



**UNIVERSIDADE FEDERAL DE SERGIPE
CENTRO DE CIÊNCIAS BIOLÓGICAS E DA SAÚDE
DEPARTAMENTO DE FARMÁCIA**

JANARA SANTOS COSTA

**α -TERPINEOL REDUCES CANCER PAIN VIA MODULATION
OF OXIDATIVE STRESS AND INHIBITION OF iNOS**

SÃO CRISTÓVÃO-SE
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Trabalho de Conclusão de Curso apresentado à
Coordenação do Curso de Farmácia do Centro de
Ciências Biológicas e da Saúde da Universidade
Federal de Sergipe, como requisito parcial para
obtenção do título de Bacharel em Farmácia.

Orientadora: Profa. Dra. Adriana Gibara
Guimarães

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Trabalho de Conclusão de Curso, famoso TCC, não significa apenas um requisito obrigatório da graduação, simboliza a finalização de várias etapas importantes em minha vida, nas quais pude evoluir como ser humano e profissional.

Agradeço a Deus pela oportunidade da vida, saúde e força para superar as dificuldades. Obrigada a minha família pelo carinho, apoio e incentivo. Agradeço a universidade da qual faço parte por ter me recebido e ter proporcionado dias de aprendizagem muito ricos, sendo um ambiente propício à evolução e conhecimento. Ao corpo docente pelos recursos e ferramentas que me permitiram evoluir um pouco mais todos os dias, obrigada pelo conhecimento compartilhado!

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Muito obrigada!

Janara Costa



α -Terpineol reduces cancer pain via modulation of oxidative stress and inhibition of iNOS



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ABSTRACT

α -Terpineol (TP) is present in a wide range of essential oils of the genus *Eucalyptus*, with recognized potential for a range of biological effects, such as analgesic. Hence, our study aimed to investigate the effect of TP on cancer pain induced by sarcoma 180 in Swiss mice. Our results showed that TP reduced significantly mechanical hyperalgesia and spontaneous and palpation-induced nociception, improved paw use without reducing tumor growth and grip strength. Importantly, no evident biochemical and hematological toxicity was observed. Furthermore, TP increased the tissue antioxidant capacity due to ferric-reducing antioxidant power (FRAP) and glutathione (GSH). TP also reduced inducible nitric oxide synthase (iNOS) immuncontent in the tumors. Molecular docking estimated that TP binds within the same range of iNOS regions (other iNOS inhibitors), such as N-Nitroarginine methyl ester (L-NAME). These data provide strong evidence that TP may be an interesting candidate for the development of new safe analgesic drugs that are effective for cancer pain control.

1. Introduction

The prevalence of cancer is increasing globally, with 17 million new cases predicted for 2020, whereby pain is one of the most prevalent, costly and distressing symptoms experienced by cancer patients [1]. Oncologic pain affects 75–90% of patients with advanced disease stage and can be considered as a significant factor for life quality impairment [2,3].

Oncologic pain presents a complex and multifactorial neurobiology. The excessive proliferative status of cancer cells is considered as a fundamental property of cancer [4]. Besides, the external microenvironment that surrounds the cancer cells can also be altered by the activation of the immune system, with the recruitment of macrophages, neutrophils and T-cells and the consequent production of inflammatory mediators [5]. Inflammation is often accompanied by increased reactive species, revealing that the oxidative microenvironment surrounding tumor cells is highly associated with increased oxidative

stress, either directly by cancer cells or indirectly by the activation of the immune system [6].

Because of its pathophysiological complexity, up to 15% of nearly 7 million chronic pain patients can not be completely relieved by the conventional management of this symptom. That occurs largely due to the side effects associated with the treatment such as constipation, nausea, vomiting, sedation, respiratory depression, dependence, tolerance, bleeding, gastrointestinal ulceration, renal toxicity, hypotension, cardiotoxicity, among others [5,6]. In this sense, a systematic review has showed that medicinal plants have historically proved their therapeutic potential and, today, still stand out as an important grouping for the identification of new analgesic drugs [7,8].

α -terpineol (TP) is an alcoholic monoterpene found in the essential oil of several species belonging to the genus *Eucalyptus* [9]. This monoterpene has peripheral-mediated antinociceptive effect due to its ability to inhibit the cyclooxygenase enzyme and the production of inflammatory mediators and cytokines such as prostaglandin E2

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(PGE₂), interleukin-1 β (IL-1 β) and nuclear factor kappa B (NF- κ B) [10–15]. This substance also modulates the levels of nitric oxide (NO), an important marker of oxidative stress [16]. However, studies that show the effect of TP on chronic pain, such as cancer pain, are scarce.

Considering the need for new therapeutic options for oncologic pain and the therapeutic potential of monoterpenes, especially TP, this study aimed to evaluate the effect of TP on nociceptive responses induced by sarcoma 180 (S180) in rodents.

2. Methods

2.1. Chemicals

α -Terpineol (96% purity), cremophor, sodium chloride, tripan blue, nitrobenzoic acid, glycerol, β -mercaptoethanol, bromophenol blue, tween, thiobarbituric acid, Tetraethoxypropane (TEP), tripyridyl-triazine (TPTZ), dinitrobenzoic acid (DTNB) and glutathione (GSH) were purchased from Sigma (USA). Morphine and lactated ringer's solution were purchased from Cristália (São Paulo, São Paulo, Brazil). Ketamine and Xylazine were purchased from Cristália (Itabira-SP, Brazil). Acetic acid, hematoxylin and eosin were purchased from Synth and EDTA from Neon. Protease inhibitors were derived from Sigma (FAST), primary anti-iNOS and anti- β -actin antibodies (Santa Cruz Biotechnology Inc - Santa Cruz, CA, USA). Anti-rabbit IgG-HRP and IgG-HRP anti-mouse secondary antibodies were purchased from Sigma (St. Louis, MO).

2.2. Animals

Male Swiss mice used (28–32 g; 2–3 months of age) were randomly housed in appropriate cages at 22 \pm 2 °C on a 12 h light/dark cycle with free access to food (Purina®, Brazil) and water. Experimental protocols were approved by the Animal Care and Use Committee (CEPA/UFS 05/14) at the Universidade Federal de Sergipe, and all handling procedures were in accordance with the International Association for the Study of Pain (IASP) guidelines for the use of animals in pain research.

2.3. Tumor cell and implantation

S180 tumor cells that had been maintained in the peritoneal cavity of Swiss mice were obtained from the Laboratory of Clinical and Experimental Oncology at the Federal University of Sergipe. A suspension of 10⁶ viable S180 cells per 25 μ l of lactated Ringer's solution was implanted subcutaneously into the plantar region of mice. Animals of the sham group received only 25 μ l of lactated Ringer's solution [17,18].

2.4. Treatment

Twenty-four hours after administration of S180, animals (n = 08/group) were treated daily with vehicle (saline + cremophor 0.4% v/v), TP (12.5, 25 or 50 mg/kg) or morphine (15 mg/kg) via subcutaneous route until the fifteenth day and were then submitted to behavioral evaluation on alternate days. The route of administration of the treatments was subcutaneous so that the hepatic first-pass effect was avoided. The animals were randomly distributed between the groups and the evaluations were performed blindly in order to reduce the assessment bias.

