nonparametric Kruskal-Wallis test supplemented with a *post-hoc* Dunn's test for multiple comparisons. For the comparison of each treatment depending on the time (0 vs 40 min post i.p.) a Shapiro-Wilk normality test was performed. When the normality test is passed, we used the paired t test and when the normality test doesn't pass we used the Wilcoxon matched pairs signed rank test. For the area under the curve (AUC) we tested the hypothesis of normality with a Shapiro-Wilk normality test. If it passed the normality test, we used an ordinary one-way ANOVA completed with a Holm-Sidak's test for *post-hoc* multiple comparisons. Otherwise we performed a nonparametric Kruskal-Wallis test supplemented with a post-hoc Dunn's test for multiple comparisons. Results were considered to be statistically significant if p values were below 0.05 (*), 0.01 (**), and 0.001 (***).

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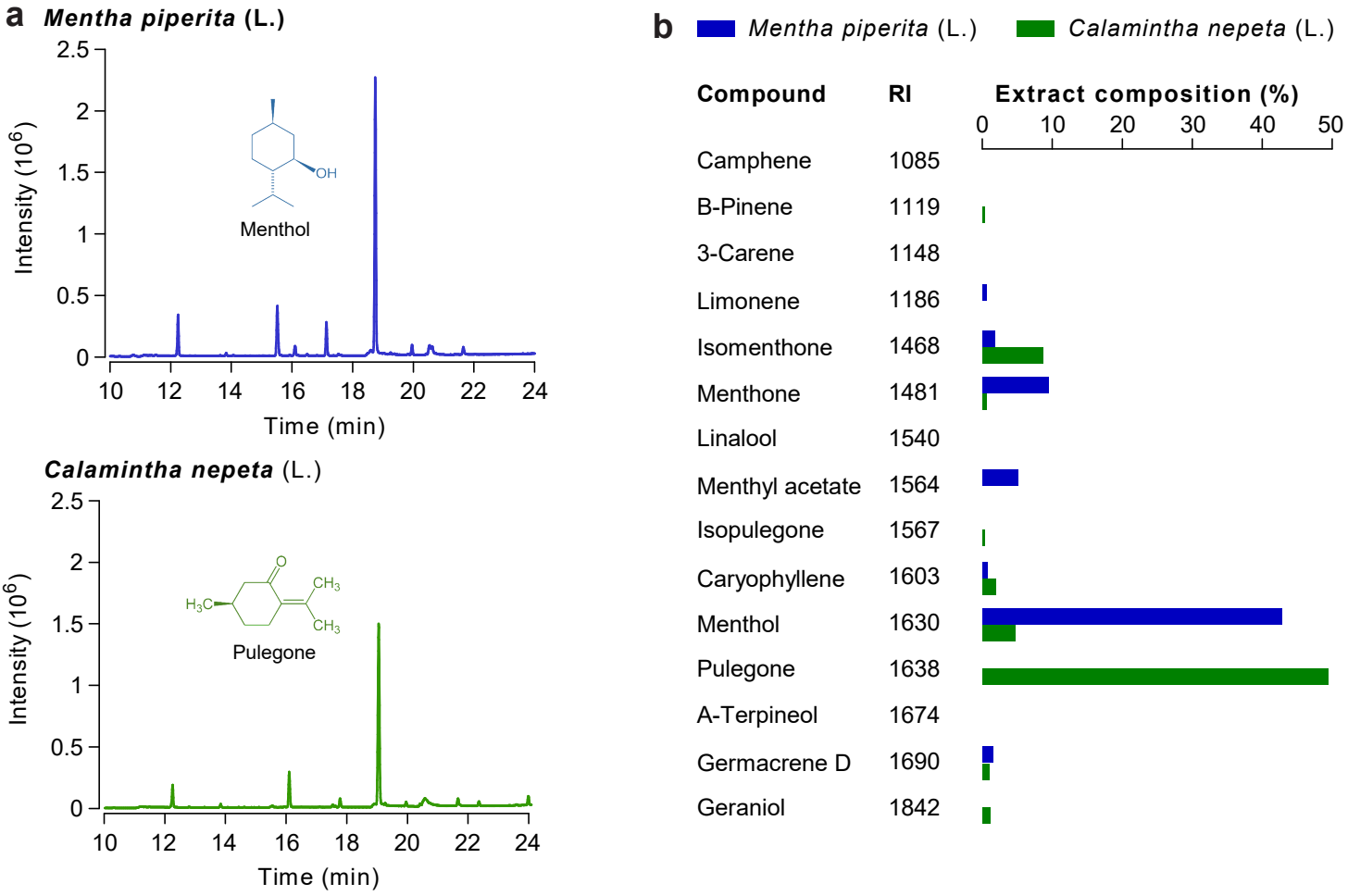
a
Mentha piperita (L.)
Intensity (10^6)
Time (min)
Menthol
OH
Calamintha nepeta (L.)
Pulegone
O
CH3
H3C
b
Mentha piperita (L.)
Calamintha nepeta (L.)
Compound
RI
Extract composition (%)
Camphene 1085
B-Pinene 1119
3-Carene 1148
Limonene 1186
Isomenthone 1468
Menthone 1481
Linalool 1540
Menthyl acetate 1564
Isopulegone 1567
Caryophyllene 1603
Menthol 1630
Pulegone 1638
A-Terpineol 1674
Germacrene D 1690
Geraniol 1842

