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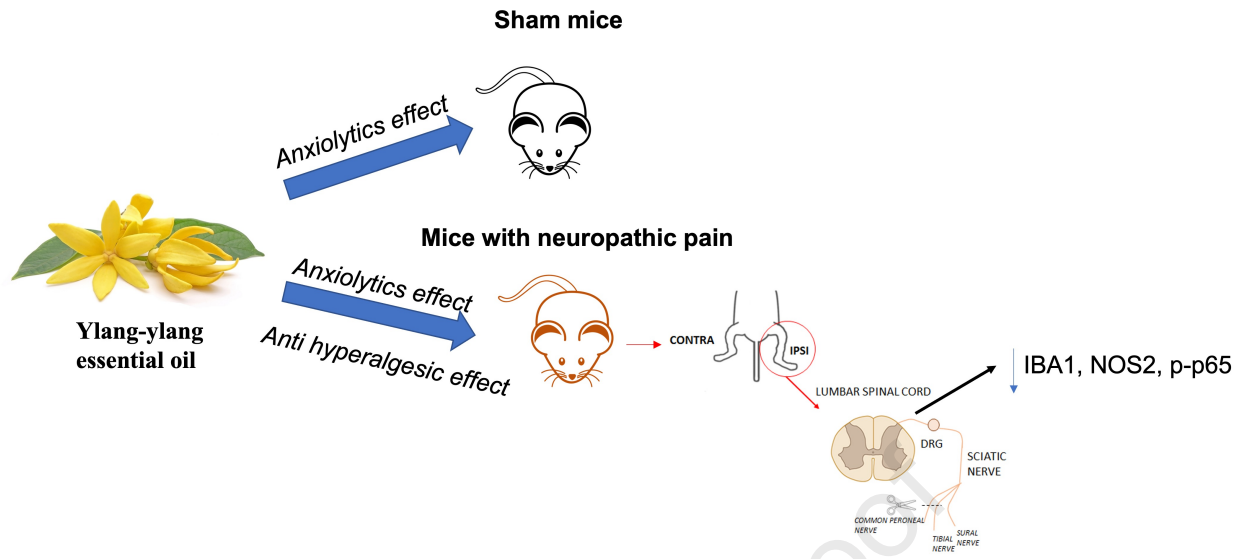
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2 **Ylang-ylang (*Cananga odorata* (Lam.) Hook. f. & Thomson) essential oil reduced neuropathic-**
3 **pain and associated anxiety symptoms in mice**

4

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21

22 Abstract

23 **Ethnopharmacological relevance:** Ylang-ylang essential oil (YEO), obtained from the flowers of
24 the tropical tree *Cananga odorata* (Lam.) Hook. f. & Thomson (family *Annonaceae*), has been largely
25 used in the traditional medicine with many uses, including anxiety and altered neuronal states.
26 Neuropathic pain is a chronic pain condition with a high incidence of comorbidities, such as anxiety,
27 depression, and other mood disorders, that drastically affect the patient's quality of life. The currently
28 available drugs used for the management of neuropathic pain are inadequate due to poor efficacy and
29 tolerability, highlighting the medicinal need of a better pharmacotherapy. Several clinical studies
30 have reported that massage or inhalation with selected essentials oils reduces symptoms associated
31 to pain and anxiety.

32 **Aim of the study:** The aim of this study was to investigate the analgesic properties of YEO and its
33 efficacy in reducing neuropathy-associated mood alterations.

34 **Materials and methods:** The analgesic properties were tested in the spared nerve injury (SNI) model
35 using male mice. Anxiolytic, antidepressant, and locomotor properties were also evaluated using
36 behavioural tests. Finally, the YEO mechanism of action was investigated in the spinal cord and
37 hippocampus of neuropathic mice.

38 **Results:** Oral administration of YEO (30 mg/kg) reduced SNI-induced neuropathic pain and
39 ameliorates pain-related anxiety symptoms that appeared 28 days after surgery. YEO reduced the
40 expression of MAPKs, NOS2, p-p65, markers of neuroinflammation, and promoted normalizing
41 effect on neurotrophin levels (BDNF).

42 **Conclusions:** YEO induced neuropathic pain relief and ameliorated pain-associated anxiety,
43 representing an interesting candidate for the management of neuropathic pain conditions and pain-
44 related comorbidities.

