Ameliorative Potential of Standardized Extract of *Condalia buxifolia* Reissek in Attenuating the Postoperative Pain: Evidence for the Inhibition of AMPA and Kainate in the Spinal Cord

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**Abstract**

Acute pain post-surgery is one of the major problems of public health, and its treatment is still a challenge. In this study, it was investigated the analgesic effect of a standardized methanolic extract of *Condalia buxifolia* (MECb) (30 and 100 mg/kg, i.g.) in a plantar incision surgery (PIS) model in mice, and the possible mechanism that underlying its effect. The effect of MECb was evaluated on the hyperalgesia (mechanical, cold and heat stimuli), and in this same model, it was analyzed the effect of MECb (100 mg/kg, i.g.) on the concentration of cytokines (TNF-α, IL-1β, IL-10) and nerve growth factor (NGF) in the paw and spinal cord. Further we investigated the effects of MECb (100 mg/kg, i.g.) on the nociceptive behaviour (spontaneous pain and hyperalgesia) induced by AMPA and kainate. Moreover, the safety profile of prolonged treatment with MECb (100 and 300 mg/kg, i.g.) and involvement of C fibers sensitive to capsaicin in its effect (100 mg/kg, i.g.) was evaluated. MECb showed a marked reduction on mechanical and heat hyperalgesia, and reduced the concentrations of cytokines (TNF-α, IL-1β, IL-10) and nerve growth factor (NGF). The treatment with MECb prevented the nociceptive behaviour and central sensitization caused by AMPA and kainate. Moreover, the analgesic effect of MECb was not affected by the ablation of the central afferent C fibers, and it showed reduced toxicity, indicating good safety and efficacy. The current data showed, for the first time, the analgesic and anti-inflammatory effect of MECb in an animal model of postoperative pain. The results support and suggest the use of MECb as an alternative treatment and a possible source of analgesics substances to postoperative pain.

**1 Introduction**

Millions of surgeries are performed annually worldwide, as a result of any pathology, accidents or aesthetic. Acute pain is among the most common outcome after surgery being a major problem of public health that decreases the quality of life, limits activity, and reduces functional capacity1. More than 73 million of surgeries are performed annually in the USA, and up to 75%
of patients experience pain after surgery. An Australian study estimated that the economic burden in the treatment of chronic pain, which develops from acute postoperative pain in individuals aged 30 years, over a lifetime, is approximately one million dollars. Acute postoperative pain is followed by persistent pain in 10–50% of individuals who have undergone common surgeries. Because chronic pain can be severe in 2–10% of these patients, persistent postoperative pain represents a main, largely unrecognized clinical problem.

In general, postoperative pain (PP) is related to a secondary inflammation and nerve damage, induced by incision itself. Remarkable signs of inflammation are seen on the site of incision including local edema, hyperthermia, hyperemia and pain, as indicative of release of proinflammatory mediators. There are studies that demonstrated the involvement of proinflammatory cytokines interleukin 1β (IL-1β) and tumor necrosis factor alpha (TNF-α), and the nerve growth factor (NGF) in the induction and maintenance of pain in injured peripheral tissue (as found in surgeries). These proinflammatory substances play important roles in mediating exaggerated pain conditions. Otherwise, it has been extensively showed that central sensitization in plantar incision surgery (PIS) model is maintained for glutamatergic non-NMDA receptors and by a role of Aβ and A-δ fibers in the mechanical hyperalgesia in this model.

In this way, provision of effective and safe PP management should be one of the top priorities of any healthcare center where surgical procedures are carried out, since patients who have well-controlled pain have an improved health-related quality of life and an overall greater satisfaction with their experience. Unfortunately, despite the introduction of new standards, guidelines, and educational efforts, data from around the world suggest that PP continues to be managed inadequately. Conventional drugs such as nonsteroidal anti-inflammatory drugs (NSAIDs), opioids, and local anesthetics have been used to control PP; however, they have a multitude of side effects such as respiratory distress, nausea, itching, gastrointestinal bleeding, and renal and liver failure. Recent studies have indicated interest in using complementing therapies such as medicinal plants, since natural products continue to be important sources for the discovery of new drugs.

Condalia buxifolia Reissek belongs to the Rhamnaceae family, which is commonly found in American continent, is used in traditional medicine, and presents critical antipyretic and anti-inflammatory effects. Furthermore, previous researches demonstrated that standardized methanol extract of Condalia buxifolia (MECb) displays anti-inflammatory and antinoceptive (analgesic) effects in rodents. Moreover, it was also shown that MECb reduces nocifensive behaviour caused by prostaglandin E2 (PGE2), transient receptor potential vanilloid type 1/acid-sensing ion channels (TRPV1/ASICs) and protein kinase A (PKA) signaling pathways and glutamate.

In this sense, although there are a large number of analgesic drugs, the PP is still affecting the quality of life in affected individuals, showing the need to seek more effective therapeutic alternatives. In this context, this study was undertaken to evaluate the effect of MECb (i) in a model of PP; (ii) on the concentrations of TNF-α, IL-1β, IL-10 and NGF; (iii) in prevent the nocifensive behaviour (spontaneous pain) and central sensitization (hyperalgesia) produced by AMPA and kainate; (iv) on the ablation of the central afferents fibers sensitive to capsaicin (TRPV1-nociceptors), and (v) finally, its safety profile by prolonged treatment.

2 Materials and methods

2.1 Drugs and reagents

The following substances were used: L-glutamic acid hydrochloride (glutamate), capsaicin and Tween 80 (Sigma-Aldrich, Saint Louis, MO, USA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), kainic acid (kainate) (Tocris, Cookson Inc. Ellisville, USA), ethanol (Vetec, Rio de Janeiro, RJ, Brazil). For the cytokines and neurotrophin analysis, the R&D Systems Kits (Minneapolis, MN, USA) were used. Biochemical analyses were performed using Gold Analisa Kits [Belo Horizonte, MG, Brazil (the LDL analysis was made using Friedewald equation)]. The standardized methanolic extract of Condalia buxifolia (MECb) was prepared from the plant root bark at the Department of Chemistry, Federal University of Santa Maria (UFSM), Brazil as previously described. All drugs were dissolved in saline solution (0.9% NaCl), except MECb which was dissolved in 5% ethanol/0.25% NaOH 1M in saline and capsaicin in 10 % ethanol/10% Tween 80 in saline. The final concentration of ethanol and Tween 80 did not cause any effect when it was administered alone (data obtained from open field test, not shown).

