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

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ABSTRACT

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

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

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

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

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

1. Introduction

Neuropathic pain (NP) is a chronic condition that occurs due to an injury or disease of the somatosensory system. Generally, NP affects 7%–10% of the world's population and its prevalence is likely to increase with ageing, cancer survival and other chronic disease (Calvo et al., 2019). The currently available drugs used for the management of NP are inadequate due to poor efficacy and tolerability (Finnerup et al., 2015). Anxiety, depression and other mood disorders are comorbidities that characterise about 34% of patients with NP, extensively affecting the patient's quality of life. However, there are no effective and safe treatments that can deal with the symptoms and comorbidity of NP (Radat et al., 2013). Indeed, existing therapies are characterised by several side effects that impede their continued use. Thus, there is an urgent need to develop new and more effective therapies. In particular, the induction of an analgesic activity together with an antidepressant/anxiolytic effect could improve the patient's overall quality of life. An increasing number of patients choose alternative medicine to relieve the symptoms of various pathological processes, including pain and aromatherapy, consisting in the medicinal uses of essential oils extracted from aromatic

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

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plants, is one of the most used (Cooke and Ernst, 2000). Several clinical studies have reported that massage or inhalation with selected essentials oils reduced symptoms associated to pain and anxiety (Dehghanmehr et al., 2017; Tabatabaeichehr and Mortazavi, 2020).

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

2. Material and methods

2.1. Animals

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

2.2. Chemicals and drug administration

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

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

2.3. Evaluation of antinociceptive activity

2.3.1. Hot plate test (HPT)

The hot plate test was performed following the protocol described by Borgonetti and co-workers (Borgonetti et al., 2020d). Mice were placed on a hot plate (Ugo Basile Biological Research Apparatus, Varese, Italy), with the temperature adjusted to 52.5 ± 0.1 °C.

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

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

2.4. Evaluation of anxiolytic-like effect

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

2.4.1. Light dark box (LDB)

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

2.4.2. Marble-burying test

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

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

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

2.6. Evaluation of antidepressant activity

2.6.1. Tail suspension test (TST)

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

2.7. Evaluation of locomotor behaviour

2.7.1. Rotarod test

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

2.7.2. Hole board test

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

2.8. Western blotting analysis

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

Protein samples (30 μg of protein/sample) were separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were then blotted onto nitrocellulose membranes (90 min at 110 V) using standard procedures. Membranes were blocked in PBST (PBS with 0.1% Tween) containing 5% non-fat dry milk for 90 min and incubated overnight at 4 °C with primary antibodies p-p38 (1:750; Santa Cruz Biotechnology, Dallas, TX, USA), p-JNK1 (1:1000; Santa Cruz Biotechnology),p-ERK1/2 (1:1000; Cell Signaling Technology, Danvers, MA, USA), NOS2 (1:500; Cell Signaling Technology), p-p65 (1:1000; Santa Cruz Biotechnology), BDNF (1:500; Santa Cruz Biotechnology), IBA-1 (1:500; Santa Cruz Biotechnology), GFAP (1:500; Santa Cruz Biotechnology). The day after, blots were rinsed three times with PBST and incubated for 2 h at room temperature with HRP-conjugated mouse anti-rabbit (1:3000) (Santa Cruz Biotechnology) and goat anti-mouse (bs-0296G,1:5000) (Bioss Antibodies, MA, USA) and then detected by chemiluminescence detection system (Life Technologies Italia, Monza, Italy). Signal intensity (pixels/mm2) was quantified using ImageJ (NIH). The signal intensity was normalized to that of GAPDH (1:5000 Santa Cruz Biotechnology).

2.9. Statistical analysis

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

3. Results

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

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

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

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

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

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

In the LDB test YEO-treated naïve mice spent more time in the light chamber in comparison to VEH-treated mice (Fig. 1a). Consistently,

