Pain neuromodulation exerted by Ruta graveolens aqueous extract in experimental models of nociception

Neuromodulação da dor exercida pela Ruta graveolens extrato aquoso em modelos experimentais de nocicepção

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ABSTRACT
Introduction: The use of medicinal plants for therapeutic purposes has been common practice since antiquity. *Ruta graveolens L.*, commonly known as rue, has been shown to have antiparasitic, antioxidant, antibacterial and allelopathic activity. Objective: The objective was to investigate the antinociceptive effect of rue, as well as the mechanisms behind this effect. Materials and Methods: The sample consisted of 40 male Norvegicus (Wistar) rats, randomly divided into a positive control and three treatment groups administered *Ruta graveolens L*. aqueous extract at the following doses: 50 mg/kg, 100 mg/kg or 500 mg/kg, *p.o*. The experimental models of nociception used in this study to assess effectiveness of the treatments were the formalin and capsaicin tests. Five days prior to nociceptive challenges, the tail immersion assay was conducted to determine baseline pain threshold. Results: Antinociceptive activity was observed at Ruta graveolens L. aqueous extract concentrations of 50 mg/kg and 100mg/kg. 500 mg/kg induced pro-nociceptive activity with activation of the L-arginine-oxide-nitric system. Conclusion: These results suggest Ruta graveolens L. aqueous extract antinociceptive activity, and possible antagonism towards receptors.

Keywords: Ruta, Nociception, Pain Measurement.

RESUMO
Introdução: O uso de plantas medicinais para fins terapêuticos tem sido uma prática comum desde a antiguidade. Ruta graveolens L., comumente conhecida como rue, tem demonstrado ter atividade antiparasitária, antioxidante, antibacteriana e alelopática. Objetivo: O objetivo era investigar o efeito antinociceptivo da arruda, assim como os mecanismos por trás deste efeito. Materiais e Métodos: A amostra consistiu de 40 ratos Norvegicus (Wistar) machos, divididos aleatoriamente em um controle positivo e três grupos de tratamento administraram extrato aquoso de Ruta graveolens L. nas seguintes doses: 50 mg/kg, 100 mg/kg ou 500 mg/kg, p.o. Os modelos experimentais de nocicepção utilizados neste estudo para avaliar a eficácia dos tratamentos foram os testes de formalina e capsaicina. Cinco dias antes dos desafios nociceptivos, o ensaio de imersão da cauda foi conduzido para determinar o limiar de dor de base. Resultados: Atividade antinociceptiva foi observada em Ruta graveolens L. concentrações de extrato aquoso de 50 mg/kg e 100mg/kg. 500 mg/kg de atividade pró-nociceptiva induzida com ativação do sistema L-arginina-oxida-nítrico. Conclusão: Estes resultados sugerem a atividade antinociceptiva do extrato aquoso de Ruta graveolens L., e possível antagonismo em relação aos receptores.

Palavras-chave: Ruta, Nocicepção, Medição da dor

1 INTRODUCTION

Ruta graveolens L., commonly known as rue, common rue or herb-of-grace, is a perennial species of the Rutaceae family that forms bushes of branches and leaves of a bluish green color, with small yellow flowers[1].

The chemical composition of Rutagraveolens L. includes flavonoids, such as rutin and quercetin, as well as volatile substances with calming properties. Rutin and quercertin can be used to fight parasites, in addition to promoting the absorption of vitamin C, with corresponding antioxidant, antibacterial and allelopathic activities[2].

Since ancient Greece, rue was known as a magic plant, used in rituals to protect against evil forces, such as the "evil eye", menstrual disorders, skin inflammation, cramps, ear and toothache. Recent pharmacological assessments have proven many interesting activities, i.e., as a febrifuge and sweating agent, as an emmenagogue, antiparasitic, spasmolytic, photosensitizing, anti-inflammatory, anti-rheumatic, antiulcerogenic, anthelmintic, as well as an abortive[1].