2.5. Behavioral studies

The mechanical hyperalgesia was assessed by means of digital von Frey (Model: EFF-301, Insight®, Brazil) through hind paw flexion reflex, which corresponds to the paw withdrawal followed by clear flinching movements. In order to evaluate the spontaneous nociception, mice

were placed scattered in boxes and allowed to acclimate for 10 min. Afterwards, the flinching behaviors were observed during a 10-min period. Non-noxious palpation of the tumor-bearing paw was performed during 2 min and the number of flinching behaviors was quantified for 2 min to determine the palpation-induced nociception [19]. The use of the limb was evaluated as previously described by Luger et al. [20], through the observation of the mouse while walking in a continuous movement. The limb and/or guard behavior of the right hind limb (treated with sarcoma) was evaluated in the following scale: 0 = complete lack of use, 1 = partial limb use in locomotor activity, 2 = limb and guard behavior, 3 = substantial limping and 4 = normal walking [20].

2.6. Measurement of forelimb grip strength

In order to check for possible changes in neuromuscular function, such as the myorelaxant effect, we measured the tension force of limbs using the commercial grip strength meter (Insight®, Brazil) before the treatment (s.c.) of tumor-free animals with vehicle or TP (12.5, 25 or 50 mg/kg) and 30, 60 and 120 min after treatment [21].

2.7. Measurement of paw volume

The effect of TP on tumor growth of S180 was evaluated through right paw volume, which was measured using plethysmometer (Insight®, Brazil) before (time zero) and on every other day up to 15 days.

2.8. Toxicity

Toxicity assessment was performed by means of the weight control of the animals and behavioral changes prior to tumor inoculation daily until the 15th day of the experiment. On the last day, the biochemical and hematological analyses were performed to evaluate glutamic-oxalacetic transaminase, glutamic-pyruvic transaminase, urea, creatinine, the total and differential leukocyte count, red blood cells, hemoglobin, hematocrit and platelets. The weights of vital organs such as heart, lung, brain, liver and kidneys were checked after the euthanasia of the mice, which were macroscopically observed. The relative weight of the organs was calculated as follows: Relative weight = [(body weight/body weight) x 100]. After that, the organs were submitted to histopathological analysis after staining with hematoxylin and eosin. Finally, the slides were analyzed in an optical microscope and documented in a photo microscope (Olympus®) using standard and polarized polychromatic light.

2.9. Histology

Different groups of animals were euthanized with excessive sedation on the 15th day after sarcoma inoculation. The paws were then submitted to fixation with 10% formalin solution, decalcified in 10% EDTA (pH 7.4) for two weeks and submitted to conventional histological processing. Sections were cut in the sagittal plane and stained with hematoxylin and eosin for optical microscopic visualization of the histopathological characteristics of the tumor [22].

2.10. Antioxidant capacity of tissues

On the 15th day, tumor, spinal cord and brain were homogenized using turrax-type homogenizer in ice bath and 0.1 M potassium phosphate buffer pH 7.0. The homogenate was centrifuged at 15,000 rpm for 30 min at 4 °C. The supernatant was used for the determination of reducing power (FRAP), glutathione (GSH) and thiobarbituric acid reducing substances (TBARS).

For the FRAP assay, in a 96-well plate, a 9- μ l aliquot of the homogenate was mixed with 27 μ l of distilled water and 270 μ l of the FRAP

fresh reagent and incubated at 37 °C [23]. After 30 min, the absorbance was read at 595 nm. A ferrous sulfate curve was prepared under the same conditions and results were expressed in mM ferrous sulfate/ mg tumor protein, spinal cord and brain.

For GSH, the homogenates were resuspended in a solution of EDTA (10–3 M) in 5% TCA [24]. Subsequently, they were centrifuged at 15,000g for 15 min at 4 °C and the supernatant was collected. For the assessment, 275 µl of phosphate buffer pH 8.0 and 20 µl of the GSH-containing supernatant were placed in a 96-well plate. Then, 10 µl of DTNB (5 mM, dissolved in ethanol) was added and after 5 min, the absorbance was read at 412 nm. The GSH concentration was quantified using a standard GSH curve. The results were expressed in mM GSH/g tumor protein, spinal cord and brain.

The assessment of TBARS was performed by the method of Ohkawa, Ohishi and Yagi [52] with some modifications: in tubes, there was the addition of 100 µL of homogenate, 350 µL of 20% acetic acid (pH 3.5) and 600 µL of thiobarbituric acid (TBA 0.5%, dissolved in acetic acid). The tubes were incubated in a thermostated bath for 1 h at 85 °C. They were then cooled in an ice bath and centrifuged at 10,000 rpm for 15 min at 4 °C and the absorbance was measured at 532 nm. A tetraepoxypropane (TEP) curve was made and the results were expressed in µM of TEP/g tumor protein.

2.11. Western blot

Immunoblotting was performed as previously described. Forty micrograms of protein was fractionated by SDS-PAGE and transferred to nitrocellulose membranes with 0.45-µm pore (Bio-Rad, CA, USA). Protein loading and transferred efficiency were verified through Ponceau S staining. The membranes were washed with TTBS (100 mM Tris-HCL, pH 7.5, containing 0.9% NaCl and 0.1% Tween-20) and then were blocked for 2 h in Tris-buffered saline-Tween 20 containing 5% non-fat dry milk at room temperature. After, the membranes were incubated overnight at 4 °C with rabbit polyclonal and monoclonal antibodies anti-β-actin (1:300, sc-32233) and anti-iNOS (sc-8310), from Santa Cruz biotechnology. After that, they were washed and incubated for 2 h at room temperature with peroxidase-conjugated secondary antibody (1:2000, anti-rabbit and anti-mouse IgG-HRP, Sigma, MO, USA). Immunodetection was performed using enhanced chemiluminescence (Luminata strong™ - Western HRP substrate, Merck-Millipore, MA, USA). Digitalized images were analyzed through densitometry using the ImageJ 1.40 software (NIH).

2.12. Docking studies

The structure of the Nitric Oxide Synthase enzyme (ID PDB 1m7z) was downloaded from Protein Data Bank (<http://www.rcsb.org/pdb/home/home.do>). Using the Hyperchem v. 8.0.3, the chemical structure of the binders was designed: TP, aminoguanidine, dexamethasone, N-nitro-L-arginine methyl ester (L-NAME) and their geometry was optimized using MM + force field.

Following, a new optimization of the geometry based on the semi-empirical method AM1 (Austin Model 1) was performed. The optimized framework underwent conformational analysis using the Spartan software for Windows 10.0. The random search method was selected with 1000 interactions, 100 optimization cycles and 10 minimum low-energy shapers. The dihedrals were evaluated through rotation according to the standard (absence) conditions of the program, in which the number of simultaneous variations was from 1 to 8, the acyclic chains were rotated from 60 to 180° and the torsion rings were in the range of 30 to 120 °C.

The ligands underwent molecular anchoring using the Molegro Virtual Docker v. 6.0.1 (MVD). All water compounds were eliminated from the receivers and the method was prepared using predefined parameters in the same software: 15 Å radius GRID. Moldock score [GRID] algorithm was used as the score function and the search

algorithm was Moldock.

2.13. Statistical analysis

The data obtained were evaluated by means of one- and two-way analyses of variance (ANOVA) followed by Bonferroni's or Tukey's test. Kruskal–Wallis followed by Dunn's test was applied to limb use. In all cases, differences were considered significant if $p < 0.05$, using the Graph Pad Prism (v 5.00) software (San Diego, CA, USA). The percent of inhibition was determined using the following formula: Inhibition% (PI) = 100 (control – experiment) / control, with the data obtained from the area under the curve (AUC).