45

46 Keywords

47 Ylang-ylang; essential oils; neuropathic pain; anxiety; aromatherapy; neuroinflammation

48

49 Abbreviations

50 BDNF, brain-derived neurotrophic factor; GFAP, glial fibrillary acidic protein; IBA-1, ionized
51 calcium binding adapter protein 1; HPT, Hot plate test; LDB, light dark box; MAPK, mitogen-
52 activated protein kinase; MORPH, morphine; NOS2, nitric oxide synthase 2; NP, neuropathic pain;
53 NSFT, novelty suppressed feeding test; p-ERK, phosphorylated extracellular signal-regulated
54 kinases; p-JNK1, phosphorylated Jun N-terminal kinase 1; PREG, pregabalin; SNI, spared nerve
55 injury; TST, tail suspension test; VEH, vehicle; YEO, ylang-ylang essential oil

56

57 **1. Introduction**

58

59 Neuropathic pain (NP) is a chronic condition that occurs due to an injury or disease of the
60 somatosensory system. Generally, NP affects 7% - 10% of the world's population and its prevalence
61 is likely to increase with ageing, cancer survival and other chronic disease (Calvo et al., 2019). The
62 currently available drugs used for the management of NP are inadequate due to poor efficacy and
63 tolerability (Finnerup et al., 2015). Anxiety, depression and other mood disorders are comorbidities
64 that characterise about 34% of patients with NP, extensively affecting the patient's quality of life.
65 However, there are no effective and safe treatments that can deal with the symptoms and comorbidity
66 of NP (Radat et al., 2013). Indeed, existing therapies are characterised by several side effects that
67 impede their continued use. Thus, there is an urgent need to develop new and more effective therapies.
68 In particular, the induction of an analgesic activity together with an antidepressant/anxiolytic effect
69 could improve the patient's overall quality of life. An increasing number of patients choose alternative
70 medicine to relieve the symptoms of various pathological processes, including pain and
71 aromatherapy, consisting in the medicinal uses of essential oils extracted from aromatic plants, is one
72 of the most used (Cooke and Ernst, 2000). Several clinical studies have reported that massage or
73 inhalation with selected essential oils reduced symptoms associated to pain and anxiety
74 (Dehghanmehr et al., 2017; Tabatabaeichehr and Mortazavi, 2020).

75 Ylang-ylang essential oil (YEO) is obtained from the flowers of the tropical tree *Cananga odorata*
76 (Lam.) Hook. f. & Thomson (family *Annonaceae*). It is generally used as fragrance and is approved
77 for food use by US Food and Drug Administration. The chemical composition of YEO has been
78 reported in several phytochemical studies and the main constituents of YEO include monoterpenes,
79 sesquiterpenes, and phenylpropanoids (Tan et al., 2015). Traditionally, different parts of *C. odorata*
80 plants have been used to treat fever, asthma, and inflammatory pain and it is commonly used in
81 aromatherapy for improving cognitive function and reducing anxiety (Zhang et al., 2016). However,
82 the possible effect of YEO on NP and NP-related symptoms has not been reported. The aim of this
83 study was to investigate the analgesic properties of YEO in a mouse model of NP and the widely
84 known sedative effects of YEO gave us the opportunity to investigate its possible application in
85 reducing neuropathy-associated mood alterations, such as anxiety and depression. Finally, the YEO
86 mechanism of action was investigated in the spinal cord and hippocampus of neuropathic mice.

87

88

89 2. Material and methods

90

91 2.1. Animals

92 CD1 male mice (4-6 weeks of age) weighting approximately 22–24 g (Envigo, Varese, Italy) were
93 housed in the Ce.S.A.L. (Centro Stabulazione Animali da Laboratorio, University of Florence)
94 vivarium and used one day after their arrival. Mice were housed in standard cages, kept at 23 ± 1 °C
95 with a 12-h light/dark cycle, light on at 7 a.m., and fed with standard laboratory diet and tap water *ad*
96 *libitum*. 24 h before the behavioural test, the animals were acclimatized by placing the cages in the
97 experimental room. All tests were conducted during the light phase. The experimental protocol was
98 approved by the Institution's Animal Care and Research Ethics Committee (University of Florence,
99 Italy), under license from the Italian Department of Health (54/2014-B). Mice were treated in
100 accordance with the relevant European Union (Directive 2010/63/EU, the council of 22 September
101 2010 on the protection of animals used for scientific purposes) and international regulations (Guide
102 for the Care and Use of Laboratory Animals, US National Research Council, 2011). All studies
103 involving animals are reported in accordance with the ARRIVE guidelines (Lilley et al., 2020). The
104 experimental protocol was designed to minimize the number of animals used and their suffering. The
105 G power software was used to perform a power analysis to choose the number of animals *per*
106 experiment (Charan and Kantharia, 2013)

107

108 2.2. Chemicals and drug administration

109 Ylang-ylang essential oil (YEO) was kindly supplied by Pranarom International (Belgium). The oil
110 was obtained by distillation of the flowers of *Cananga odorata* from Madagascar (batch number
111 OF23435). According to the GC-FID analyses, the main constituents were: germacrene D (12,34%),
112 linalool (10,19%), benzyl acetate (9,89%), β -caryophyllene (7,57%), geranyl acetate (7,29%), benzyl
113 benzoate (6,62%), methyl benzoate (4,98%), α -farnesene (4,02%), cinnamyl acetate (3,72%),
114 methyl-p-cresol (3,40%), farnesyl acetate (2,85%), benzyl salicylate (2,57%). The oil was liquid, light
115 yellow and with floral odour.