Pain, considered a "fifth vital sign" by the American Academy of Pain Medicine (AAPM), is characterized as a subjective phenomenon which involves physical, psychological and cultural mechanisms[3].

Due to its complexity, pain may also be defined a psychophysical problem of interest to psychology, as this discipline is concerned with the relationship between the physical properties of stimuli and behavioral responses and sensory perceptions [4]. The International Association for the Study of Pain (IASP) defined pain as an unpleasant sensory and emotional experience associated with real or potential tissue damage[5].

This phenomenon can be classified as acute or chronic. Acute pain is brief and acts as a defense mechanism. It starts with an injury, followed by the synthesis and release of algogenic substances, which stimulate nerve fiber nociceptors. In a normal state pain is expected to regress, however situations in which several neuronal pathways are activated in a prolonged way, acute pain can become chronic[6].

Chronic pain may be present in chronic diseases, as a consequence of intense and prolonged trauma as well as during subsequent scarring, but also in the case of cancer or neuropathies. Adversely, acute pain is generally associated with acute tissue or organ trauma or burns; or originated from inflammatory or infectious processes. Therefore, acute pain is mostly considered a symptom of a disease, while chronic pain, may sometimes be classified as a disease in itself, independent of the generating stimulus[6].

One of the oldest forms of medicinal practice is the use of medicinal plants to treat, cure and prevent diseases. The World Health Organization (WHO) defines traditional herbal medicines as naturally occurring, plant-derived substances with minimal or no industrial processing that have been used to treat illness within local or regional healing practices. In addition to its availability and low cost, combined with the fact that a large amount of the global population lacks medical and pharmaceutical assistance, the use of traditional herbal medicines is still very significant for the treatment of various types of diseases[7,8].

In this context, the objective of this study was to evaluate, through a pharmacological approach, the antinociceptive effect of Ruta graveolens L. aqueous extract, as well as investigate some of the mechanisms behind this effect.

2 METHODOLOGY

40 male Norvegicus (Wistar) rats, weighing between 180g and 200g, were obtained from the Central Animal Hospital of the State University of Maringá - UEM, where they were acclimated to 22 ± 2oC, in a light / dark cycle of 12/12 hours and treated with chow and water ad libitum. The animals were acclimatized to the laboratory 1 hour before the experiments which were conducted according to the Normative Resolution Conselho Nacional de Controle de Experimentação Animal (CONCEA) #38, of April 17, 2018.

Preparation of Ruta graveolens L. aqueous extract

Identification and acquisition

Leaves of wild Ruta graveolens L. were acquired and taken to the Neuroanatomy and Neurophysiology laboratory of Unicentro University for the preparation of the aqueous extract.

Drying

First the botanical material was dried at room temperature (22 ± 2oC) for hours.

Hydrodistillation

The sample was then subjected to hydrodistillation (steam distillation) using a Clevenger-type apparatus. 200g of dry material and 1000 ml of distilled water were boiled for 60 minutes. The aqueous extract supernatant was collected and filtered.

Dosing

The doses used in this study were 50 mg/kg, 100 mg/kg, 500 mg/kg. Administration route was P.O.

Experimental groups

The animals were divided into 4 groups as follows:

Control Group: Consisting of 10 animals submitted to Formalin and Capsaicin challenges and treated with 500mg/kg saline solution (orally).

Treatment Group 50 mg/kg: Consisting of 10 animals submitted to Formalin and Capsaicin challenges and treated with 50 mg/kg Ruta graveolens L. aqueous extract (orally).

Treatment Group 100 mg/kg: Consisting of 10 animals submitted to Formalin and Capsaicin challenges and treated with 100 mg/kg Ruta graveolens L. aqueous extract (orally).

Treatment Group 500 mg/kg: Consisting of 10 animals submitted to Formalin and Capsaicin challenges and treated with 500 mg/kg Ruta graveolens L. aqueous extract (orally).

Anesthesia

The animals were anesthetized (intraperitoneally, 80 mg/Kg) with a solution of Ketamine Hydrochloride (Ketamine, 10 ml flask, 80 mg/kg) and Xylazine Hydrochloride (Dopaser, 10 ml flask, 15 mg/kg).