3. Results and discussion

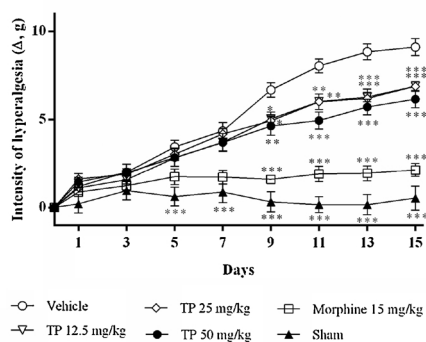
In this study, TP was able to attenuate the nociception induced by the inoculation of S180 cells into the paw of mice. For so, different methods of evaluation were used that imitate the different modalities of pain experienced by cancer patients. The first one was hyperalgesia, which indicates increased sensitivity to painful stimuli with the involvement of C and A-δ fibers [25]. In our study, S180 cells promoted a significant increase of this hyperalgesia in vehicle-treated animals compared to animals of the sham group ($p < 0.001$) on the 9th day, as observed in previous studies [17,18].

The treatment of the animals with TP in doses of 12.5 and 25 mg/kg was able to reduce significantly ($p < 0.05$ on day 9, $p < 0.01$ on day 11 and < 0.001 on days 13 and 15 in both doses) mechanical hyperalgesia from the 9th to the 15th day after administration of the tumor cells, with percentages of inhibition of 24.72% and 30.63%, respectively. TP at 50 mg/kg also reduced hyperalgesia ($p < 0.01$ on day 9 and $p < 0.001$ as of day 11) when compared to vehicle-treated animals, with a percentage of inhibition of 35.17%. Morphine (15 mg/kg) also promoted the relief of the hyperalgesic response ($p < 0.001$) from the 9th to 15th day of tumor induction, with 76.34% inhibition (Fig. 1A). In other studies, TP also triggered an antihyperalgesic effect on chronic muscular pain, probably evoked by the modulation of the opioid and serotonergic (5HT3) systems and pain models induced by carrageenan, TNF-α, PGE₂ and dopamine, with reduction of leukocytes and production of nitric oxide [14,26]. In fact, the modulation of these systems has been the treatment strategy to reduce cancer pain currently worldwide [27].

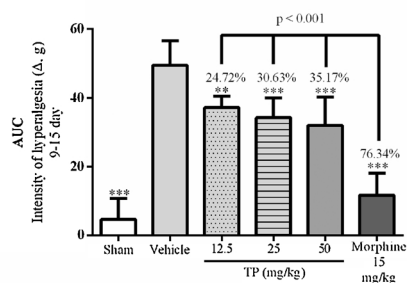
Besides hyperalgesia, we also evaluated the spontaneous nociception, a behavioral response associated with sensitization of C-fiber nociceptors and studied in several pain models [28]. Animals treated with the vehicle had an increase in the number of flinches when compared to animals of the sham group ($p < 0.05$ on day 9, $p < 0.001$ on day 11 to 15). TP 12.5 ($p < 0.05$ on day 15) and TP 25 ($p < 0.01$ at day 15) and TP 50 mg/kg ($p < 0.05$ on day 9, $p < 0.001$ from day 11) were able to significantly reduce the number of spontaneous nociceptive responses (flinches), with percentage inhibition of 38.24; 50.71 and 99.65%, respectively. Treatment with TP 50 mg/kg presented inhibition similar to morphine (99.32%) (Fig. 1B).

In order to mimic allodynia, we measured the nociception evoked by palpation in animals with cancer, which is defined as pain in response to a non-nociceptive stimulus induced by low-threshold fibers, such as A-β fibers [25]. In this study, the intraplantar injection of S180 cells promoted a significant increase in the number of flinches after palpation. As shown in the Figure (Fig. 1C), TP was able to decrease the number of flinches when compared to the vehicle ($p < 0.05$ on day 9, $p < 0.01$ on day 11 and $p < 0.001$ on the 13th and 15th day). The reduction of nociception induced by non-nocice palpation was observed in mice treated with TP 12.5 mg/kg ($p < 0.05$ at day 15), TP 25 mg/kg ($p < 0.001$ on the 15th day) and TP 50 mg/kg ($p < 0.05$ on day 9, $p < 0.01$ on day 11 and $p < 0.001$ on days 13 and 15) when compared to vehicle group. These doses presented inhibition percentages of 16.62, 30, 57 and 95.54%, respectively. There was no significant

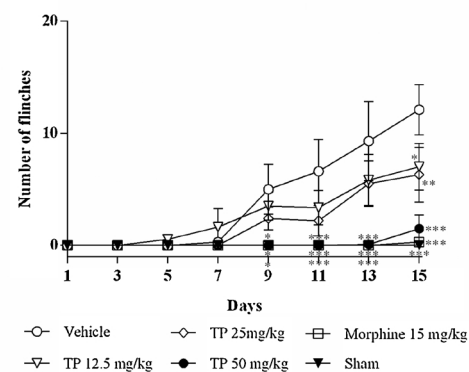
A.1



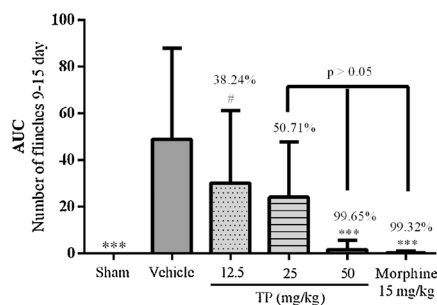
A.2



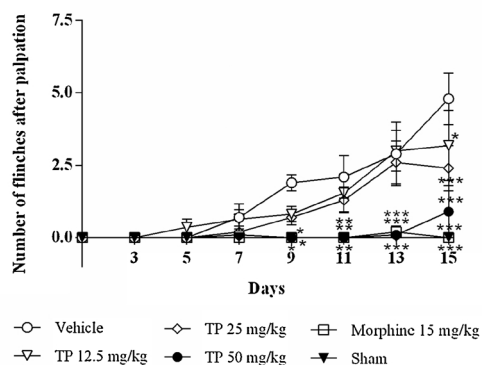
B.1



B.2



C.1



C.2

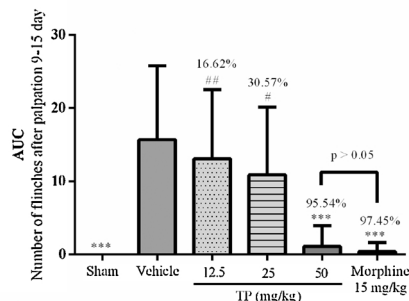


Fig. 1. Effect of α -terpineol (TP, 12.5–50 mg/kg, s.c.) on the mechanical hyperalgesia (A), spontaneous (B) and palpation-induced nociception (C) in mice with S180 (n = 8/group). *p < 0.05, **p < 0.01 and ***p < 0.001 vs. the control group, #p < 0.05, ##p < 0.01, ###p < 0.001 vs. morphine (one-way ANOVA followed by Tukey's test).

difference between the group treated with TP 50 mg/kg and the group that received morphine as treatment, which reduced the allodynia in 97.45% (Fig. 1C).