116 Mice were randomly assigned to each treatment group by a researcher and an operator. YEO was
117 diluted in 5% DMSO and administered p.o. 30 min before testing at the dose of 30 mg/kg for all
118 experiments, except for dose-response curve where YEO has been administered at doses ranging from
119 0.1 to 60 mg/kg. The control group (Naïve) received equivalent volume of vehicle (DMSO 5% in
120 saline solution). Pregabalin (30 mg/kg i.p.) (Sigma-Aldrich, Milan, Italy) and morphine
121 hydrochloride (7 mg/kg i.p.) (SALARS, Como, Italy) were dissolved in saline solution and
122 administered 3 h and 15 min before testing, respectively.

123

124 **2.3. Evaluation of antinociceptive activity**

125

126 **2.3.1. Hot plate test (HPT)**

127 The hot plate test was performed following the protocol described by Borgonetti and co-workers
128 (Borgonetti et al., 2020d). Mice were placed on a hot plate (Ugo Basile Biological Research
129 Apparatus, Varese, Italy), with the temperature adjusted to $52.5 \pm 0.1^\circ\text{C}$.

130

131 **2.3.2. Spared nerve injury (SNI) procedure and von Frey filaments**

132 The spared nerve injury model is an established mono-neuropathy model, which was performed as
133 previously described. (Borgonetti et al., 2020c). The mechanical threshold was recorded at day 21
134 from surgery by delivering a mechanical stimulus using grade-strength von Frey monofilaments
135 (0.07, 0.16, 0.4, 0.6, 1.0, 1.4, 2.0 g) both ipsilateral and contralateral sides. Monofilaments were
136 delivered to the plantar surface of the hind paw of the mouse, starting with filament of 0.07 g and a
137 response was established by a paw withdrawal response to any three of five repeating stimuli.

138

139 **2.4. Evaluation of anxiolytic-like effect**

140 Tests to assess anxiolytic-like activity were carried out 21-28 days after the operation.

141 **2.4.1. Light dark box (LDB)**

142 The light-dark box was performed as previously reported (Borgonetti et al., 2021). The time spent in
143 the light portion was used as a signal of the level of anxiety of each animal.

144

145 **2.4.2. Marble-burying test**

146 The marble-burying behavioural test was performed as previously described (Borgonetti et al.,
147 2020d). The number of buried marbles (at least two thirds) was measured in 30 minutes, which is a
148 measure of the animal's anxiety.

149

150 **2.5. Novelty suppressed feeding test and evaluation (NSFT) of food consumption**

151 The NSFT test was performed as previously described (Borgonetti et al., 2020a). The fasting latency
152 and the pellet quantity eaten, measured in mg, was recorded in 5 min. To eliminate an effect on
153 appetite, we conducted the feeding test, in which the animals were kept fasting for 4 h, with water ad
154 libitum. The difference between the weight of the pellet given and the weight of the pellet left 15, 30
155 and 60 min after feeding was recorded.

156

157 **2.6. Evaluation of antidepressant activity.**

158

159 **2.6.1. Tail suspension test (TST)**

160 The TST was performed as described by Borgonetti (Borgonetti et al., 2020b). The test was conducted
161 for a total of 6 minutes and depression-like behaviour was set in the last 4 minutes, when the mice
162 were passively hanging and completely immobile. This test was performed 21-28 days from surgery
163

164 **2.7. Evaluation of locomotor behaviour**

165

166 **2.7.1 Rotarod test**

167 The onset of motor side effects induced by treatment was evaluated with rotarod test, as previously
168 described (Borgonetti et al., 2020c).

169

170 **2.7.2. Hole board test**

171 The hole-board test is commonly used to verify the effect of a drug on the spontaneous mobility and
172 exploratory activity (Borgonetti et al., 2020d).