2.2 Animals

The experiments were performed with 292 females and 15 males Swiss mice 2-month-old (25–35 g) obtained from the animal facility of the Federal University of Santa Catarina (Florianopolis, SC, Brazil). The animals were housed in groups of 5 per cages at 22 ± 2°C and humidity (60–80%) under a 12-hours light/dark cycle (lights on at 06:00 h), with ad libitum access to standard laboratory diet (BioBase®, Águas Frias, SC, Brasil) and water filtered for fecal coliform and heavy metals (Jojacó® model J190, Capivari de Baixo, SC, Brazil). Animals were habituated at least a week in the sectorial facility and to laboratory conditions for at least 1 hour before testing, and all experiments were performed during the light phase of the cycle. The animals were randomly distributed between the experimental groups (5-10 animals per group), and all experiments reported in this study were carried out in UK J Pharm & Biosci, 2017: 5(1); 25
accordance with current guidelines for the care of laboratory and ethical guidelines for investigation of experimental pain in conscious animals29. This study was approved by the Ethics Committee for Animal Research of the Federal University of Santa Catarina (protocol number PP00745). Each animal was used only once for each group (vehicle and MECb) and was euthanized by cervical dislocation or decapitation (when it was necessary to collect the spinal cord) immediately after the completion of the experiment under isoflurane anesthesia. The number of animals used and the intensity of the noxious stimuli were the minimum necessary to obtain reliable data. Female blinded observer conducted all behavioural experiments.

2.3 Postoperative pain model produced by plantar incision surgery

The plantar incision surgery (PIS) was performed as previously described30. Briefly, mice were anesthetized with 1%–2% isoflurane delivered via a nose cone. After sterile preparation of the right hind paw, a 5 mm longitudinal incision was made through skin and fascia of the plantar surface using a number 11 scalpel blade. The incision started 2 mm from the proximal edge of the heel and extended toward the toes. The underlying muscle was elevated with curved forceps, leaving the muscle origin and insertion intact. After wound homeostasis, the skin was sutured with a 6.0 mm nylon mattress suture, and the wound was covered with 10 % povidone–iodine solution. Sham mice were anesthetized, but no incision was made. Animals were allowed to recover in their cages after surgery. All animals (sham or operated groups) were analyzed in mechanical and thermal (hot and cold stimulus) hyperalgesia. The Fig. 1 summarizes the treatments and the tests performed in this study.

![Fig 1: Experimental arrangement and time points in days or minutes. Showing the time points of events and the animal arrangement in 6 separate experimental processes: (A) Evaluation of mechanical and thermal (heat and cold) hyperalgesia in PIS model; (B) Evaluation of spontaneous nociceptive induced by glutamate, AMPA and Kainate; (C) Evaluation of mechanical hyperalgesia induced by AMPA and kainate; (D) On day 2 after PIS, mice were killed and samples tissues were collected to ELISA; (E) Evaluation of ablation of C fibers by capsaicin intrathecally in PIS model; (F) Evaluation of the safety profile of MECb and after 30 days the mice were killed. Also, were collected blood and organs for hematological and biochemical analysis. B: baseline; PIS: Plantar incision surgery; MECb: standardized methanolic extract of Condalia buxifolia; Veh: Vehicle; Glu: Glutamate; KA: Kainate; Cap: Capsaicin; l.t.: intrathecal route.](image-url)
2.4 Assessment of mechanical hyperalgesia

The mechanical hyperalgesia in PIS model was measured as described previously. Mice were acclimated in individual clear boxes (9x7x11 cm) on an elevated wire mesh (6 mm) platform (70x40 cm) to allow access to the ventral surface of the hind paws. The frequency method was used to analyze the mechanical hyperalgesia, in which the right hind paw is stimulated with a constant pressure of 0.4 g von Frey filaments (VFF) (Stoelting, Chicago, USA). Preliminary studies carried out in our laboratory (data not shown) have shown that 0.4 g of von Frey hair produces a mean withdrawal response frequency of about 20%, which was considered an adequate value for the measurement of mechanical hyperalgesia. Therefore, only 0.4 g of von Frey hair was used in our experiment and only animals that showed a response rate around 20% in the baseline response were selected. The response frequency to 10 applications was taken as the nocifensive behaviour and the results were expressed as the percentage of withdrawal response. One day before surgery, the animals were subjected for testing to characterize the baseline response (B). After baseline measurements, the animals were randomly divided into the following groups: sham + vehicle, sham + MECb, PIS + vehicle, and PIS + MECb, which received vehicle (10 mL/kg, i.g.) or MECb (30 and 100 mg/kg, i.g.). The treatments started only 24 hours after surgery. Mechanical hyperalgesia response was recorded before surgery (B), immediately before treatment (0 hour, PB), and after treatment (1, 2, 3, and 4 hours) to verify the time-course effect of MECb, as described below. These doses were chosen based on a previous study (formalin-induced pain) since both (formalin and PIS) are inflammatory models. The dose of 100 mg/kg was chosen because it was the most effective, and the dose of 30 mg/kg (a non-effective dose) was chosen to demonstrate that the effect of MECb is dose-dependent. To investigate the effects of repeated treatment with MECb on the mechanical hyperalgesia response, MECb (100 mg/kg, i.g.) was administered once daily for 6 consecutive days, and its antinociceptive effect was examined in 1 to 2 hours after treatment, which corresponds to the maximal effect observed during the time-course experiment.