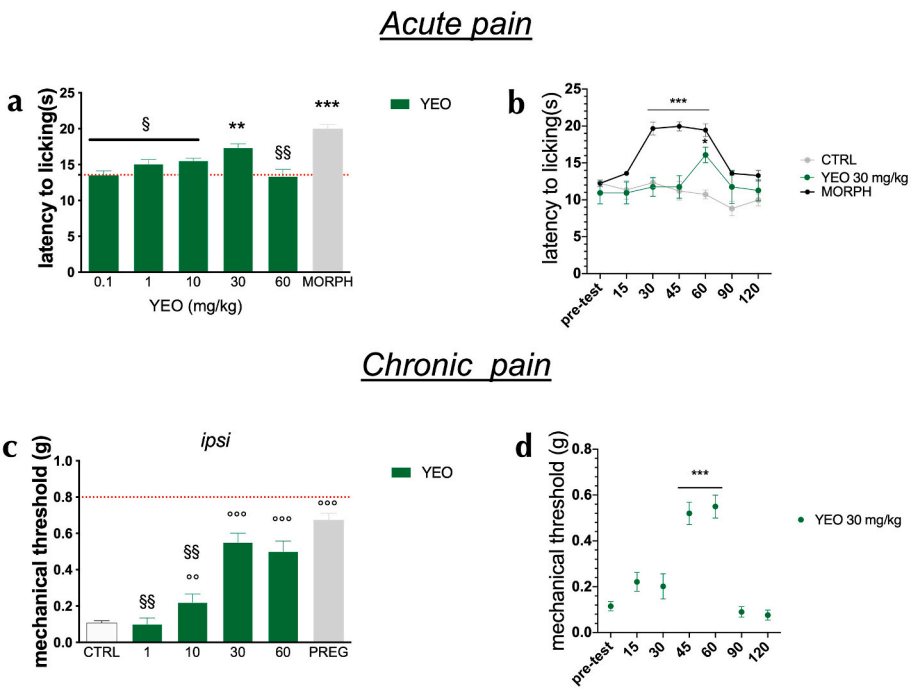

Fig. 1. Antinociceptive profile of YEO. **a)** A dose-response curve showed antinociceptive activity of YEO (0.1–60 mg/kg p.o.) against an acute thermal stimulus (hot plate test). (One-way ANOVA, *p < 0.01 ***p < 0.001vs CTRL; §§p < 0.01§p < 0.05 vs MORPH). Red dashed line represents the CTRL group. **b)** Time-course experiments with YEO (30 mg/kg p.o.) in comparison with morphine (7 mg/kg i.p.) in the hot plate test. (Two-way ANOVA, treatment ***p < 0.0001; *p < 0.05 vs CTRL). **c)** Mice that underwent spared nerve injury (SNI) showed mechanical allodynia in the ipsilateral side in comparison with the contralateral side (red dashed line), on day 7 after surgery. YEO (30 mg/kg p.o.) prevented mechanical hypersensitivity. Pregabalin (30 mg/kg i.p.) was used as reference drugs (One-way ANOVA, °p < 0.001, °p < 0.01 vs. contralateral side; §§p < 0.01 vs. pregabalin). **d)** Time-course experiments with YEO (30 mg/kg p.o.) (One-way ANOVA, *p < 0.01 ***p < 0.001vs pre-test). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

YEO-treated mice buried a lower number of marbles, compared to control group (Fig. 1b). These results were confirmed in the NFST, where the latency time to feed is reduced in mice treated with YEO (Fig. 1c).

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

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

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

3.4. Lack of locomotor behaviour impairments

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

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

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

MAPKs represent an important target involved in neuropathic pain. As previously reported (Sanna,2015), 28 days after surgery an increase of p-ERK1/2 (Fig. 4a), p-p38 (Fig. 4b) and p-JNK1 (Fig. 4c) protein expression in the ipsilateral side of SNI spinal cord mice is observed, compared to the CTRL group (dashed red line). The oral administration of YEO 30 mg/kg did not significantly alter ERK1/2 phosphorylation (Fig. 4a). Conversely, YEO reduced the up-regulation of p-p38 (Fig 4b), and p-JNK1(Fig. 4c) in SNI-mice. These results might indicate a prominent effect on glia cells compared to neuronal cells.