173

174 **2.8. Western blotting analysis**

175 The Western Blotting analysis was performed as previously reported (Sanna et al., 2019). The
176 dissected spinal cord and hippocampus tissue of animal with neuropathy at the 28 days were
177 homogenized in a lysis buffer containing 25 mM Tris-HCl pH (7.5), 25 mM NaCl, 5 mM EGTA,
178 2.5 mM EDTA, 2 mM NaPPi, 4 mM PMSF, 1 mM Na₃VO₄, 1 mM PMSF, 20 µg/ml leupeptin, 50
179 µg/ml aprotinin, 0.1% SDS (Sigma-Aldrich). The homogenate was centrifuged at 12000 x g for 30
180 min at 4 °C and the pellet was discarded.

181 Protein samples (30 µg of protein/sample) were separated by 10% SDS-polyacrylamide gel
182 electrophoresis (SDS-PAGE). Proteins were then blotted onto nitrocellulose membranes (90 min at
183 110 V) using standard procedures. Membranes were blocked in PBST (PBS with 0.1% Tween)
184 containing 5% non-fat dry milk for 90 min and incubated overnight at 4°C with primary antibodies
185 p-p38 (1:750; Santa Cruz Biotechnology, Dallas, TX, USA), p-JNK1 (1:1000; Santa Cruz
186 Biotechnology), p-ERK1/2 (1:1000; Cell Signaling Technology, Danvers, MA, USA), NOS2 (1:500;
187 Cell Signaling Technology), p-p65 (1:1000; Santa Cruz Biotechnology), BDNF (1:500; Santa Cruz
188 Biotechnology), IBA-1 (1:500; Santa Cruz Biotechnology), GFAP (1:500; Santa Cruz
189 Biotechnology). The day after, blots were rinsed three times with PBST and incubated for 2 h at room
190 temperature with HRP-conjugated mouse anti-rabbit (1:3000) (Santa Cruz Biotechnology) and goat

191 anti-mouse (bs-0296G,1:5000) (Bioss Antibodies, MA, USA) and then detected by
192 chemiluminescence detection system (Life Technologies Italia, Monza, Italy). Signal intensity
193 (pixels/mm²) was quantified using ImageJ (NIH). The signal intensity was normalized to that of
194 GAPDH (1:5000 Santa Cruz Biotechnology).

195

196 **2.9. Statistical analysis**

197 Behavioural test: results are given as mean \pm SEM; eight mice per group were used. One-way and
198 two-way analysis of variance, followed by Tukey and Sidak post hoc test, respectively, were used for
199 statistical analysis. When appropriate, student's t-test was also used. *In vitro* experiments: results are
200 given as the mean \pm SEM of three independent triplicate. One-way ANOVA, followed by Tukey post
201 hoc test, was used for determining the differences between each experimental group and a P value
202 lower than 0.05 was considered significant. All statistical analyses were performed using GraphPad
203 Prism version 5.0 (GraphPad Software, San Diego, CA, USA).

204

205 **3. Results**

206 **3.1. Antinociceptive activity of YEO in acute and chronic pain models**

207 The antinociceptive activity of YEO was evaluated in both acute and chronic pain conditions.

208 In the acute pain model, a thermal stimulus was applied to the hind paw of mice. The dose response
209 curve revealed a bell-shaped trend of activity for YEO. Even though not significant, doses ranging
210 between 0.1 and 10 mg/kg showed a trend to an increase of pain threshold that peaked at 30 mg/kg
211 and returned to basal levels at the dose of 60 mg/kg (Fig. 1a). Time-course experiments showed that
212 the peak of the effect is observed 60 min after oral administration, with an intensity comparable to
213 morphine (MORPH), used as positive control drug (Fig 1b).

214 In the chronic pain model, SNI mice showed a strong mechanical allodynia after 7 days from surgery
215 on the ipsilateral side compared to the contralateral uninjured side (mean value represented by the
216 red dashed line). These results are consistent with those obtained in the acute model. Indeed, the anti-
217 hyperalgesic activity peaked at 30 mg/kg with an efficacy comparable to that of pregabalin (PREG),
218 used as reference drug (Fig. 1c). Time-course studies showed a peak in the anti-hyperalgesic effect
219 between 45- 60 minutes from oral administration (Fig. 1d).

220

221 **3.2. Anxiolytic-like effect of YEO in naïve and SNI mice with neuropathy**

222 To evaluate whether YEO could ameliorate neuropathic pain-associated comorbidities, the
223 anxiolytic-like and antidepressant-like activities were investigated after treatment with YEO
224 analgesic doses.

225 In the LDB test YEO-treated naïve mice spent more time in the light chamber in comparison to VEH-
226 treated mice (Fig. 1a). Consistently, YEO-treated mice buried a lower number of marbles, compared
227 to control group (Fig. 1b). These results were confirmed in the NFST, where the latency time to feed
228 is reduced in mice treated with YEO (Fig.1c).