2.5 Assessment of thermal hyperalgesia

To assess thermal hyperalgesia to cold and hot stimulus in PIS model, the Cold/Hot Plate Analgesia Meter (AVS system, cold-hot plate, São Paulo, SP, Brazil) was used according to a minor modification of the method described previously. Mice were placed in clear plastic chambers (7x9x11 cm) on the surface of the apparatus and the time (s) between placement and the shaking or licking of paws or jumping was recorded as the index of nocifensive behaviour. Mice were placed either on the cold or hot plate to analyze cold (10 ± 1 ºC) or heat (48 ± 1º C) thermal hyperalgesia, respectively. Mice were treated with MECb (100 mg/kg, i.g.) or vehicle (10 mL/kg, i.g.) 1 hour before thermal analyzes. The nocifensive behaviour was evaluated by the right hind paw withdrawal latency. The cut-off latency for cold plate test was 120 seconds and for hot plate was 60 seconds. The nocifensive behaviour was tested 48 hours (heat) and 72 hours (cold) after PIS. The evaluations were carried out on different days for two reasons: (i) for the animals not to be submitted to different stimuli (mechanical and thermal to heat and cold) on the same day and, (ii), for the evaluations to be performed within the most effective period of MECb.

2.6 Quantification of cytokines concentration

In another set of experiments, the PIS model and mechanical assessments were performed as described above. Twenty-four and forty-eight hours after surgery, animals received MECb (100 mg/kg, i.g.) or vehicle (10 mL/kg, i.g., control group) 1 hour before von Frey test (using the same protocol above). Two hours after last MECb administration (second day), mice were anesthetized with isoflurane and killed by decapitation and then the skins of the right planter surface and spinal cord (L4-L6) were removed. The tissues were homogenized with a PBS solution containing Tween 20 (0.05%), 0.1 mM phenylmethylsulphonyl fluoride (PMSF), 10 mM EDTA, 2 ng/mL aprotinin, and 0.1 mM benzethonium chloride and centrifuged at 3000 g for 10 min at 4 ºC. The supernatant obtained was immediately stored at -80º C and the total protein content were measured using the Bradford method. The concentrations of TNF-α, IL-1β, IL-10 and NGF were measured using ELISA kits, according to the instructions of the manufacturer. All results are expressed as pg/mg of protein. The second day post-incision was selected for euthanasia of the animals and collection of tissues, based on the analgesic effect of MECb in the paw incision model made earlier.

2.7 Involvement of the central afferent fibers sensitive to Capsaicin

In another set of experiments, to explore the role of capsaicin-sensitive fibers in the postoperative pain model, mice were maintained anesthetized with isoflurane 1-2 % and intrathecally treated with capsaicin (10 μg/site) or vehicle (5 μL/site). After 24 hours, the animals were submitted to paw withdrawal latency in the hot plate test (50 ºC, cut-off 30 s) to verify the efficacy of desensitization. Then, the animals were grouped randomly and then were submitted to the PIS model. Forty-eight hours after surgery, animals were treated with MECb (100 mg/kg, i.g.) or vehicle (10 mL/kg, i.g.) and behavioural mechanical hyperalgesia was performed as described above.

2.8 Nocifensive behaviour induced by glutamatergic receptor agonist (glutamate, AMPA and kainic acid)

In order to investigate the potential action of MECb over the nociception induced by glutamatergic receptor agonists, the animals were pretreated with MECb (100 mg/kg, i.g.) or vehicle (10 mL/kg, i.g.) 1 hour before the injection of agonists.
Glutamate (an excitatory amino acid, 175 nmol/site)43, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA, a selective agonist of AMPA subtype of ionotropic glutamate receptors, 135 pmol/site)35 or kainic acid (kainate, a selective agonist of kainate subtype of ionotropic glutamate receptors, 110 pmol/site)35 were injected intrathecally in a volume of 5 μL of each drug per animal. To be performed intrathecal (i.t.) injection, animals were manually restrained, and a 30-gauge needle connected by polyethylene tubing to a 25 μL Hamilton gas-tight syringe (Hamilton, Birmingham, UK), was inserted through the skin and between the vertebras into the subdural space of the L5–L6 spinal segments, as previously described by Hylden and Wilcox36. The tail reflex movement was considered as indicative of success of administration and the injections were given over a period of 5 s. The animals were treated with MECb (100 mg/kg, i.g.) or vehicle (10 mL/kg, i.g.) 1 hour before the injection of the agonists. Immediately after i.t. injection of the substances, mice were individually placed in observation chambers and the amount of time (seconds) the animal spent biting, licking or scratching the caudal region (flanks, hind limbs and tail) was taken as index of nocifensive behaviour. The ceiling for observation of nocifensive behaviour was variable to the different agonists: glutamate 3 min; AMPA 1 min and kainic acid 4 min34,35.

2.9 Assessment of mechanical hyperalgesia Induced by AMPA and kainate

Since MECb inhibited the spontaneous nociceptive response induced by glutamatergic agonists, our next step was to evaluate the effect of MECb on mechanical hyperalgesia induced by intrathecal (i.t.) injection of the agonists of AMPA and kainate receptors, which are known to be involved in the maintenance of postoperative pain12,13,14. Previously, in a separate set of experiment, a dose-response curve for AMPA (67.5 or 135 pmol/site) and kainate (55 or 110 pmol/site) administered intrathecally, as described above, was standardized to be assessed in mice at 5, 15, 30, 45 and 60 min after the agonist injection. According to the results, the best dose of AMPA (135 pmol/site, i.t.) and kainate (110 pmol/site, i.t.) were chosen in order to highlight the mechanical hyperalgesia. Of note, the mechanical hyperalgesia was performed after the complete disappearance of the nocifensive behaviour. In another group of experiments, mice were treated with MECb (100 mg/kg, i.g.) or vehicle (10 mL/kg, i.g.) 1 hour before i.t. injection of AMPA (135 pmol/site) or kainate (110 pmol/site). The mechanical hyperalgesia was evaluated as described above at the same time of previously test of effect of AMPA and kainate.