In the tumor environment, cancer and immunological cells release several inflammatory mediators and cell factors that sensitize A and C fibers, promoting changes in nervous transmission and leading to the perception of different types of pain, including hyperalgesia and allodynia [5]. In our study, TP reduces all nociceptive responses, reinforcing its antinociceptive activity. This compound may reduce the pain thanks to central or peripheral mechanisms, as demonstrated in a study published by Quintans-Júnior et al. [15]. In fact, several studies have showed TP modulation of neurotransmitters [14,26], ion channels [29], decrease in pro-inflammatory cytokines as TNF- α [30], IL-6 [11], IL-1 β [31], increase in IL-10, an important anti-inflammatory cytokine, and

inhibitors of the expression of COX-2 and iNOS [32], via NF-kB and ERK pathways [31,32].

The tumor also caused impairment of limb use evidenced by changes in normal ambulation in animals treated with vehicle compared to the sham group (p < 0.01 on days 9 and 11, p < 0.001 at 13 and 15). The treatments with TP 50 mg/kg (p < 0.01 at day 9 and day 11, p < 0.001 at day 13 and p < 0.05 at day 15) and with morphine (p < 0.01 at day 9 and day 13, p < 0.05 at 11 and 15 days) attenuated the effects induced by sarcoma 180 (Fig. 2A). Animals treated with vehicle increased paw volume when compared to the non-tumor sham group (p < 0.01 on the 9th day and p < 0.001 as of the 11th day). TP was not able to reduce the tumor growth (Fig. 2B), showing that the antinociceptive and antihyperalgesic activity of TP does not depend on the decrease of the tumor mass. In previous studies, TP

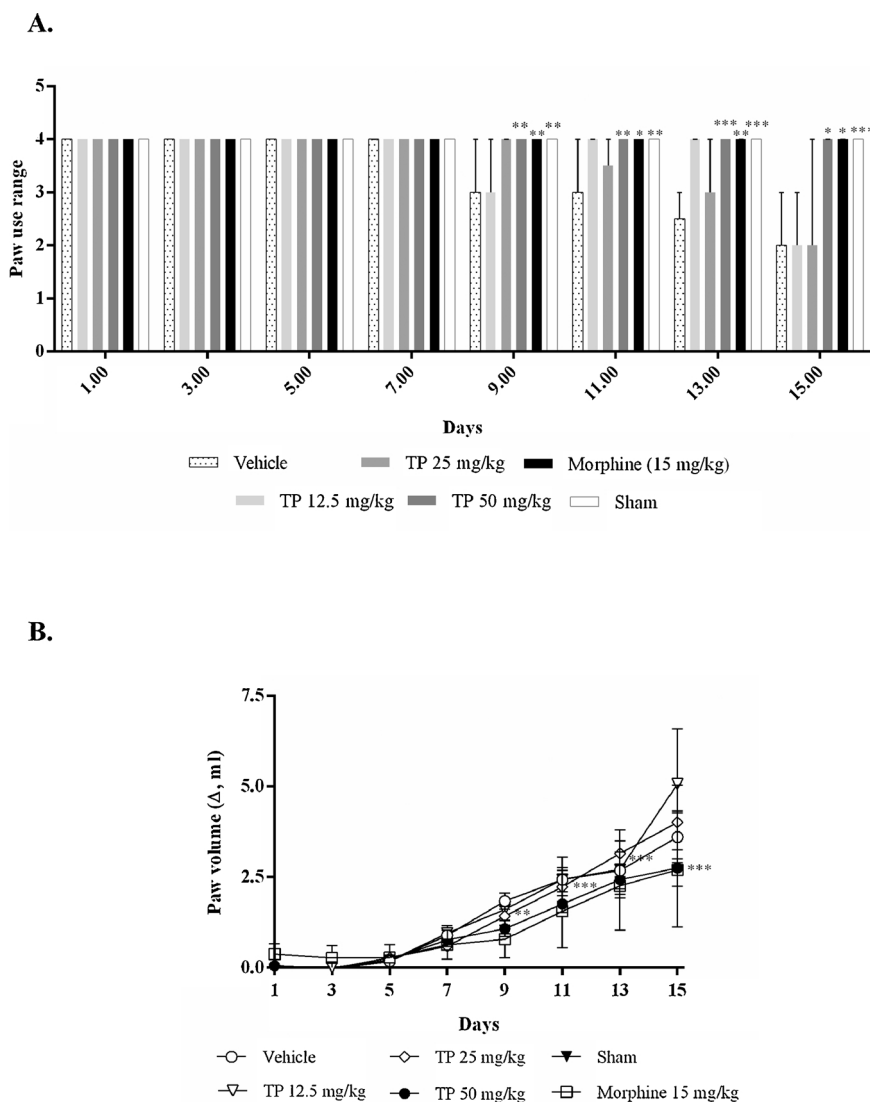


Fig. 2. Effect of terpineol (TP, 12.5–50 mg/kg, s.c.) on the paw use (A) and the tumor growth (B) in mice with S180 ($n = 8/\text{group}$). * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ vs. the control group (Paw use: median with interquartile range, Kruskal–Wallis followed by Dunn's test. Paw volume: mean \pm SEM, two-way ANOVA followed by Bonferroni's test).

reduced the growth of epithelial malignancies and, in an in vitro study, inhibited the growth of tumor cells from blood samples of patients with chronic lymphocytic leukemia [10,33].

Tumor presents an oxidative environment with extracellular release of glutamate, which is responsible for stimulating nerve transmission, causing painful conditions in humans [34,35]. For this reason, the present study also aimed to evaluate the effect of TP on the oxidative stress induced by S180, through the evaluation of the antioxidant capacity in the tumor, brain and spinal cord using the oxidative stress markers FRAP, GSH and TBARS.

In order to evaluate the influence of the tumor and the treatment with TP 50 mg/kg on the oxidative stress, the antioxidant capacity of the tissues was evaluated by the FRAP method. Treatment with TP (50 mg/kg) increased the concentration of substances capable of reducing iron in the tumor when compared to groups receiving vehicle ($p < 0.01$) and morphine ($p < 0.01$) as treatment. This evaluation was not performed with the sham group due to the absence of tumor mass in the animals belonging to this group (Fig. 3A). Similarly, the brain homogenate of animals treated with TP (50 mg/kg) also increased the tissue-reducing ability ($p < 0.05$) when compared to vehicle (Fig. 3B). In contrast, the spinal cord homogenate of animals treated with TP did not show significant differences (Fig. 3C) in FRAP when

compared to the vehicle and morphine groups, although it has showed a tendency to do so. Thus, the reducing capacity of TP, highlighted in FRAP, can contribute to the decrease in pain, once reactive species have an important role in the development of peripheral and central sensitization of the various pain etiologies [36]; also, the oxidation also sensitizes peripheral nociceptors by increasing the excitability of voltage-operated sodium channels [37].

In order to assess tissue redox status, the GSH concentration was determined. Increased GSH concentration was observed in the tumor homogenate ($p < 0.001$ versus vehicle; $p < 0.001$ versus morphine) and the brains ($p < 0.05$ versus vehicle) belonging to the TP-treated group, as shown in Fig. 4A and B, respectively. The homogenates obtained from the spinal cord of animals treated with TP did not present significant differences in GSH concentrations when compared with the vehicle and morphine groups (Fig. 4C). These findings suggest that TP reduces the oxidative stress induced by S180 by preserving GSH levels, preventing the depletion of total antioxidant capacity and oxidative stress, as well as contributing to the reduction of nociceptive responses induced by tumor cells.