Baseline Evaluation - Tail immersion assay

All animals underwent the tail immersion assay 5 days before Formalin and Capsaicin challenges to access baseline pain threshold.

The test consisted of exposing the animal's tail to hot water (51-55°C). The time it took the animals to withdraw the tail from the water was recorded as indicative of nociception. A cut-off time of 180 seconds was observed to avoid injury.

The animals were used in two moments during the research, i.e., in the Formalin and Capsaicin challenges. A resting period of 10 days between nociceptive models was observed.

Formalin-induced pain model

The procedure used was adapted from Hunskaar and Hole[9]. The animals were injected with 50 µL of 2.5% formalin in the subplantar region of the right hind paw.

Footprint evaluation

After formalin injection, the footprints of the hind legs were obtained using a 5-megapixel digital camera, according to the method proposed by DeMedinaceli and collaborators[10] and modified by Lowdon and collaborators[11].

The animals were placed on a 1-meter-long by 10 cm wide and 70 cm high glass walkway, where they walked to the stop point. The photos containing the footprints were filed in separate folders, stored and analyzed with the ImageJ program. Data for each animal were individually identified, in order to allow follow-up over time. The length of the second to fourth finger, with smaller openings being considered indicative of pain, was measured in naïve/normal (N) and formalin-injected (F) paws.

Capsaicin-induced pain model

Each animal was injected 20 µl of capsaicin solution, injected in the tail region. 30 minutes after Ruta graveolens aqueous extract treatment nociceptive threshold was assessed by the tail immersion assay as described above.

Analysis of the underlying mechanisms involved in Ruta graveolens aqueous extract antinociceptive activity

For the analysis of the underlying mechanisms involved in Ruta graveolens aqueous extract antinociceptive activity, the most effective dose was used, i.e. 100 mg/Kg. The neuromodulation pathways studied in the present study were: opioid, cholinergic and nitric oxide. The pharmacological model chosen to study the pathways and their possible influences on the antinociceptive effect of Ruta graveolens aqueous extract was the model in which the most effective results were obtained. The protocol is represented below, through the flowchart.

Pretreatment with antagonists and / or controls (ip) === (15 min wait) === Pretreatment with agonists and / or controls (ip) === (30 min wait) === assessment (20 min)

A) Involvement of the opioid system in the antinociceptive effect of Ruta graveolens aqueous extract

The animals were pretreated with the μ opioid receptor antagonist, naloxone (1 mg/Kg, i.p.), 15 minutes before the administration of the agonist (Ruta graveolens aqueous extract). Control group received only the antagonist. 30 minutes after treatment with the agonist, the possible Involvement of the opioid system on the antinociceptive effect of Ruta graveolens was analyzed with the tail immersion assay as previously described.

B) Involvement of the Nitric Oxide-L-Arginine system in the antinociceptive effect of Ruta graveolens aqueous extract

The animals were pre-treated with L-arginine (NO precursor, 600 mg/Kg, i.p.), 15 minutes before the administration of the agonist (Ruta graveolens aqueous extract). Control group received only the antagonist. 30 minutes after treatment with the agonist, the possible Involvement of the Nitric Oxide-L-Arginine system on the antinociceptive effect of Ruta graveolens was assessed.

C) Involvement of the Cholinergic system in the antinociceptive effect of Ruta graveolens aqueous extract

The animals were pre-treated with the non-selective cholinergic antagonist atropine (1 mg/Kg, i.p.), 15 minutes before the administration of the agonist (Ruta graveolens aqueous extract). Control group received only the antagonist. 30 minutes after treatment with the agonist, the possible Involvement of the Cholinergic system on the antinociceptive effect of Ruta graveolens was analyzed with the tail immersion assay as previously described.