229 These findings encouraged us to evaluate the activity of YEO in mood alterations associated to
230 neuropathic pain. 28 days after surgery, SNI mice developed anxiolytic-like symptoms compared to
231 naïve uninjured mice, as demonstrated by the reduced time in the light chamber in the LDB (Fig. 2a)
232 and the reduced latency to feed in NFST (Fig. 2c; 2d). No differences were registered for the MBT,
233 indeed the number of marbles buried from naïve, and SNI VEH-treated mice were comparable. YEO
234 30 mg/kg was able to reduce the anxiety-related response in SNI mice in all paradigms evaluated
235 (Fig. 1a,b,c). Feeding consumption cumulative curves showed a comparable amount of food eaten by
236 mice treated with YEO and the vehicle-treated control group, excluding a possible anorexiant effect
237 by treatment that might lead to a misinterpretation of the results (Fig 2e).

238

239 **3.3. Lack of antidepressant-like effect of YEO in naïve and SNI mice with neuropathy**

240 SNI mice showed a more marked immobility time in the TST than control mice, indicating the
241 presence of a depressant-like behaviour. However, YEO, administered at analgesic dose, did not
242 modify immobility time showed no antidepressant-like activity in either group (Fig 2f).

243

244 **3.4. Lack of locomotor behaviour impairments**

245 To investigate possible locomotor alterations, specific tests were conducted. As expected, SNI mice
246 showed an increased number of falls from the rotating rod in the rotarod test (Fig. 3a). YEO treatment
247 did not alter locomotor behaviour in either group in comparison to control groups (Fig. 3a).

248 The hole board test was used for measuring the spontaneous mobility (Fig. 3b) and exploratory
249 activity (Fig. 3c) of mice. No differences were observed between naïve and mice with neuropathy
250 regarding the spontaneous mobility. YEO treatment increased both spontaneous mobility and
251 exploratory activity in SNI mice without any effect in the naïve control group. These results let
252 hypothesize that the increased exploratory activity in SNI is related to a reduction of pain
253 hypersensitivity rather than to an induction of side effects by the treatment since (Fig. 3b,c).

254

255 **3.5. YEO reduced p-p38 and p-JNK1 protein expression in spinal cord 28 days after surgery**

256 MAPKs represent an important target involved in neuropathic pain. As previously reported
257 (Sanna,2015), 28 days after surgery an increase of p-ERK1/2 (Fig 4a), p-p38 (Fig. 4b) and p-JNK1
258 (Fig.4c) protein expression in the ipsilateral side of SNI spinal cord mice is observed, compared to

259 the CTRL group (dashed red line). The oral administration of YEO 30 mg/kg did not significantly
260 alter ERK1/2 phosphorylation (Fig 4a). Conversely, YEO reduced the up-regulation of p-p38 (Fig
261 4b), and p-JNK1 (Fig 4c) in SNI-mice. These results might indicate a prominent effect on glia cells
262 compared to neuronal cells.

263

264 **3.6. YEO reduced NOS2 and p-p65 protein expression in spinal cord tissue of SNI-mice**

265 To confirm the effect on glia cells we evaluated specific microglia and astrocytes-activated markers.
266 SNI mice showed a strong glia activation in spinal cord tissue compared to the control group, as
267 indicated by an increase of the expression of NOS2 (Fig. 5a) and p-p65 (Fig. 5b), marker of microglia
268 activation, and GFAP (Fig. 5c), an astrocyte marker. YEO reduced the expression of NOS2 (Fig.5a)
269 and p-p65 (Fig. 5b) without affecting GFAP expression (Fig.5c), thus showing microglia as a
270 prominent site of action.

271 **3.7. YEO reduced microglia neurotoxicity normalizing BDNF level in hippocampus and BV-** 272 **2 cells**

273 Lastly, we aimed to investigate if modulation of microglia activation could be involved in YEO
274 anxiolytic-like effects. We first detected the effect of YEO on IBA-1 expression in the hippocampus
275 of SNI mice. 28 days after surgery, SNI mice developed a strong microglia activation, with increased
276 levels of IBA-1, which was reverted by YEO (Fig. 6a). BDNF is typically produced by microglia
277 cells to adjust the normal synapse activity, but when its production is dysregulated, it can lead to
278 important neurotoxicity (Phillips, 2017). SNI mice notably increased BDNF expression (Fig. 6b) that
279 was reduced by YEO treatment, normalizing BDNF protein levels. ERK1/2 represents an important
280 pathway involved in neurogenesis that promotes an increase of BDNF levels as sign of protection of
281 the neuron activity. However, YEO did not reduce the increased levels of p-ERK1/2 in SNI mice,
282 demonstrating that the effect on BDNF levels was not related to a modulation of ERK activation (Fig.
283 6c). These data are consistent with a prominent microglial activity of YEO, being ERK mainly
284 expressed in the neuronal cells (Borges et al., 2015).