Similar results have been observed for other natural products, such as quercetin, which is able to reduce cancer pain with the modulation of GSH levels, indicating that the prevention of GSH depletion may be an

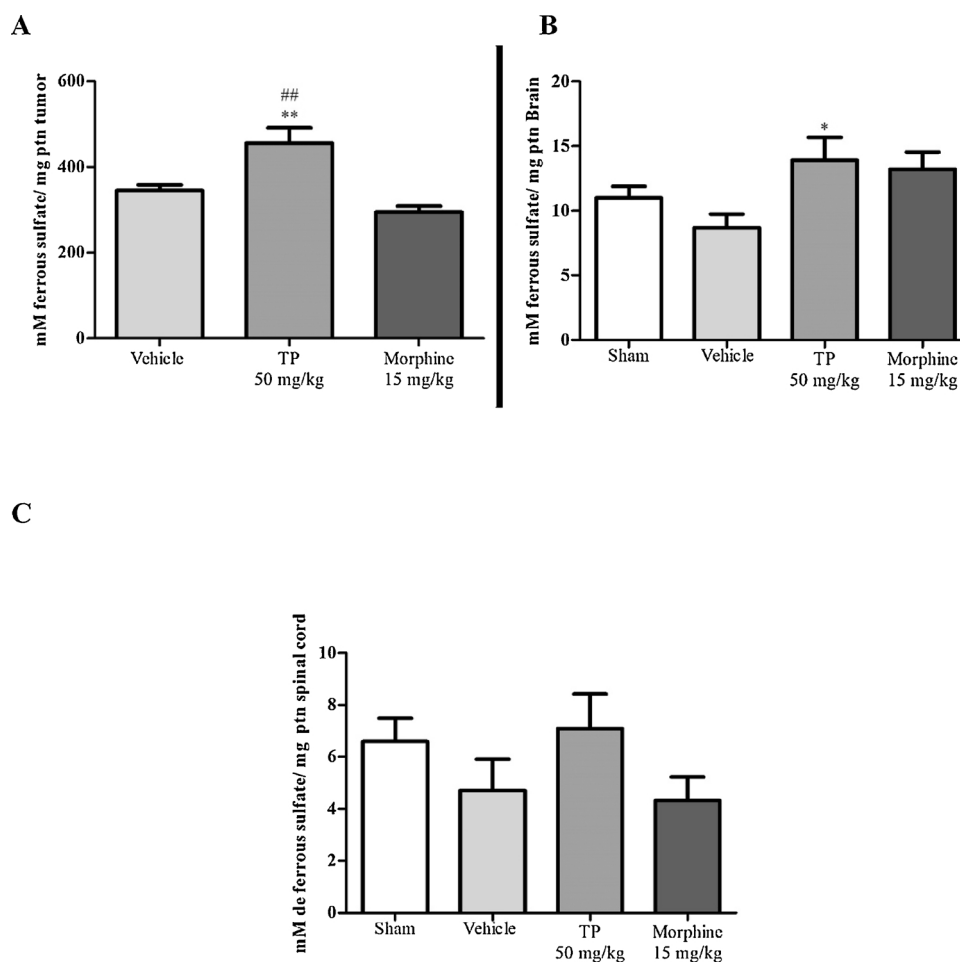


Fig. 3. Influence of TP on the antioxidant capacity (FRAP method) of tumors (A), brain (B) and spinal cords (C) in mice containing S180. * $p < 0.05$, ** $p < 0.01$ versus vehicle group and ^{##} $p < 0.01$ versus morphine group (one-way ANOVA followed by Tukey's post-test).

important analgesic mechanism, as it has been demonstrated [38]. Besides, a recent study shows that the administration of GSH suppressed mechanical allodynia and central sensitization in rats with chronic post-ischemia pain [39]. It is worth noting that rises in GSH levels seem to be more expressive in the tumor and in the brain. On the other hand, changes in glutamate neurotransmission at medullary levels have been most strongly associated with the redox imbalance associated with the tumor [6].

However, no significant differences were found between groups tested for TBARS concentrations in the tumor, an important marker of lipid damage in oxidative stress (Figure S2). That differs from the finding in another study, which shows that TP suppresses lipid peroxidation in the hippocampus, improving cerebral ischemia-related memory impairment in rats [40]. This contradiction can be associated to tissue characteristics, once that the brain is rich in lipids, being a target of peroxidation [41], different from the tumor evaluated.

It is well known that the redox status also influences threshold NO levels within the tumor microenvironment [42]. Indeed, nitric oxide (NO) may act as an important neurotransmitter affecting spinal nociceptive processing in several pain models [43,44]. The isoform iNOS stands apart as it generates more NO than do the constitutive members, expressed after cytokine exposure and involved in the development and maintenance of central and peripheral sensitization in inflammatory and neuropathic pain [45,46]. Several studies have provided consistent evidence that iNOS levels within tumor microenvironment plays a pivotal role in the maintenance of cancer pain [42,43] due to nociceptive sensitization. In accordance, we demonstrated that TP was able to attenuate of immunoccontent of iNOS ($p < 0.01$) induced by tumor-

bearing S180 when compared with vehicle group (Fig. 5).

Besides, molecular docking was performed in order to verify the energy of complex formation of the enzyme nitric oxide synthase together with TP and with inhibitors of this enzyme, such as L-NAME, aminoguanidine and dexamethasone (Fig. 6). TP presented a hydrogen bond (blue lines) and a steric binding with THR324 (red lines), with energy binding close to the energy presented by dexamethasone and L-NAME and lower than the energy of aminoguanidine. Dexamethasone showed three hydrogen bonds: two with the residue THR324 and one with ARG243. In addition, there were steric bonds between apolar atoms with ARG243, THR324 and TRP325. L-NAME presented three hydrogen bonds: with ARG243, THR324 and TRP325 (blue lines) of the enzyme NOS and steric connections with residues ARG243, TRP325, MET236 and PRO329 (red lines). Aminoguanidine showed two hydrogen bonds with the TRP234 residue. In fact, a previous study demonstrated that TP can modulate the nitric oxide pathway, attenuating the dependence and tolerance to morphine by NO production, an effect that was potentiated by L-NAME and antagonized by L-arginia [47].

The modulation of the production of reactive oxygen species seems to be an important target to control pain, since compounds with scavenger effects have showed suppressed action of mechanical allodynia in a rat model of cancer-induced bone pain [48]. In fact, nitric oxid produced by iNOS enzyme in pathologic conditions activates TRPV1 and TRPA1 channels promoting nociception in mice [44] and causing central pain sensitization [49].