2.10 Evaluation of the safety profile of MECb: determination of hematological and biochemical parameters

As postoperative pain represents a class of persistent painful conditions, which requires long-term treatment, was performed an experiment to evaluate possible toxic effects of prolonged administration for 30 consecutive days of MECb. In this way, the protocol of toxicity was performed as previously described36 that requires the use of both sex and the higher doses that presented effect. Male and female were treated, once a day, with vehicle or MECb (100 or 300 mg/kg, i.g.) (n=5 per group). The dose of 100 mg/kg was chosen since it was the dose used in the behavioural tests performed in this study, and the dose of 300 mg/kg showed efficacy in previous study37,38. Fifteen hours after the last administration of MECb, the animals were anesthetized with isoflurane. The blood was collected from infraorbital plexus, to perform biochemical and hematological analysis. To biochemical assay the blood was transferred to 1.5 mL microtubes, centrifuged at 4,000 x g for 10 min at 23 °C, and then the serum was assayed for the determination of gamma-glutamyl transferase (GGT), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities and the levels of cholesterol (total, HDL and LDL), triglycerides, creatinine, urea, uric acid and glycermia, which were used as indicators of hepatic, renal and pancreas lesion, by the use of specific commercial kits (Gold Analisa, Belo Horizonte, MG, Brazil). To hematological assay the blood was transferred to 1.5 mL microtubes containing anticoagulant (10% solution of potassium EDTA (w/v) in distilled water), for determination of lymphocytes, monocytes, granulocytes, erythrocytes, hemoglobin, hematocrit and platelets. In addition, vital organs (heart, lung, liver, kidney, spleen, mesenteric lymph nodes, thymus, adrenal and salivary (right) gland, uterus, ovary and testicle) of all animals were excised, weighed, and carefully examined by macroscopic observation with respect to general appearance, shape, consistency and color. Finally, the intake of food and drink and the percentage of weight of the organs in relationship to body weight were calculated.

2.11 Statistical analysis

Data are presented as the mean ± SEM of 5-10 mice per group. Statistical analysis of the results was carried out by using, unpaired t test and one-way or two-way ANOVA. For one-way ANOVA was considered as an independent variable the treatment and for two-way were considered time and treatment. For the statistical analysis of toxicity profile of MECb, was performed two-way ANOVA comparing treated group with respective sex control group and comparing female x male with respective group treatment. Following significant ANOVA multiple post hoc comparisons were performed using Student-Newman–Keuls or Bonferroni test, respectively. The area under the curve in AMPA and kainate hyperalgesia from 5 to 60 min (AUC5–60 min) was calculated using the trapezoidal rule. In all cases, differences were considered significant when P<0.05. Statistical parameters were computed and graphics made using GraphPad Prism (Graph Pad Software 5.1, Inc.).
Results

3.1 MECb reduces mechanical hyperalgesia after plantar incision surgery

Mechanical hyperalgesia produced by PIS was detected from 1st to 5th day post-surgery compared to sham groups, which is indicated by the increase in frequency of withdrawal of the paw of the mouse (Fig. 2A and B). In addition, the treatment of mice with MECb (30 and 100 mg/kg, i.g.) caused a significant inhibition (100%; F(4, 45) = 235.5; p<0.0001) of mechanical hyperalgesia (frequency of response) at dose of 100 mg/kg as compared to the vehicle-treated group. The effect was maintained by 3 hours after the treatment and the maximum inhibition was achieved at first hour (Fig 2A).

![Fig 2: MECb administered by intragastric route decreases postoperative pain. Panel A presents the time-course of the analgesic effect of MECb on the mechanical hyperalgesia. Panel B represents the analgesic effect of repeated administration of MECb (100 mg/kg, i.g.) on the mechanical hyperalgesia. MECb (100 mg/kg, i.g.; administered 60 min beforehand) decreases heat (48 hours, Panel C) and cold (72 hours, Panel D) hyperalgesia after PIS. Each point or column represents the mean of the values obtained in 10 animals and the vertical lines indicate the S.E.M. **P<0.01 and ***P<0.001 when compared to PIS + vehicle group or ### P<0.001 when compared with sham groups (One-way ANOVA followed by Student-Newman–Keuls test or Two-way ANOVA followed by Bonferroni test). B: baseline withdrawal threshold; PB: post-baseline withdrawal threshold (24 hours after PIS); PIS: plantar incision surgery.](image)

Furthermore, repeated treatment of mice with MECb (100 mg/kg, i.g.) once a day for five days completely reduces the mechanical hyperalgesia caused by plantar incision when compared with the PIS group that received vehicle (inhibition of 94 to 100%; F(3, 36) = 137.1; p < 0.001) (Fig. 2B).

3.2 MECb reduces mechanical hyperalgesia after plantar incision surgery

Mice from PIS group showed reduction to heat and cold threshold compared to sham group, which is indicated by the decrease in latency of withdrawal of the mouse paw (Fig 2 C and D). Intra-gastric treatment with MECb (100 mg/kg) completely reduced the heat thermal hyperalgesia induced by plantar incision when compared with PIS group that received vehicle (inhibition 97±1%, F(1, 36) = 22.89; p < 0.001) (Fig 2 C).

Conversely, MECb treatment were not able to reduce the cold hyperalgesia when compared with the PIS group that received vehicle (F(1, 36) = 0.0578; p > 0.05) (Fig 2 D).

3.3 MECb decreases the concentrations of proinflammatory cytokines (IL-1 β, TNF-α) and neurotrophin (NGF) in PIS model

PIS produced a significant increase in the concentration of TNF-α, IL-1β, and NGF, while decreased the concentration of IL-10, in the skin of the hind paw when compared with the sham + vehicle group (Fig 3 A, B, C and D). Meanwhile, PIS does not promote alterations in the concentration of TNF-α, IL-1β, NGF and IL-10 in spinal cord when compared with the sham + vehicle group (data not shown). Treatment with MECb (100 mg/kg, i.g.) significantly prevented increase the concentration of TNF-α, IL-1β, and NGF when compared with the PIS group that
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...received vehicle, with inhibitions of 100%, 71 ± 12%, 88 ± 9%, respectively (Fig 3A, B and C). However, MECb treatment did not prevent the reduction in the concentration of IL-10 caused by plantar incision when compared to the PIS group that received vehicle (Fig 3D).

Fig 3: Effect of treatment with MECb (100 mg/kg, i.g.) on TNF-α (panel A), IL-1β (panel B), NGF (panel C) and IL-10 (panel D) concentration in the right hind paws of the animals submitted to the PIS model. Each column represents the mean of the values obtained in 5-7 animals and the vertical lines indicate the S.E.M. * P<0.05 when compared with PIS + vehicle group or # P<0.05 when compared with sham + vehicle group (One-way ANOVA followed by Student-Newman–Keuls test)

3.4 MECb analgesia not dependent on central afferent fibers sensitive to Capsaicin

Intrathecal administration of capsaicin (acting on TRPV1 receptor) results in an ablation of central terminals followed by a desensitization of TRP⁺ C fibers and impairment in the nociceptive transmission from periphery. Here, we confirmed previous findings and demonstrated that an intrathecal injection of capsaicin (27.18 ± 1.12 s) significantly (p < 0.001) increased the pain latency in the hot plate test compared to vehicle (18.12 ± 0.73 s) group (Fig 4A).