285

286 4. Discussion

287 Neuropathic pain is a major socio-economic problem in the world due to the lack of effective and
288 safe therapies. Moreover, the development of co-morbidities in chronic pain, such as anxiety and
289 depression, severely decrease patients' quality of life. Thus, the inappropriate effect of analgesic drugs
290 still presents an urgent problem in the management of neuropathic pain.

291 Complementary and alternative therapies offer an alternative method to decrease pain and improve
292 quality of life (Hamlin and Robertson, 2017). Aromatherapy, the use of essential plant-based oils for
293 medicinal purposes, is one of the main alternative medicines, notably through the use of massage and
294 inhalation (Mansfield and Keene, 2012). To find new therapeutic options for neuropathic pain relief,
295 we investigated the analgesic effect of a ylang-ylang essential oil in the SNI model of peripheral
296 neuropathy.

297 YEO dose-dependently increased the pain threshold of naïve mice in a condition of acute thermal
298 nociception, highlighting an antinociceptive activity of the extract. Moreover, a singular oral
299 administration of YEO reversed mechanical allodynia in the SNI model, a neuropathic pain model,
300 increasing the pain threshold with an intensity comparable to pregabalin, used as reference drug. At
301 the analgesic doses, we also observed anxiolytic-like effects in both naïve and neuropathic mice.
302 Indeed, it has been reported that after 4-6 weeks SNI mice show marked anxious behaviours, showing
303 a close link on chronic neuropathic pain and chronic anxiety (Sieberg et al., 2018). For this reason,
304 finding novel treatments able to control the main symptoms associated to neuropathic pain and mood
305 comorbidities could lead to an ideal therapeutic strategy for increasing patients' quality of life.
306 Several studies reported the anxiolytic-like efficacy of aromatherapy inhalation of YEO in naïve mice
307 (Zhang et al., 2016) and this was also confirmed in clinical studies (Pujiarti R, 2012). To the best of
308 our knowledge, this is the first observation of an analgesic and anxiolytic effects of YEO in mice with
309 neuropathy after oral administration.

310 YEO did not alter the locomotor and cognitive activity of mice at the active dose, which is an
311 important improvement compared to the common therapy used for neuropathic pain that are endowed
312 with relevant side effects (Derry et al., 2019).

313 MAPK activation is a fundamental pathway in the spinal cord of SNI mice for the evolution of
314 pathological properties of neuropathic pain (Borgonetti et al., 2020c). YEO reduced the activation of
315 p-p38 and p-JNK1, while not affecting p-ERK1/2 levels. These results indicate a prominent role of
316 glia activation in the cellular effects of YEO. Indeed, p38 is strongly involved in the transcriptional
317 activity of microglia genes in neuropathic-pain mice (Bhatia et al., 2017), while JNK is mainly
318 expressed in astrocytes cells in the spinal cord of SNI mice (Gao et al., 2009). To better elucidate if
319 the effect of YEO could be on microglia/astrocytes or in both cell type, we tested its effect on the

320 most known targets of these cells. In inflammatory condition NOS2 is detected in microglia, where
321 it induces an increase of NO production (Béchéde et al., 2014). NOS2 is also involved in chronic
322 pain, as an increase in this factor has been detected in mice with neuropathy (Hervera et al., 2010).
323 YEO totally reverted the SNI-induced increase of NOS2. Another important marker of microglia
324 activation is represented by p-p65, which is a transcriptional factor that promotes transcription of
325 genes associated to inflammation. Inhibitor of this pathway are considered good candidate for the
326 management of neuropathic pain symptoms (Wang et al., 2020). YEO reduced the activation of p-
327 p65, returning its expression to basal levels.

328 We already described the increase of GFAP, a marker of reactive astrocytes, in the spinal cord of
329 animal with neuropathy (Li et al., 2019), and in SNI-mice. Contrary to microglia marker, no effects
330 were observed on GFAP expression in the spinal cord tissue of YEO treated mice. These results
331 suggest that the analgesic activity of YEO is mainly related to a modulation of microglia activation
332 with a marginal involvement of astrocyte modulation.

333 The *in vitro* activity of YEO on peripheral inflammation in macrophage cells, through inhibition of
334 NOS2 expression, has been previously reported (Choi and Hwang, 2005). Besides the *in vitro* studies
335 mentioned above, the anti-inflammatory activity of YEO has been also recently evaluated in animal
336 model, such as the carrageenan induced paw oedema model (Tan et al., 2015).