During the 15 days of experiment, an increase was observed in the weight of the animals treated with TP 12.5 mg/kg ($p < 0.01$ on the 12th and 15th days; $p < 0.05$ on the 14th day) and TP 50 mg/kg

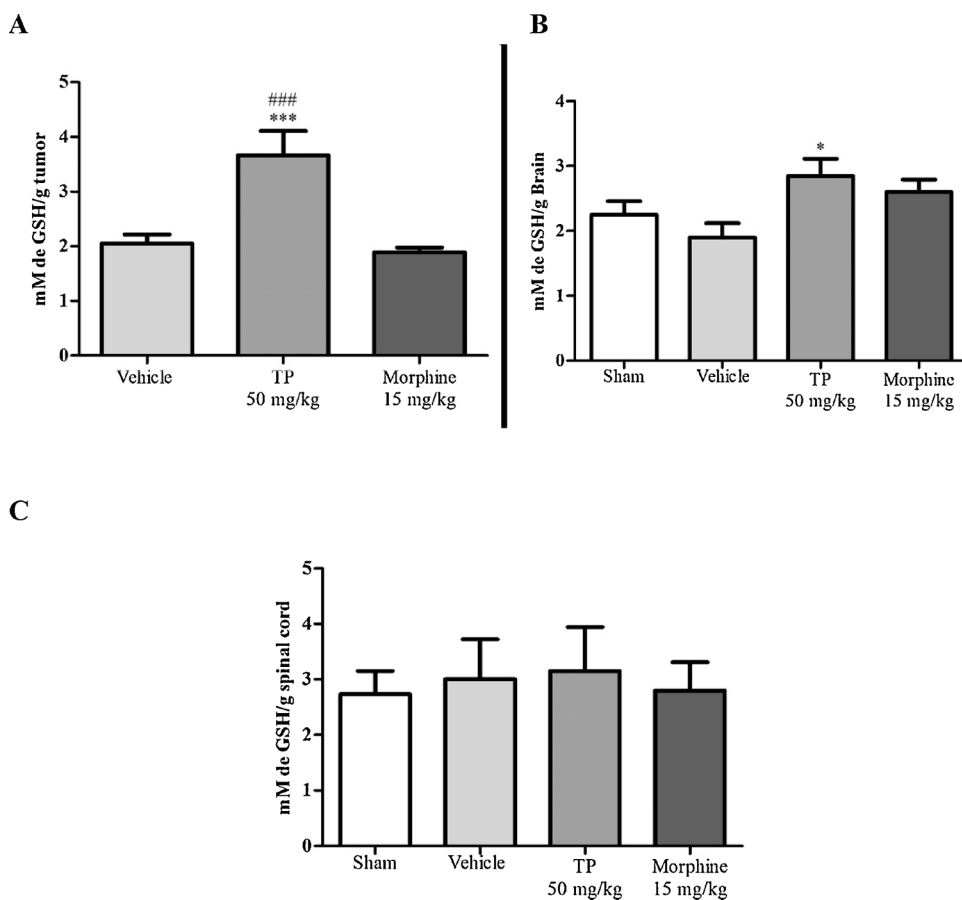


Fig. 4. Effect of chronic administration of TP on the GSH concentrations in mM per gram of homogenate of tumors (A), brain (B) and spinal cords (C) in mice containing S180 (n = 5/group). * p < 0.05, *** p < 0.001 versus vehicle group and ### p < 0.001 versus morphine group (one-way ANOVA followed by Tukey post-test).

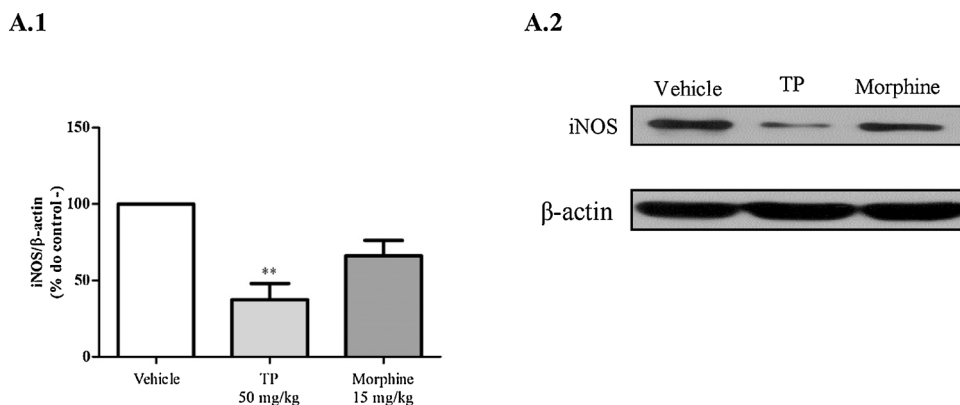


Fig. 5. Effect of TP on the tumor-bearing S180 induced by iNOS immunocent in the paw. Representative western blot and densitometric analyses. Data are expressed as the mean ± SEM, n = 5 animals/group. ** p < 0.01 versus the vehicle group (one-way ANOVA followed by the Dunnett's post-test).

(p < 0.05 on the 9th, 12th and 13th days, p < 0.01 on the 10th and 14th days), when compared to the vehicle-treated group (Figure S3). This weight gain may be related to the animal welfare in relation to the treatment with TP, once that according to OECD [53], a significant body weight loss can be an indicator of deteriorating animal condition. Although we did not notice apparent changes in feed consumption, other studies can assess the feeding behavior through the evaluation of the feed consumption for a better understanding of this effect. In addition, no behavioral changes suggestive of TP toxicity were evidenced. No changes suggestive of toxicity were observed in the hematological and biochemical parameters (Table S1), in the necropsy of the animal organs and histopathological analysis of the liver and kidneys (Figure S3). These findings corroborate Api et al. who in a short review summarized several evidences about the safety of terpineol [50].

In contrast, all animals treated with morphine presented reducing palpebral and auricular reflexes, as well as reaction of Straub's tail, a central excitatory effect of this drug caused by the contraction of the dorsal sacrococcygeus muscle [51]. Animals treated with morphine had a significant weight reduction (p < 0.05 on the 13 and 15th days and p < 0.01 on the 14th days) when compared with those from the vehicle group (Figure S3). It was verified that the animals with the tumor treated with morphine presented increase (p < 0.01) in the weight of the encephalous when compared with the animals of the vehicle group. This increase was not observed in the animals of the other groups, which presented values close to the animals without the tumor (Table S2).

However, animals treated with morphine showed an increase in brain size when compared to the vehicle group. The