Figure 1

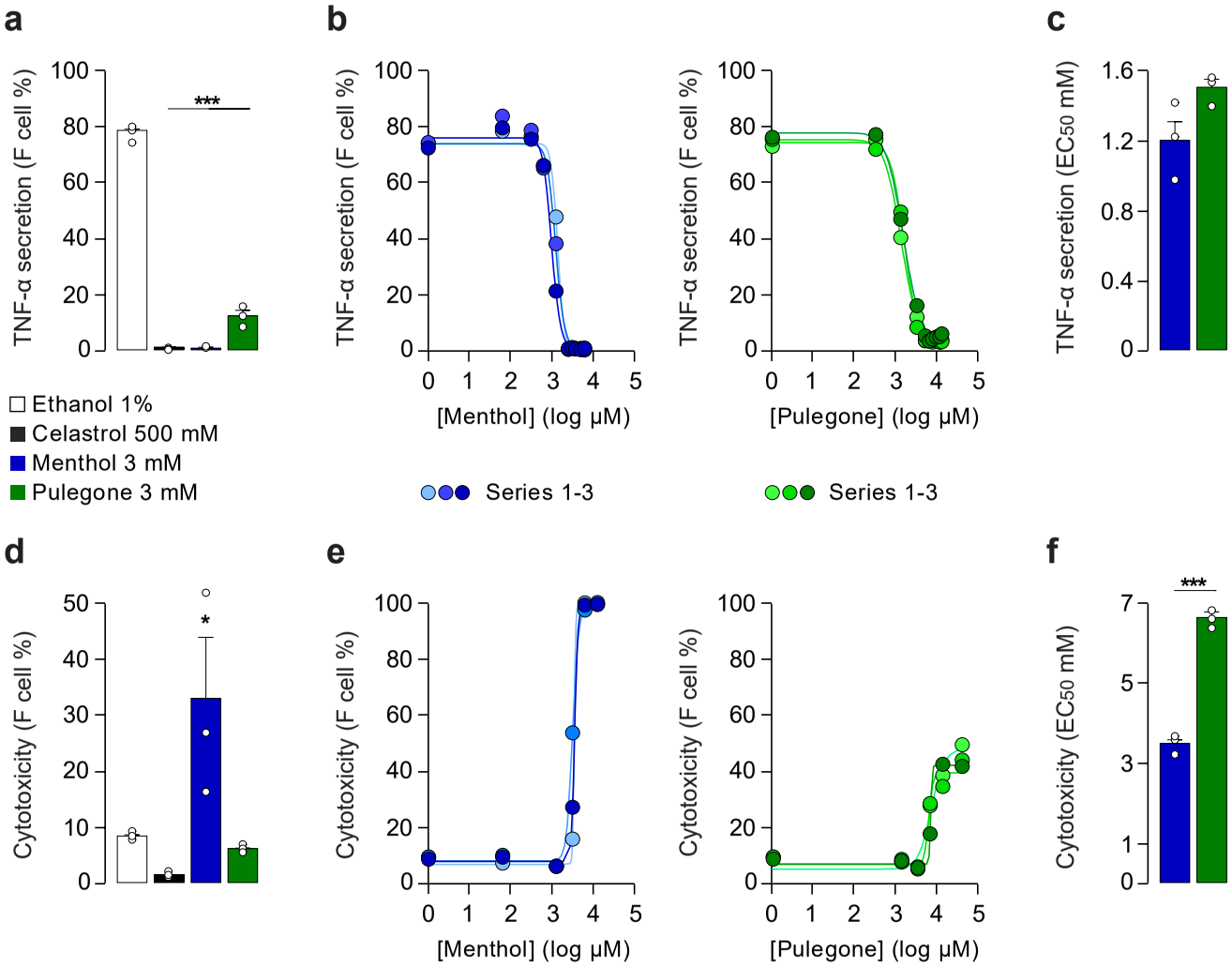
a
b
c
d
e
f
TNF-α secretion (F cell %)
***
Ethanol 1%
Celastrol 500 mM
Menthol 3 mM
Pulegone 3 mM
[Menthol] (log µM)
[Pulegone] (log µM)
Series 1-3
Series 1-3
TNF-α secretion (EC50 mM)
Cytotoxicity (F cell %)
*
[Menthol] (log µM)
[Pulegone] (log µM)
Cytotoxicity (EC50 mM)
***