Fig 4: Involvement of central afferent fibers sensitive to capsaicin injected intrathecally (i.t.) in the analgesic effect of MECb in mice. Panel A shows the increase of latency in hot plate test. Each column represents the mean of the values obtained in 8 animals and the vertical lines indicate the S.E.M. Significance levels when compared before and after the desensitization are indicated by *** P < 0.001 (paired t test). Panel B represents animals submitted to PIS model after the desensitization and evaluate the response on withdrawal of the right hind paw when treated with MECb (100 mg/kg, i.g.). Each column represents the mean of the values obtained in 7-10 animals and the vertical lines indicate the S.E.M. Significance levels when compared to vehicle group are indicated by *** P < 0.001 or ### P < 0.001 (Two-way ANOVA followed by Bonferroni test for multiple comparisons)
Figure 4B shows that there was no change in the mechanical hyperalgesia response induced by PIS in the vehicle i.g. groups, pretreated intrathecally with capsaicin or vehicle (F1, 30 = 0.1177; p > 0.05). Further, the analgesic effect of MECb (100 mg/kg, i.g.) (F1, 30 = 118.9; p<0.001) has not changed significantly between groups pretreated intrathecally with capsaicin or vehicle (p > 0.05) with inhibition of 76 ± 6% and 68 ± 8%, respectively (Fig 4B). Thus, this data suggests that MECb acts on other subtypes of nociceptive fibers to promote inhibition of mechanical hyperalgesia in PIS model, since TRP+ C fibers are not involved in this experimental condition.

3.5 MECb decrease nocifensive behaviour induced by glutamate, AMPA and kainate
Intrathecal injection of glutamate (175 pmol/site), AMPA (135 pmol/site) and kainate (110 pmol/site) caused marked nocifensive behaviour in mice (Fig 5A–C).

Fig 5: Effect of pretreatment with MECb (100 mg/kg, i.g.) on spontaneous nociception induced by intrathecal injection of glutamate (panel A), AMPA (panel B) or kainate (panel C). Each column represents the mean of the values obtained in 8-9 animals and the vertical lines indicate the S.E.M. ** P < 0.01 and *** P < 0.001 when compared to vehicle group (unpaired t test)

Treating animals with MECb (100 mg/kg, i.g., 1 hour before testing) inhibited the nocifensive behaviour induced by the intrathecal injection of glutamate (p < 0.001), AMPA (p< 0.001) and kainate (p < 0.01) when compared to group that received vehicle, with significant inhibitions of 50 ± 7%, 79 ± 9%, and 52 ± 12%, respectively (Fig 5A–C).

3.6 MECb prevent mechanical hyperalgesia induced by AMPA and kainate
The results of Fig 6 and 7 show that intrathecal injection of AMPA (135 pmol/site) and kainate (110 pmol/site) induced significant mechanical hyperalgesia compared with the vehicle group for up to 60 and 30 minutes, respectively (Fig 6A and 7A). In contrast, intrathecal injection of low dose of AMPA (67.5 pmol/site) and kainate (55 pmol/site) did not cause mechanical hyperalgesia when compared with group that received vehicle (Fig 6A and 7A). MECb (100 mg/kg, i.g.; 1 h before testing) inhibits partially, but significant (inhibition of 46 ± 20 % to 82 ± 6 %; F1, 12=15.02; p < 0.001), and markedly (inhibition of 86 ± 9 % to 89 ± 8 %; F1, 14=9.938; p < 0.001) the mechanical hyperalgesia when compared with group AMPA and kainate, respectively (Fig 6B and 7B). AUC analysis showed that mechanical hyperalgesia in MECb group was significantly lower than AMPA (p < 0.05, Fig. 6C) and kainate (p < 0.001, Fig 6C) group.

3.7 Evaluation of the safety profile of MECb
To investigate whether repeated treatment with MECb (100 and 300 mg/kg, i.g.) induces toxicity compared to control group treated with vehicle, the whole blood and serum were assayed for determination of hematological and biochemical parameters, and selected organs (heart, lung, liver, kidney, spleen, mesenteric lymph nodes, thymus, adrenal and salivary (right) gland, uterus, ovary and testicle) for macroscopic analysis of the animals. After 30 days of treatment with MECb, animals did not present any alteration in overall behaviour (i.e., body and organ weight, food and water intake), analyzed hematological and biochemical parameters, or observed macroscopic organs when compared with vehicle-treated animals and between the different doses tested with respective sex control group (Table 1, 2 and 3). Nevertheless, when comparing female and male with respective group treatment, it was observed that in male groups food intake increased in all doses tested and the body weight was higher, only in the dose of 300 mg/kg. These data are expected since males exhibit physiologically higher feed intake and consequently, higher body weight than females. Therefore, the results indicate that MECb do not cause mortality or produce any remarkable hematological, biochemical and body and organs weight adverse effects in chronic toxicity studies in mice.

4 Discussions
A suitable analgesic approach in the management of postoperative pain can reduce or avoid significantly the pain followed the procedure, thus improving the quality of life of the patients. However, more than 50% of patients experience significative pain after surgery39. The treatment with NSAIDs and opiates required to control postoperative pain is often associated with adverse side effects40,41. Thus, we proposed to study the effect of MECb in an animal model of postoperative
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pain, which exhibits similarities to the human postoperative pain syndrome, of note, heat and mechanical hyperalgesia. This was the first study that demonstrated acute and prolonged treatment of mice with MECB was able to reduce mechanical (1 to 5 days) and heat hyperalgesia (in the second day) in a mouse postoperative pain model.