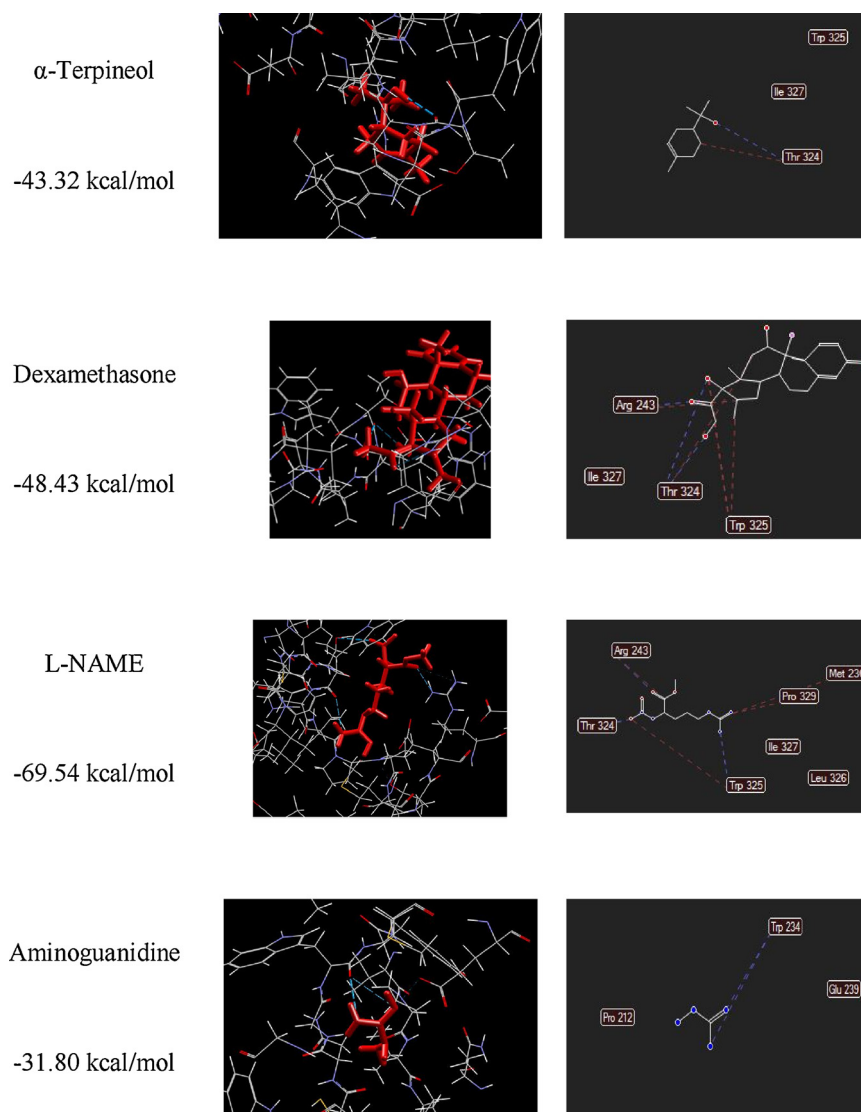


Fig. 6. Complex formation energy (ligand + receptor) and binder maps to Nitric Oxide Synthase – α -terpineol (A), Nitric Oxide Synthase – Dexamethasone (B), Nitric Oxide Synthase – L-NAME (C), and Nitric Oxide Synthase – Aminoguanidine (D). Blue lines - Hydrogen bonds and red lines - Steric interactions.

anatomopathological analysis of this organ showed no degeneration or cellular alteration of the tissues, what suggests that the administration of morphine did not cause functional toxic damage to the encephalon (Figure S5). The structures of the encephalous of the other groups were also preserved, with no apparent changes in the cortical, hippocampal and cerebellum regions. Finally, we observed that mice treated with vehicle had a reduction of muscle strength when compared to the animals of the sham group ($p < 0.001$ on the 13th and 15th days). TP did not promote changes in grip strength (Fig S1), corroborating Oliveira et al. [26], who show TP did not develop alterations in motor coordination in Rota rod test. Therefore, the antihyperalgesic effect of TP observed in this study is not entirely due to muscle relaxation or an inhibitory CNS effect.

In conclusion, the present study demonstrates that TP reduces the nociception induced by sarcoma 180 in rodents, without promoting myorelaxant effect. This antinociceptive effect of TP probably occurs thanks to the mechanisms related to the modulation of oxidative stress, with maintenance of the endogenous antioxidant substances and reduction of iNOS levels. This study also suggests that chronic administration of TP does not trigger evidence of preclinical toxicity. The analgesic dose of TP did not alter tumor growth, demonstrating that its analgesia does not depend on the reduction of tumor mass.

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Conflict of interest

The authors declare no competing financial interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.biopha.2018.06.027>.

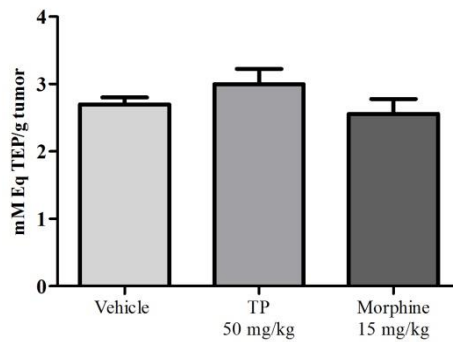
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1 **Supplemental material**

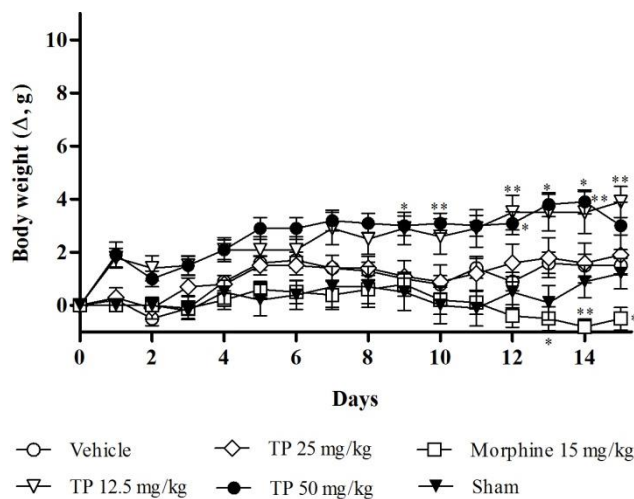
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4 **Figure S1.** Effect of chronic administration of TP on tetraepoxypropane (TEP)
5 concentrations. The results were expressed in $\mu\text{M TEQ Eq}$ per gram of S180 tumor ($n =$
6 5/group) (one-way ANOVA followed by Tukey's post-test).

7



8

9 **Figure S2.** Weight evolution of sham animals and those treated with vehicle, TP and
10 morphine for 15 days ($n = 8$ /group). * $p < 0.05$ and ** $p < 0.01$ versus the control group
11 (two-way ANOVA followed by the Bonferroni post-test).

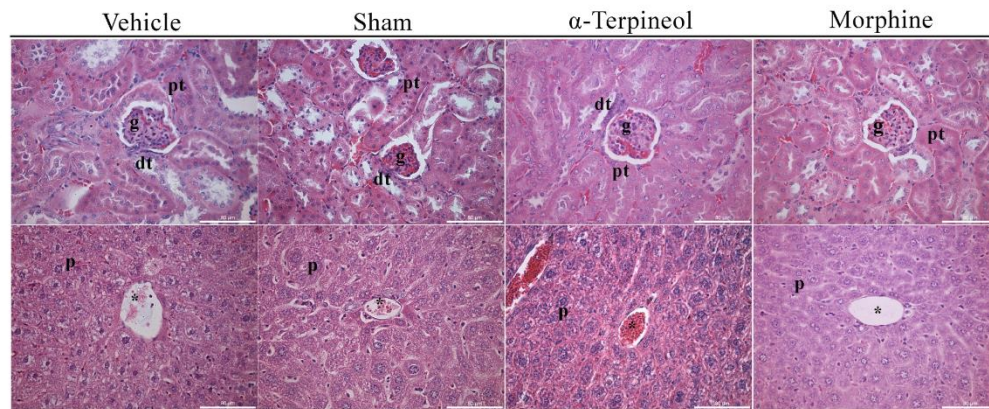
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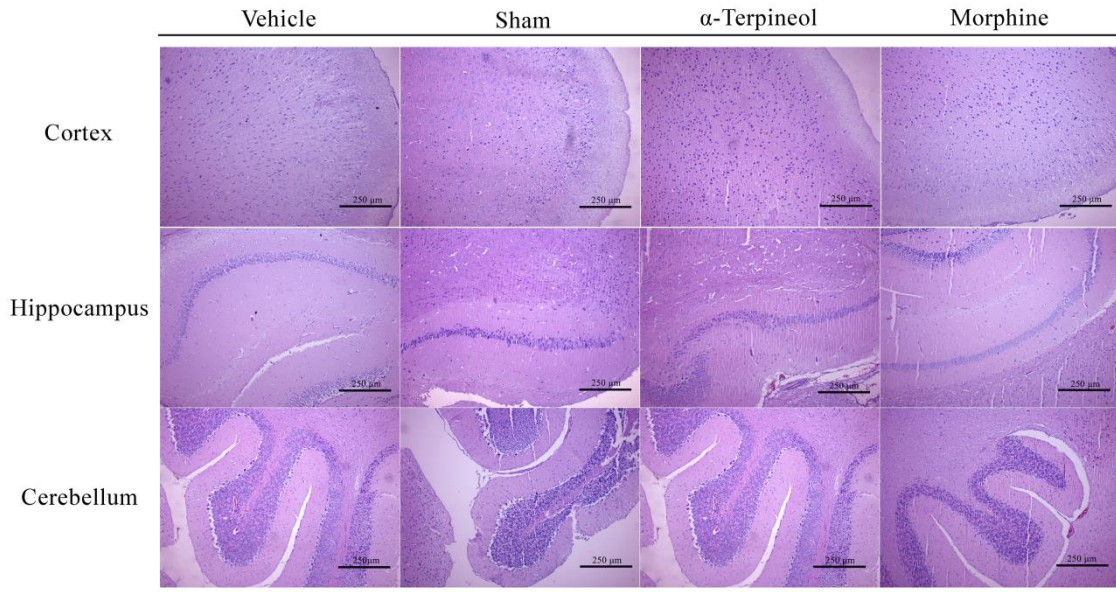
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23 **Figure S3.** Histological analysis of the Kidney and liver sections stained-H&E (40x
24 objective), showing respectively conserved glomeruli (g), distal tubule (dt) and
25 proximal tubule (pt), such as well-preserved liver plates (p) and hepatic portal vein (*).
26 Liver presented normal-looking hepatic parenchyma, preserved lobes, central vein,
27 hepatocyte cords and triad portal, without the presence of inflammatory infiltrate,
28 fibrosis or hemorrhagic spots on the hepatic lobe. The cortical and medullary regions of
29 kidneys were normal, with glomeruli, renal tubules and preserved capillaries.