337 To confirm the modulatory activity on microglia as a mechanism involved in both the analgesic and
338 anxiolytic activity, we evaluated the effect of YEO on microglia activation in the hippocampus of
339 SNI mice. 28 days after surgery SNI animals showed an increase of IBA-1 expression that was
340 completely prevented by YEO, consistently to the effect observed in the spinal cord tissue. It has been
341 reported that microglia could be involved in several mood disorders (Wohleb, 2016) and could
342 represent an important target for innovative therapeutic approach. The alteration of the physiological
343 role of microglia could influence BDNF levels. BDNF is a crucial neurotrophin for the maintenance
344 of neurons in brain systems associated with cognitive and affective function. In SNI mice we saw an
345 increased expression of BDNF compared to the control group which was totally prevented by YEO.
346 The role of BDNF in neuropathic pain and anxiety is yet controversial. (Garraway and Huie, 2016).
347 The TrkB-Erk-CREB pathway has been described to prevent microglia activation, thereby inhibiting
348 neuroinflammation by increasing BDNF signalling (Wu et al., 2020). YEO did not alter ERK
349 activation, suggesting that the other pathways might be implicated in its cellular mechanism

350

351 **5. Conclusions**

352 In this work, we demonstrated that the oral administration of YEO reduces SNI-induced neuropathic
353 pain and related symptoms in mice. Specifically, YEO, administered at analgesic doses, ameliorates

354 anxiety-related symptoms that appeared 28 days after surgery. Our data demonstrate that the
355 pharmacological effects of YEO depend on an inhibition of microglia activation, reduction of
356 neuroinflammation, and promotion of controlling effect on neurotrophin levels in spinal cord and
357 hippocampus. In conclusion YEO could represent an interesting candidate for the management of
358 neuropathic pain and pain-associated conditions.

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361 supervised the experiments, provided funding and facilities, performed the formal analysis of results as well as
362 writing, review and editing of the manuscript. V.L. supervised the work, corrected the final draft as well as
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364

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479 **Figure Legends**

480

481 **Fig. 1 Antinociceptive profile of YEO.** **a)** A dose-response curve showed antinociceptive activity
 482 of YEO (0.1–60 mg/kg p.o.) against an acute thermal stimulus (hot plate test). (One-way
 483 ANOVA, * $p < 0.01$ *** $p < 0.001$ vs CTRL; §§ $p < 0.01$ § $p < 0.05$ vs MORPH). Red dashed line
 484 represents the CTRL group. **b)** Time-course experiments with YEO (30 mg/kg p.o.) in comparison
 485 with morphine (7 mg/kg i.p.) in the hot plate test. (Two-way ANOVA, treatment *** $p < 0.0001$;
 486 * $p < 0.05$ vs CTRL). **c)** Mice that underwent spared nerve injury (SNI) showed mechanical
 487 allodynia in the ipsilateral side in comparison with the contralateral side (red dashed line), on day 7
 488 after surgery. YEO (30 mg/kg p.o.) prevented mechanical hypersensitivity. Pregabalin
 489 (30 mg/kg i.p.) was used as reference drugs. (One-way ANOVA, °° $p < 0.001$, ° $p < 0.01$ vs.
 490 contralateral side; §§ $p < 0.01$ vs. pregabalin) **d)** Time-course experiments with YEO (30 mg/kg p.o.)
 491 (One-way ANOVA, * $p < 0.01$ *** $p < 0.001$ vs pre-test).

492 **Fig. 2 Effect of YEO on anxiety and depression in naïve and in SNI mice.** Anxiolytic-like
 493 activity of LEO showed by a reduction of a) the time spent in the light chamber, b) the number of
 494 buried marbles c) and the latency and (d) the quantity of food eaten (Two-way ANOVA, * $p < 0.05$
 495 vs CTRL naïve, § $p < 0.05$ vs CTRL naïve, ° $p < 0.05$ vs CTRL SNI). e) The food consumption was
 496 evaluated as the cumulated amount of food eaten over a 90-min period in 4 h food-deprived mice.
 497 YEO (30 mg/kg p.o.) didn't alter food consumption compared to naïve group. f) YEO didn't induce
 498 antidepressant-like effects (30 mg/kg p.o.) in the tail suspension test (Two-way ANOVA, § $p < 0.05$
 499 vs CTRL naïve).

500 **Fig. 3 Lack of impairment of motor coordination a), spontaneous mobility b), and exploratory**
 501 **activity c)** in mice treated with LEO (Two-way ANOVA *** $p < 0.001$ * $p < 0.05$ vs SNI, ° $p < 0.05$ vs
 502 VEH SNI).