Figure 2

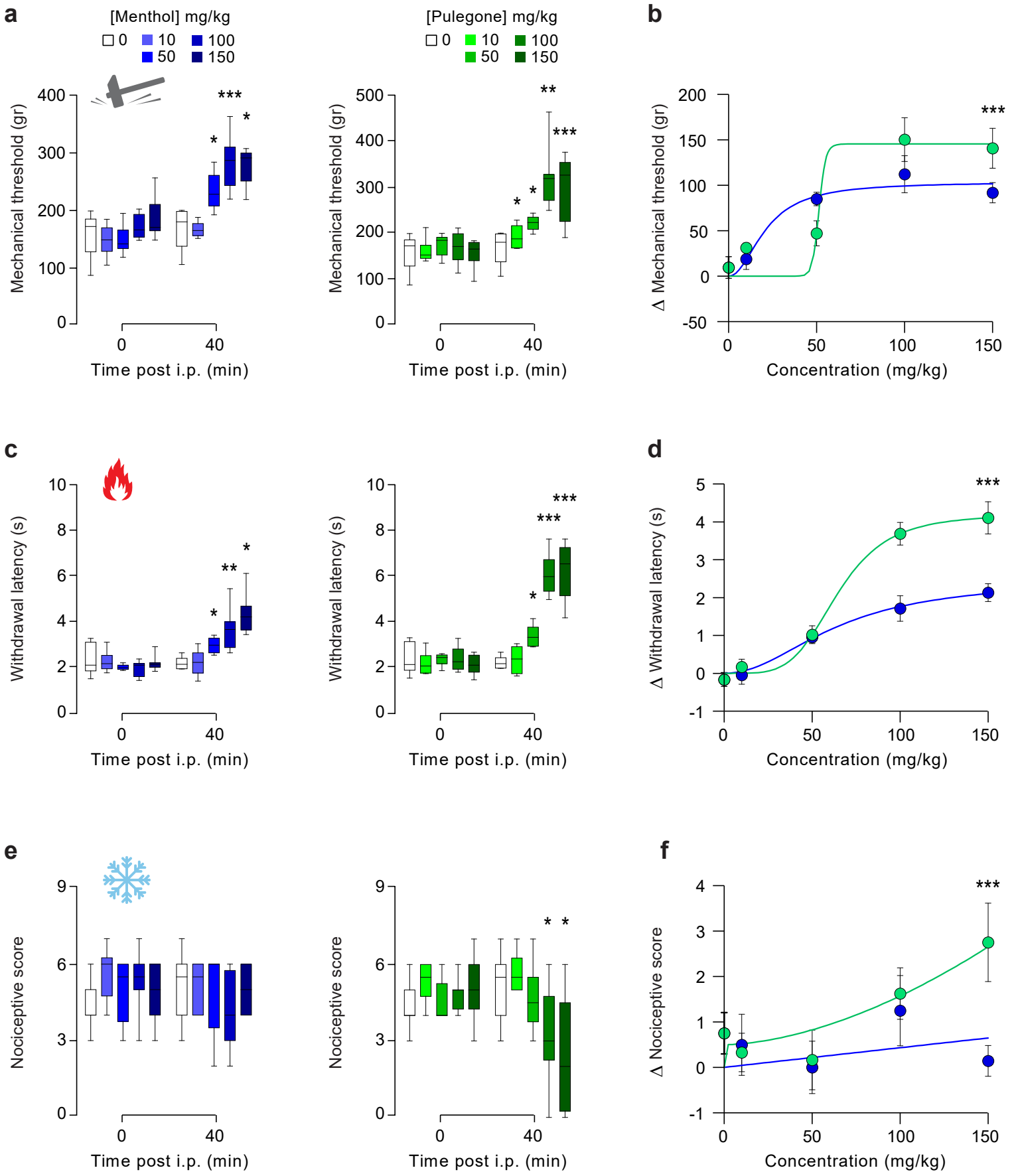
a
[Menthol] mg/kg
0
10
50
100
150
Mechanical threshold (gr)
Time post i.p. (min)
[Pulegone] mg/kg
b
Δ Mechanical threshold (gr)
Concentration (mg/kg)
c
Withdrawal latency (s)
d
Δ Withdrawal latency (s)
e
Nociceptive score
f
Δ Nociceptive score