**Fig 6: Mechanical hyperalgesia induced by intrathecal injection of AMPA.** Panel A shows the response curve of animals treated with AMPA in the doses of 67.5 pmol and 135 pmol. Panel B presents the pretreatment with MECB (100 mg/kg, i.g.) preventing the hyperalgesia induced by AMPA (135 pmol/site). Panel C represents the values of area under curve of effect of the MECB (100 mg/kg, i.g.) on mechanical hyperalgesia induced by AMPA (135 pmol/site). Each column represents the mean of the values obtained in 6-8 animals and the vertical lines indicate the S.E.M. * P < 0.05, ** P < 0.01 and *** P < 0.001 when compared with vehicle or AMPA group (Two-way ANOVA followed by Bonferroni test or unpaired t test)

**Fig 7: Mechanical hyperalgesia induced by intrathecal injection of kainate.** Panel A shows the response curve of animals treated with kainate in the doses of 55 pmol and 110 pmol. Panel B presents the pretreatment with MECB (100 mg/kg, i.g.) preventing the hyperalgesia induced by kainate (110 pmol/site). Panel C represents the values of area under curve of effect of the MECB (100 mg/kg, i.g.) on mechanical hyperalgesia induced by kainate (110 pmol/site). Each column represents the mean of the values obtained in 6-8 animals and the vertical lines indicate the S.E.M. * P < 0.05, ** P < 0.01 and *** P < 0.001 when compared with vehicle or kainate group (Two-way ANOVA followed by Bonferroni test or unpaired t test)

Moreover, peripheral inhibition of cytokines (i.e., IL-1β and TNF-α) and nerve growth factor (NGF) seems to be involved in the analgesic action of MECB. Also, MECB prevented nociceptive behaviour and central sensitization induced by AMPA and kainate.

The infusion of *Condalia buxifolia* root bark is used in traditional medicine in Brazil to treat inflammatory and painful diseases. Phytochemical study of *Condalia buxifolia* has shown high levels of cyclopeptide alkaloids (Condaline-A) which is relevant, since alkaloids are known to possess significant analgesic effect.

Acute pain resulting from plantar incision surgery (PIS) in an animal model involves primary and secondary hyperalgesia, suggesting involvement of both peripheral and central sensitization. The mechanisms of postoperative pain involve activation, modulation and modification on the peripheral, spinal and cerebral levels. Peripherally, the place of incision presents signs of inflammation and the inflammatory process is maintained through vascular and cellular events, such as the

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release, by resident cells, of inflammatory mediators (as IL-1β and TNF-α) which contribute to the induction and maintenance of the hyperalgesia45,46.

Table 1: Effect of Condalia buxifolia (1 x/day) on body weight, food and water intake under the chronic toxicity (30 days)

<table>
<thead>
<tr>
<th>Animal Parameter</th>
<th>Female (n=5 per group)</th>
<th>Male (n=5 per group)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Condalia buxifolia Reissek (mg/kg)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C 100 300</td>
<td>C 100 300</td>
</tr>
<tr>
<td>Food Intake (g)</td>
<td>5.500 ± 0.1806</td>
<td>5.480 ± 0.1879</td>
</tr>
<tr>
<td></td>
<td>5.670 ± 0.2227</td>
<td>6.300 ± 0.1523*</td>
</tr>
<tr>
<td></td>
<td>6.440 ± 0.1571**</td>
<td>6.500 ± 0.112*</td>
</tr>
<tr>
<td>Water Intake (mL)</td>
<td>5.100 ± 0.1674</td>
<td>5.140 ± 0.1762</td>
</tr>
<tr>
<td></td>
<td>5.180 ± 0.2035</td>
<td>4.930 ± 0.1191</td>
</tr>
<tr>
<td></td>
<td>4.970 ± 0.1212</td>
<td>5.100 ± 0.0875</td>
</tr>
<tr>
<td>Body Weight (g)</td>
<td>37.80 ± 1.241</td>
<td>36.20 ± 1.241</td>
</tr>
<tr>
<td></td>
<td>36.00 ± 1.414</td>
<td>42.60 ± 1.030</td>
</tr>
<tr>
<td></td>
<td>41.00 ± 1.000</td>
<td>42.80 ± 0.735**</td>
</tr>
</tbody>
</table>

The values are expressed as mean ± S.E.M. The statistical analysis was made comparing treated group with respective sex control group and comparing female x male with respective group treatment (Two-way ANOVA followed by Bonferroni test). * denote the difference of male compared with female for its respective treatment group. * P < 0.05, ** P < 0.01

Besides these cytokines, the neurotrophin NGF is also demonstrated to be released after the incision, collaborating to the painful and inflammatory condition10,45, and, together with IL-1β and TNF-α, are also crucial in the peripheral and central sensitization17-49.

At the spinal level, plantar incision model induces increased background activity in wide dynamic range (WDR) neurons in the dorsal horn, reflecting incision-induced central sensitization12. It has been suggested that this increased background activity is responsible for the pain-related guarding behaviour observed in mice after incision, a behaviour comparable to pain at rest in patients after surgery. The WDR neurons show enhanced responses to weak in von Frey filaments after incision, suggesting a role in the exaggerated behavioural responses to both punctuate and blunt mechanical stimuli55.

Fig 8: Schematic representation of the possible sites that the MEC may act to relieve postoperative pain. Experimental data suggest that MECb administered by i.g. route reduces the concentrations of pro-inflammatory cytokines TNF-α and IL-1β, and the neurotrophin NGF, peripherally. Moreover, centrally a MECb act negatively modulates the glutamatergic system through direct or indirect blocking AMPA and kainate receptor

Of note, some studies from literature showed the involvement of TNF-α, IL-1β and NGF in increasing reactions of TRPV1- evoked, all of which produce hypersensitivity to heat4,58,59. These findings are interesting since MECb presented an increase in latency on hot plate test in PIS model, which is related to TRPV1 activation. Furthermore, our data corroborate previous studies, which demonstrated the inhibitory effect of MECb on nociception induced by capsaicin, an agonist of TRPV1 channel28.