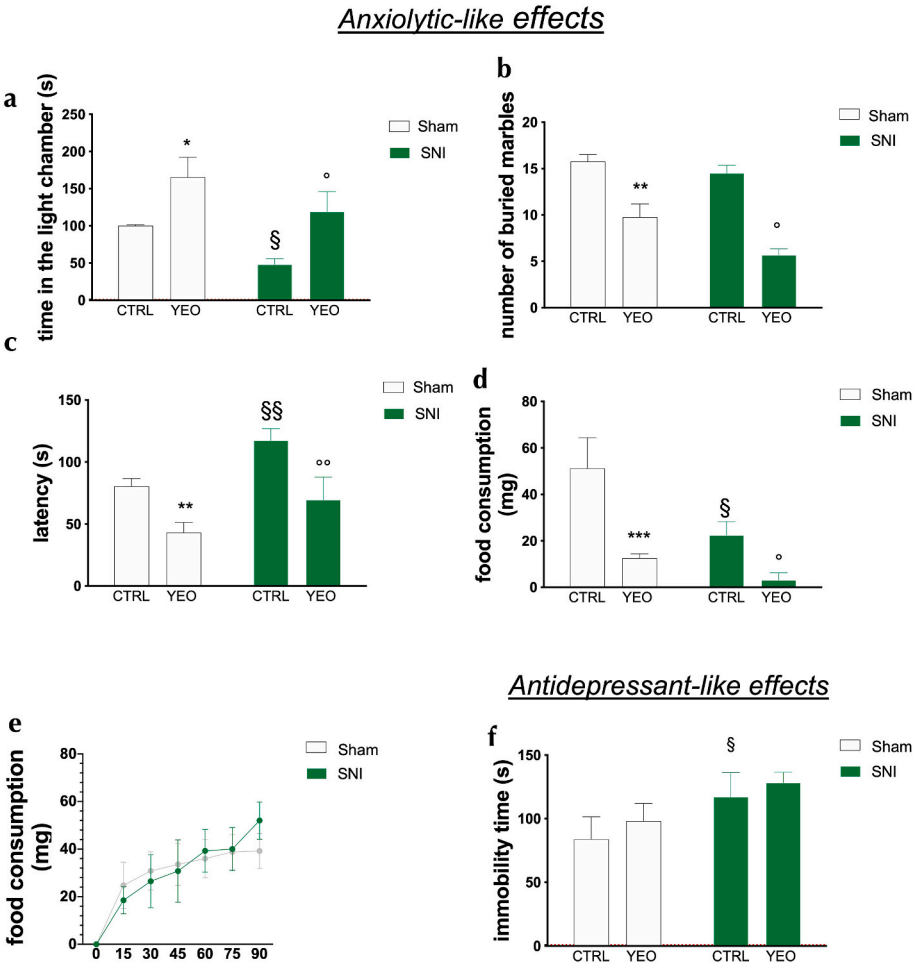

Fig. 2. Effect of YEO on anxiety and depression in naïve and in SNI mice. Anxiolytic-like activity of LEO showed by a reduction of a) the time spent in the light chamber, b) the number of buried marbles c) and the latency and (d) the quantity of food eaten (Two-way ANOVA, *p < 0.05 vs CTRL naïve, §p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

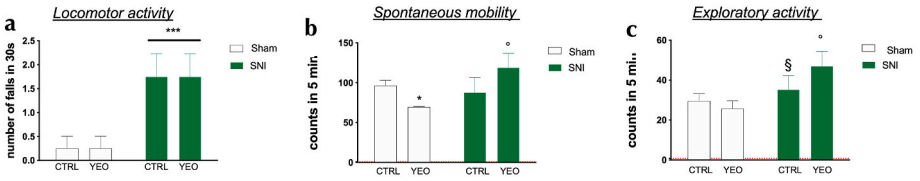

Fig. 3. Lack of impairment of motor coordination **a)**, spontaneous mobility **b)**, and exploratory activity **c)** in mice treated with LEO (Two-way ANOVA ***p < 0.001 *p < 0.05 vs SNI, °p < 0.05 vs VEH SNI).

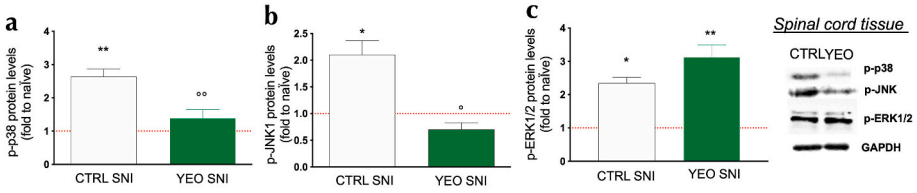

Fig. 4. Effect of YEO on MAPK phosphorylation in the spinal cord of SNI mice. YEO (30 mg/kg p.o.) prevented the increase in the phosphorylation of **a)** p38 and **b)** JNK1 but didn't alter **c)** ERK1/2 activation induced by SNI 28 days after surgery (One-way ANOVA **p < 0.01 *p < 0.05 vs sham-operated control group (red dashed line) °p < 0.01 °p < 0.05 vs VEH SNI), Representative blots were reported in each panel. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