503 **Fig. 4 Effect of YEO on MAPK phosphorylation in the spinal cord of SNI mice.** YEO (30 mg/kg
 504 p.o.) prevented the increase in the phosphorylation of **a)** p38 and **b)** JNK1 but didn't alter **c)**
 505 ERK1/2 activation induced by SNI 28 days after surgery (One-way ANOVA ** $p < 0.01$ * $p < 0.05$ vs
 506 sham-operated control group (red dashed line) °° $p < 0.01$ ° $p < 0.05$ vs VEH SNI), Representative blots
 507 were reported in each panel.

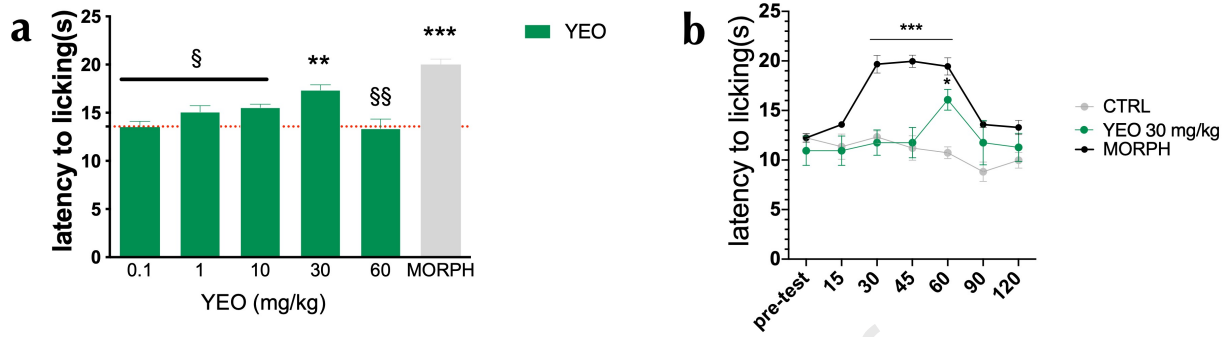
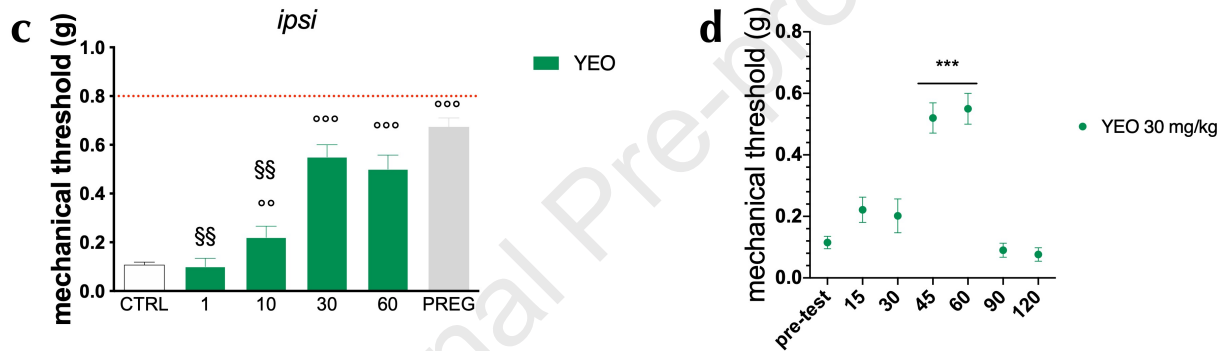
508 **Fig. 5 YEO (30 mg/kg p.o.) prevented the increased expression of a) NOS2 and b) p-p65 induced**
 509 **by SNI 28 days after surgery in spinal cord tissue. c)** Lack of effect of YEO on GFAP decreased
 510 expression compared to SNI mice (One-way ANOVA, *** $p < 0.001$ * $p < 0.05$ vs sham-operated

511 control group (red dashed line), $^{\circ\circ}p<0.01$ $^{\circ}p<0.05$ vs VEH SNI). Representative blots were reported
512 in each panel.

513 **Fig. 6** YEO (30 mg/kg p.o.) prevented the increased expression of **a)** IBA-1 and **b)** BDNF in
514 hippocampus induced by SNI 28 days after surgery. **c)** YEO increased p-ERK expression compared
515 to SNI mice (One-way ANOVA, $***p<0.001$ vs sham-operated control group (red dashed line),
516 $^{\circ\circ}p<0.001$ $^{\circ\circ}p<0.01$ $^{\circ}p<0.05$ vs VEH SNI). Representative blots were reported in each panel.

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Anxiolytic-like effects