TNF-α and IL-1β have the ability to stimulate nociceptors, through their specific receptors, increasing nociceptive behaviour. It was demonstrated that IL-1β is increased in the dorsal horn of the spinal cord of rats50 and in perincisional tissues51,52 and serum46 of postoperative human patients. IL-1 may cause hyperalgesia through the expression or release of pronociceptive compounds such as substance P53, prostaglandins, and NGF44,54,55. Our results showed an increase of TNF-α, IL-1β and NGF after postoperative model in the paw.
ECb induced a decrease in the

Table 2: Effect of Condalia buxifolia (1 x/day) on the absolute and relative organ under the chronic toxicity (30 days)

<table>
<thead>
<tr>
<th>Organ Parameter</th>
<th>Condalia buxifolia Reissek (mg/kg)</th>
<th>Female (n=5 per group)</th>
<th>Male (n=5 per group)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>C 100 300 C 100 300</td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>A. weight</td>
<td>1.573 ± 0.078</td>
<td>1.571 ± 0.092</td>
</tr>
<tr>
<td></td>
<td>R. weight</td>
<td>0.041 ± 0.001</td>
<td>0.043 ± 0.001</td>
</tr>
<tr>
<td>Kidney</td>
<td>A. weight</td>
<td>0.219 ± 0.012</td>
<td>0.206 ± 0.010</td>
</tr>
<tr>
<td></td>
<td>R. weight</td>
<td>0.005 ± 0.0001</td>
<td>0.005 ± 0.0001</td>
</tr>
<tr>
<td>Lung</td>
<td>A. weight</td>
<td>0.226 ± 0.009</td>
<td>0.220 ± 0.008</td>
</tr>
<tr>
<td></td>
<td>R. weight</td>
<td>0.005 ± 0.000001</td>
<td>0.006 ± 0.0002</td>
</tr>
<tr>
<td>Spleen</td>
<td>A. weight</td>
<td>0.138 ± 0.01</td>
<td>0.145 ± 0.003</td>
</tr>
<tr>
<td></td>
<td>R. weight</td>
<td>0.003 ± 0.0001</td>
<td>0.0039 ± 0.0001</td>
</tr>
<tr>
<td>Heart</td>
<td>A. weight</td>
<td>0.129 ± 0.008</td>
<td>0.126 ± 0.0052</td>
</tr>
<tr>
<td></td>
<td>R. weight</td>
<td>0.003 ± 0.00001</td>
<td>0.003 ± 0.00001</td>
</tr>
<tr>
<td>Mesenteric lymph node</td>
<td>A. weight</td>
<td>0.055 ± 0.010</td>
<td>0.063 ± 0.001</td>
</tr>
<tr>
<td></td>
<td>R. weight</td>
<td>0.001 ± 0.0003</td>
<td>0.001 ± 0.0001</td>
</tr>
<tr>
<td>Thymus</td>
<td>A. weight</td>
<td>0.144 ± 0.0009</td>
<td>0.099 ± 0.006</td>
</tr>
<tr>
<td></td>
<td>R. weight</td>
<td>0.002 ± 0.0003</td>
<td>0.002 ± 0.0001</td>
</tr>
<tr>
<td>Adrenal Gland (Right)</td>
<td>A. weight</td>
<td>0.006 ± 0.0004</td>
<td>0.023 ± 0.015</td>
</tr>
<tr>
<td></td>
<td>R. weight</td>
<td>0.0001 ± 0.00002</td>
<td>0.0002 ± 0.00001</td>
</tr>
<tr>
<td>Salivar Gland (Right)</td>
<td>A. weight</td>
<td>0.077 ± 0.003</td>
<td>0.066 ± 0.003</td>
</tr>
<tr>
<td></td>
<td>R. weight</td>
<td>0.002 ± 0.00001</td>
<td>0.001 ± 0.0001</td>
</tr>
<tr>
<td>Uterus and ovary</td>
<td>A. weight</td>
<td>0.242 ± 0.01</td>
<td>0.248 ± 0.036</td>
</tr>
<tr>
<td></td>
<td>R. weight</td>
<td>0.005 ± 0.0002</td>
<td>0.006 ± 0.0009</td>
</tr>
<tr>
<td>Testicle (Right)</td>
<td>A. weight</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>R. weight</td>
<td>0.03 ± 0.0006</td>
<td>0.033± 0.0003</td>
</tr>
</tbody>
</table>

The values are expressed as mean ± S.E.M. The statistical analysis was made comparing treated group with respective sex control group and comparing female x male with respective group treatment (Two-way ANOVA followed by Bonferroni test). A. weight = absolute weight (in grams, g); R. weight = Relative weight; N/A = Not Available

The anti-inflammatory cytokine IL-10 negatively modulates the production of inflammatory mediators, which in turn limit the secretion of pro-inflammatory cytokines. Moreover, in human surgical wound exudates is found an increase of this cytokine. Conversely, in our experiment the concentrations of IL-10 were decreased in PIS group, and the treatment with MECb not altered its concentrations. Thus, we can suggest that the analgesic effect of MECb in the PIS model is due to decreased production and release of pro-inflammatory cytokines and neurotrophin without an increase of the IL-10 concentrations. Our approach was limited to dosage, thus is necessary additional studies to reveal the exact regulatory effect of MECb on cytokines in this model. Additionally, no change was found in the cytokines and neurotrophin in spinal cord.

Mechanical hyperalgesia in the PIS model is maintained by a primary sensitization over all the time (up to 5 days after surgery), whereas secondary sensitization is present up to 1
day after surgery\textsuperscript{31}. It was demonstrated that after plantar incision in rats, there is an increase responsiveness of A\textsubscript{β}\textsuperscript{14} and/or A\textsubscript{δ}\textsuperscript{15} fibers, otherwise the C fibers remained unchanged.

In this sense, we performed another set of experiment in which was ablated TRPV1-positive fibers.

This method disrupts the response to noxious heat, but not the mechanical noxious response\textsuperscript{37}. Our results showed that desensitization with capsaicin did not show modification in mechanical response in the PIS model. However, more studies are required to determine the exact mechanism that the MECb may be acting on the A\textsubscript{β} and/or A\textsubscript{δ} fibers in the PIS model.