Figure 3

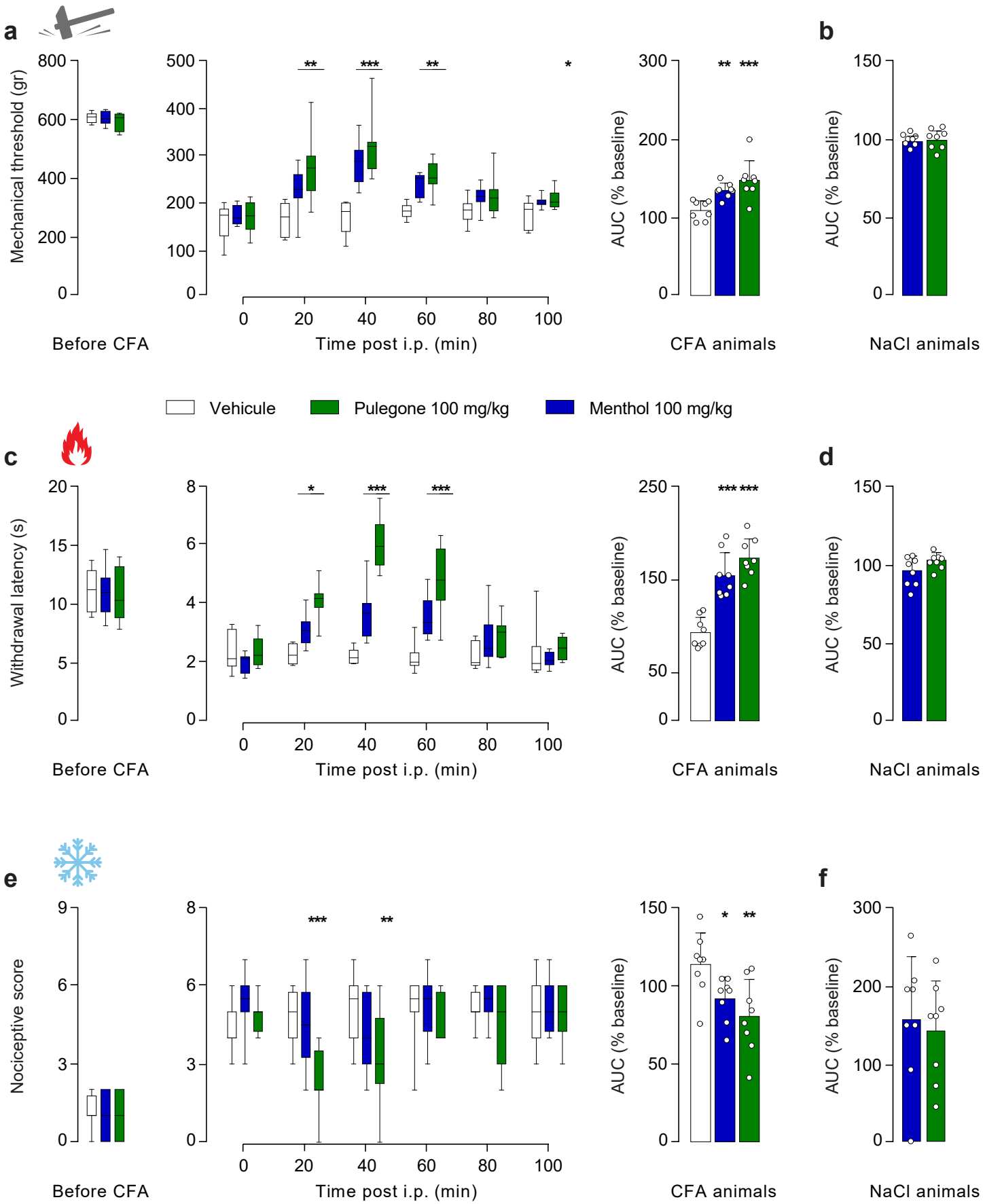
a
b
Mechanical threshold (gr)
800
600
400
200
0
500
400
300
200
100
0
**
***
**
*
0
20
40
60
80
100
Before CFA
Time post i.p. (min)
AUC (% baseline)
300
200
100
0
** ***
CFA animals
150
100
50
0
NaCl animals
Vehicule
Pulegone 100 mg/kg
Menthol 100 mg/kg
c
d
Withdrawal latency (s)
20
15
10
5
0
8
6
4
2
0
*
***
***
250
150
50
0
*** ***
e
f
Nociceptive score
9
6
3
0
***
**
150
100
50
0
* **
300
200
100
0