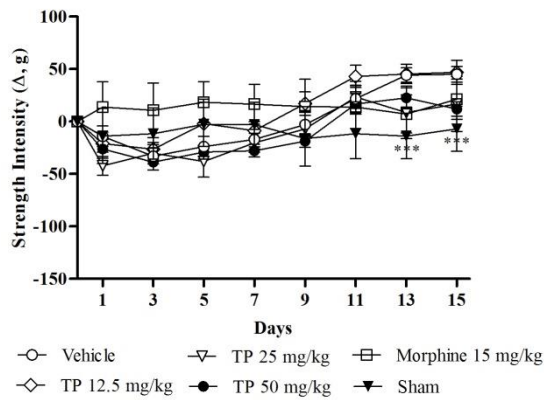
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33 **Figure S4.** Photomicrographs (250 μm) of the cerebral cortex, hippocampus and
 34 cerebellum of sham animals, vehicle, TP 50 mg/kg and morphine, 15 days after tumor
 35 induction and daily treatment.

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38 **Figure S5.** Effect of terpineol (TP, 12.5 to 50 mg/kg, s.c.) on the strength intensity in
 39 mice with S180 (n= 8/group). ***p < 0.001 vs. the control group (ANOVA two-way
 40 followed by Bonferroni's test)

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45 **Table S1.** Hematological and Biochemical analysis of euthanized animals after 15 days
 46 of TP administration (mean \pm SEM).

Hematologic parameters	Sham	Vehicle	TP (50 mg/kg)	Morphine
Blood Cells (10 ⁶ /mL)	7.49 \pm 0.65	8.69 \pm 0.31	8.50 \pm 0.33	7.77 \pm 0.45
Hemoglobin (g/dL)	12.68 \pm 1.12	13.74 \pm 0.44	13.71 \pm 0.59	13.08 \pm 0.67
Hematocrit (%)	42.33 \pm 3.21	47.47 \pm 1.99	46.91 \pm 2.33	42.25 \pm 2.13
Total leukocytes (10 ³ /mL)	5.88 \pm 1.12	6.25 \pm 0.92	9.01 \pm 2.06	5.32 \pm 0.77
Neutrophils (%)	40.58 \pm 5.32	39.33 \pm 2.88	41.20 \pm 1.78	36.00 \pm 5.95
Eosinophil (%)	1.40 \pm 1.23	0.53 \pm 0.14	0.18 \pm 0.05	0.72 \pm 0.49
Monocytes (%)	1.80 \pm 0.69	6.59 \pm 0.99	5.50 \pm 1.09	1.42 \pm 0.39
Lymphocytes (%)	55.88 \pm 6.21	50.34 \pm 3.80	52.66 \pm 1.70	61.62 \pm 5.78
Platelets (10 ³ /mL)	1187.25 \pm 208.20	1014.33 \pm 52.58	1174.00 \pm 117.94	1335.83 \pm 115.28

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Biochemical parameters	Sham	Vehicle	TP (50 mg/kg)	Morphine
TGO (U/L)	178.90 \pm 17.55	225.83 \pm 17.55	224.50 \pm 23.63	210.13 \pm 12.47
TGP (U/L)	56.90 \pm 6.22	71.25 \pm 9.41	53.08 \pm 11.22	49.63 \pm 4.22
Urea (mg/dL)	47.60 \pm 2.12	46.83 \pm 2.4	43.33 \pm 2.50	49.13 \pm 2.42
Creatinine (mg/dL)	0.35 \pm 0.02	0.42 \pm 0.02	0.40 \pm 0.03	0.34 \pm 0.01

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49 **Table S2.** Analysis of body weight. * p < 0.05 versus vehicle group (mean \pm SEM).

Organs (mg/100 mg body weight)	Sham	Vehicle	TP (50 mg/kg)	Morphine
Brain	1.44 \pm 0.06	1.25 \pm 0.04	1.48 \pm 0.02	1.63 \pm 0.03*
Heart	0.51 \pm 0.03	0.50 \pm 0.02	0.48 \pm 0.12	0.51 \pm 0.01
Lung	0.52 \pm 0.13	0.53 \pm 0.03	0.54 \pm 0.013	0.64 \pm 0.02
Liver	6.64 \pm 0.33	5.87 \pm 0.16	5.92 \pm 0.26	6.21 \pm 0.18
Kidney	1.51 \pm 0.19	1.34 \pm 0.23	1.63 \pm 0.06	1.82 \pm 0.14

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ANEXO: Aprovação do Comitê de Ética em Pesquisa com Animais



UNIVERSIDADE FEDERAL DE SERGIPE
PRÓ-REITORIA DE PÓS-GRADUAÇÃO E PESQUISA
COORDENAÇÃO DE PESQUISA
COMITÊ DE ÉTICA EM PESQUISA COM ANIMAIS (CEPA)

DECLARAÇÃO

Declaro, para os devidos fins, que o Projeto de Pesquisa intitulado **“INVESTIGAÇÃO DO EFEITO DO A-TERPINEOL SOBRE AS RESPOSTAS NOCICEPTIVAS INDUZIDAS POR CÉLULAS TUMORAIS (SARCOMA 180) EM ROEDORES.”** Sob coordenação da **Profª.Drª. Adriana Gibara Guimarães** (protocolo **CEPA 05/2014**) foi aprovado pelo Comitê de Ética em Pesquisa com Animais da Universidade Federal de Sergipe, em reunião realizada dia **21/08/2014**.

São Cristóvão, 21 de agosto de 2014.

Prof.ª. Dr.ª. FLÁVIA TEIXEIRA SILVA
Coordenadora do CEPA/UFS