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

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

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

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

4. Discussion

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

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

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

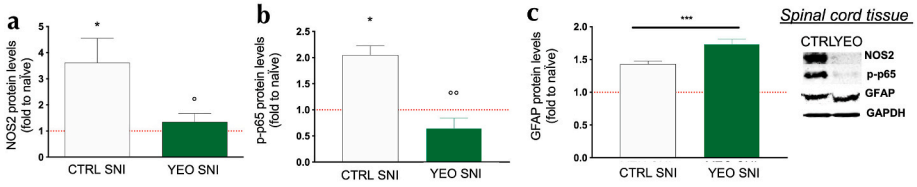

Fig. 5. YEO (30 mg/kg p.o.) prevented the increased expression of **a)** NOS2 and **b)** p-p65 induced by SNI 28 days after surgery in spinal cord tissue. **c)** Lack of effect of YEO on GFAP decreased expression compared to SNI mice (One-way ANOVA, ***p < 0.001 *p < 0.05 vs sham-operated control group (red dashed line), °p < 0.01 °p < 0.05 vs VEH SNI). Representative blots were reported in each panel. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

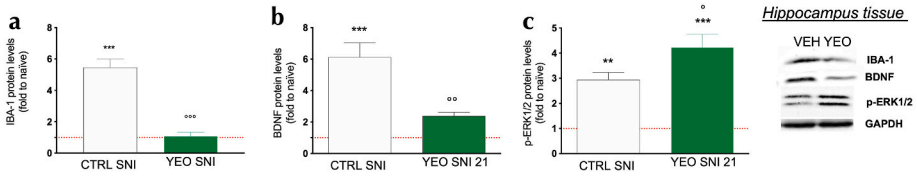

Fig. 6. YEO (30 mg/kg p.o.) prevented the increased expression of **a)** IBA-1 and **b)** BDNF in hippocampus induced by SNI 28 days after surgery. **c)** YEO increased p-ERK expression compared to SNI mice (One-way ANOVA, ***$p < 0.001$ vs sham-operated control group (red dashed line), $p < 0.001$ °$p < 0.01$ °$p < 0.05$ vs VEH SNI). Representative blots were reported in each panel. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

with neuropathy after oral administration.

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

MAPK activation is a fundamental pathway in the spinal cord of SNI mice for the evolution of pathological properties of neuropathic pain (Borgonetti et al., 2020c). YEO reduced the activation of p-p38 and p-JNK1, while not affecting p-ERK1/2 levels. These results indicate a prominent role of glia activation in the cellular effects of YEO. Indeed, p38 is strongly involved in the transcriptional activity of microglia genes in neuropathic-pain mice (Bhatia et al., 2017), while JNK is mainly expressed in astrocytes cells in the spinal cord of SNI mice (Gao et al., 2009). To better elucidate if the effect of YEO could be on microglia/astrocytes or in both cell type, we tested its effect on the most known targets of these cells. In inflammatory condition NOS2 is detected in microglia, where it induces an increase of NO production (Béchade et al., 2014). NOS2 is also involved in chronic pain, as an increase in this factor has been detected in mice with neuropathy (Hervera et al., 2010). YEO totally reverted the SNI-induced increase of NOS2. Another important marker of microglia activation is represented by p-p65, which is a transcriptional factor that promotes transcription of genes associated to inflammation. Inhibitor of this pathway are considered good candidate for the management of neuropathic pain symptoms (Wang et al., 2020). YEO reduced the activation of p-p65, returning its expression to basal levels.

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

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

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

5. Conclusions

In this work, we demonstrated that the oral administration of YEO reduces SNI-induced neuropathic pain and related symptoms in mice. Specifically, YEO, administered at analgesic doses, ameliorates anxiety-related symptoms that appeared 28 days after surgery. Our data demonstrate that the pharmacological effects of YEO depend on an inhibition of microglia activation, reduction of neuroinflammation, and promotion of controlling effect on neurotrophin levels in spinal cord and hippocampus. In conclusion YEO could represent an interesting candidate for the management of neuropathic pain and pain-associated conditions.

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Author contributions

V.B. performed the experiments and wrote the first draft of the manuscript. N.G. supervised the experimets, provided funding and facilities, perfomed the formal analysis of results as well as writing, review and editing of the manuscript. V.L. supervised the work, corrected the final draft as well as acquired funding.

Declaration of competing interest

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