Figure 4

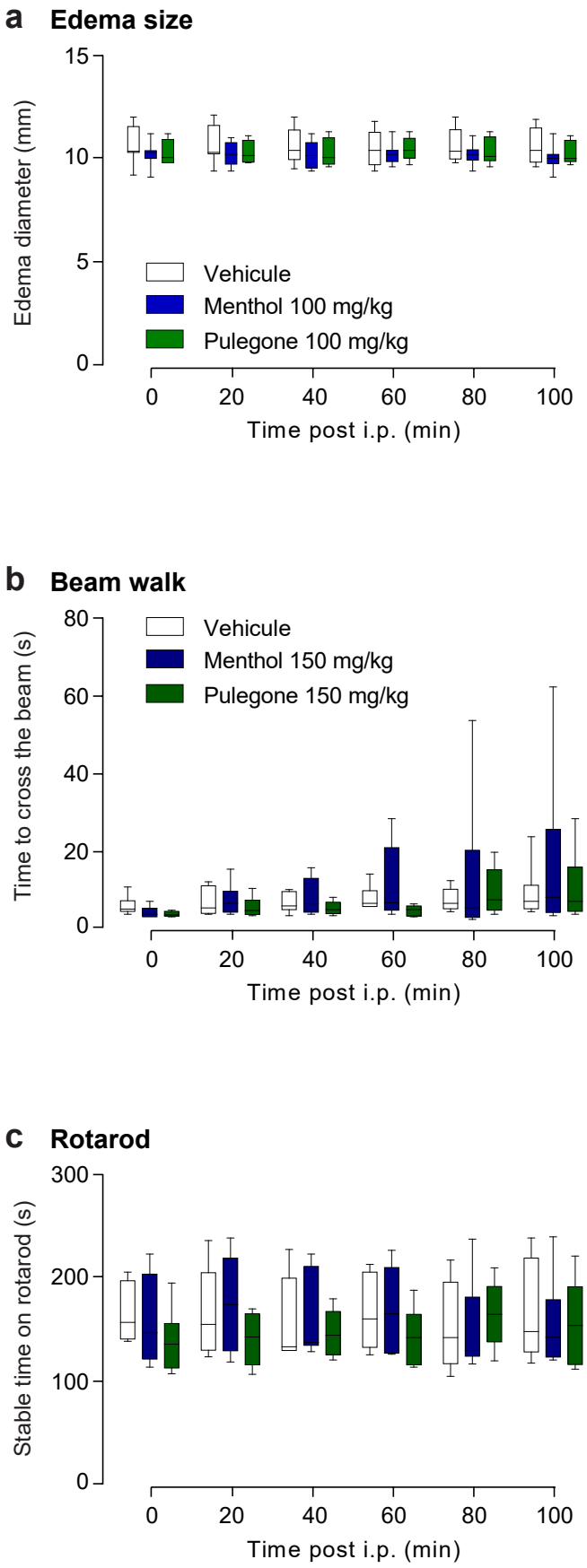
a
Edema size
Edema diameter (mm)
15
10
5
0
Vehicule
Menthol 100 mg/kg
Pulegone 100 mg/kg
0
20
40
60
80
100
Time post i.p. (min)
b
Beam walk
Time to cross the beam (s)
80
60
40
20
0
Vehicule
Menthol 150 mg/kg
Pulegone 150 mg/kg
0
20
40
60
80
100
Time post i.p. (min)
c
Rotarod
Stable time on rotarod (s)
300
200
100
0
0
20
40
60
80
100
Time post i.p. (min)

Figure 5

Supplementary Files

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