Neurotransmitters released by noxious stimuli may contribute to the enhanced excitability of nociceptive pathways at spinal cord level. Particular attention has been paid to the action of excitatory amino acids (EAA)s\textsuperscript{6}, and a number of previous works have shown the action of these neurotransmitters with postoperative model\textsuperscript{13,62-64}. There are data from literature suggesting that NMDA is not involved on mechanical hyperalgesia and sensitization in PIS model\textsuperscript{12,62}. Moreover, there are conflicts in published data according to the role of metabotropic glutamate receptor in PIS model\textsuperscript{62-64}. Meanwhile, the non-NMDA receptors possess well established its role in PIS model. Agonists of non-NMDA (AMPA and kainate) receptors were found to induce pain behaviour and the reduced withdrawal threshold that develops after an incision\textsuperscript{13}. Further, intrathecal injected non-NMDA receptor antagonist returned the withdrawal threshold nearly to pre-incision levels\textsuperscript{12,13}.

### Table 3: Effect of repeated treatment (30 days) with standardized extract of \textit{Condalia buxifolia} on the hematological and biochemical parameters in mice

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Female (n= 5 per group)</th>
<th>Male (n= 5 per group)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C</td>
<td>100</td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>100.40 ± 1.97</td>
<td>105.00 ± 7.04</td>
</tr>
<tr>
<td>Urea (mg/dL)</td>
<td>39.57 ± 4.83</td>
<td>40.26 ± 5.21</td>
</tr>
<tr>
<td>Creatinine (mg/dL)</td>
<td>0.92 ± 0.12</td>
<td>0.79 ± 0.14</td>
</tr>
<tr>
<td>Uric acid (mg/dL)</td>
<td>3.31 ± 0.08</td>
<td>3.21 ± 0.05</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>3.74 ± 0.31</td>
<td>3.44 ± 0.08</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>35.28 ± 6.43</td>
<td>40.58 ± 2.19</td>
</tr>
<tr>
<td>γGT (U/L)</td>
<td>12.69 ± 1.05</td>
<td>10.92 ± 0.85</td>
</tr>
<tr>
<td>Total cholesterol (mg/dL)</td>
<td>106.20 ± 6.58</td>
<td>100.10 ± 3.20</td>
</tr>
<tr>
<td>HDL cholesterol (mg/dL)</td>
<td>22.06 ± 1.61</td>
<td>19.48 ± 0.72</td>
</tr>
<tr>
<td>LDL cholesterol (mg/dL)</td>
<td>57.39 ± 5.64</td>
<td>52.58 ± 3.02</td>
</tr>
<tr>
<td>Lymphocytes %</td>
<td>76.40 ± 1.06</td>
<td>78.34 ± 0.63</td>
</tr>
<tr>
<td>Monocytes %</td>
<td>2.86 ± 0.18</td>
<td>2.780 ± 0.26</td>
</tr>
<tr>
<td>Granulocytes %</td>
<td>21.82 ± 0.90</td>
<td>19.72 ± 0.53</td>
</tr>
<tr>
<td>Erythrocytes (cells x 10\textsuperscript{6}/µL)</td>
<td>8.35 ± 0.26</td>
<td>8.87 ± 0.16</td>
</tr>
<tr>
<td>Hemoglobin (g/dL)</td>
<td>140.20 ± 6.93</td>
<td>147.40 ± 5.44</td>
</tr>
<tr>
<td>Hematocrit %</td>
<td>41.72 ± 2.33</td>
<td>46.16 ± 1.77</td>
</tr>
<tr>
<td>Platelets (cells x 10\textsuperscript{3}/µL)</td>
<td>633.00 ± 31.40</td>
<td>580.20 ± 29.76</td>
</tr>
</tbody>
</table>

The values are expressed as mean ± S.E.M. The statistical analysis was made comparing treated group with respective sex control group. One-way ANOVA followed by Student Newman-Keul’s test.

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It was found on this work that MECb reduces significantly the spontaneous pain elicited by intrathecal administration of glutamate, AMPA and kainate, and also prevented the mechanical hyperalgesia provoked by AMPA and kainate. In agreement with these reports, it has been demonstrated that MECb produced analgesic activity when evaluated in the glutamate animal experimental model. These results suggest a possible effect of MECb on the central neurotransmission of glutamatergic system. Of note, as it was not found an increase on central cytokines or neurotrophin, at least on the time investigated. This suggests that the increase of pain response is sustained centrally by synaptic strength and plasticity dependent of non-NMDA receptors in the PIS model.

Finally, to assess the safety profiles of MECb, toxicological analysis was performed in animals that received daily treatment. Thus, after 30 treatment sessions, animals did not present any alteration in overall behaviour (such as body and organ weight, food and water intake) and there were no changes in the macroscopic observation of some organs (such as heart, lung, liver, kidney, spleen, mesenteric lymph nodes, thymus, adrenal and salivary (right) gland, uterus, ovary and testicle). Furthermore, hematological (lymphocytes, monocytes, granulocytes, erythrocytes, hemoglobin, hematocrit and platelets) and biochemical [glucose, urea, creatinine, uric acid, AST, ALT, γGT, cholesterol (total, HDL and LDL)] parameters, in the blood, were not modified after repeated treatment (30 days) with MECb. These results may indicate that the prolonged treatment does not cause loss and/or impairment of the organ functions assessed in this study, therefore presenting good safety conditions and efficacy. Collectively, these findings extend results from literature by giving new insights about the analgesic mechanism of *Condalia buxifolia*, as proposed in Fig 8.

5 Conclusion

In conclusion, the data presented here show that the ability of MECb (directly or indirectly) reduces the nociceptive behaviour and central sensitization caused by AMPA and kainate, seems to be involved in its analgesic effect. Additionally, the analgesic effect of MECb in the PIS model suggests its potential to be used clinically as an analgesic for postoperative pain. Furthermore, MECb showed reduced toxicity, indicating good safety and efficacy.

6 Acknowledgment

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7 Conflict of interest

The authors declare that there are no conflicts of interest.

8 Author’s contributions

RRS and ISC carried out the experimental design, conducting all behavioural experiments, analysis of experimental data, the literature review and draft the manuscript. AZ and AFM participated in collection, characterization and preparation of MECb. MJF and FRMBS performed the hematological and biochemical analysis to toxicity assay. EMZ participated in review the manuscript. ARSS contributed all reagents, experimental design, analysis of experimental data, and in directing research, editing and correcting the manuscript. All authors read and approved the final manuscript.

9 References