Statistical analysis

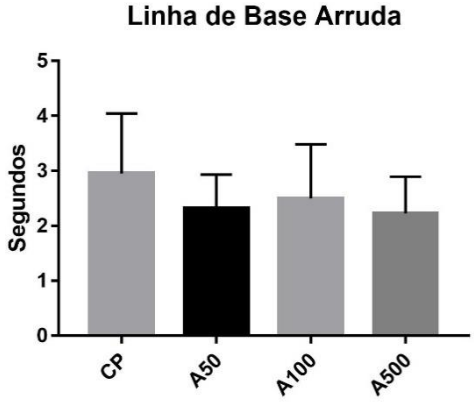
The results were presented as the mean ± standard error of the mean for each experimental group and their respective confidence intervals, at 95%. The Kruskal-Wallis test followed by Dunn's post-hoc test was used.

3 RESULTS

Baseline assessment

Baseline assessment in the in the tail immersion assay (Figure 1) indicate no statistically significant differences between groups. Average of 2.958 ± 1.092 seconds for positive control; 2.319 ± 0.6147 seconds for 50 mg/Kg group; 2.553 ± 0.9828 seconds for 100 mg/Kg group; and 2.228 ± 0.6695 seconds for 500 mg/Kg group.

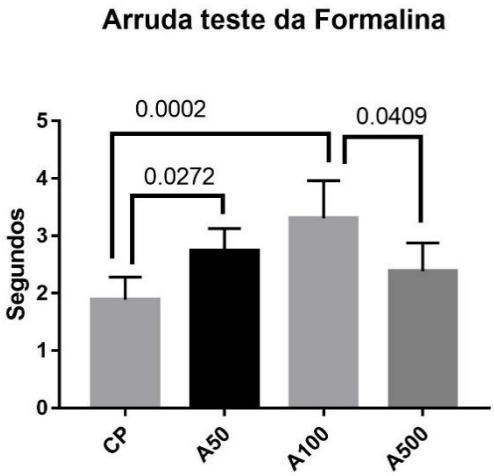
Legend for figure 1 - Baseline pain threshold of experimental groups in the tail immersion assay. Each column represents the average of the 4 groups and the vertical lines indicate the S.E.M. (Kruskal-Wallis followed by Dunn's post-hoc test).



Formalin test for nociception

Results depicted in Figure 2 indicate that 50 mg/Kg and 100 mg/Kg Ruta graveolens aqueous extract significantly increased tail withdrawal threshold following 2.5% formalin subplantar injection (50 µL) in relation to positive control (p=0.0272 and p=0.0002, respectively). 500 mg/Kg did not statistically differ from positive control.

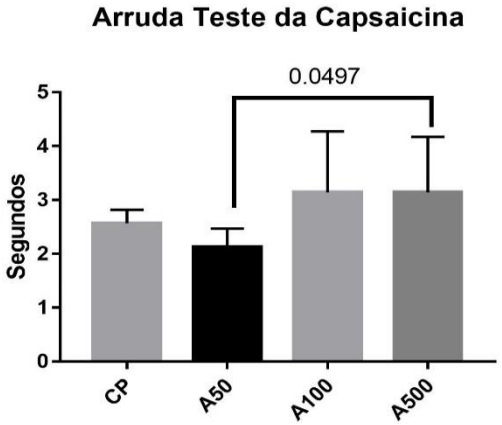
Legend for Figure 2 – Effect of Ruta graveolens aqueous extract in the tail immersion assay following 2.5% formalin subplantar injection (50 µL). Each column represents the average of the 4 groups and the vertical lines indicate the S.E.M. (Kruskal-Wallis followed by Dunn's post-hoc test).



Capsaicin test for nociception

Results depicted in Figure 3 indicate Ruta graveolens aqueous extract did not increased tail withdrawal threshold following capsaicin injected in the tail region (1.6 µg / paw) in relation to positive control.

Legend for Figure 3 - Effect of Ruta graveolens aqueous extract in the tail immersion assay following capsaicin subplantar injection (1.6 µg / paw). Each column represents the average of the 4 groups and the vertical lines indicate the S.E.M. (Kruskal-Wallis followed by Dunn's post-hoc test).



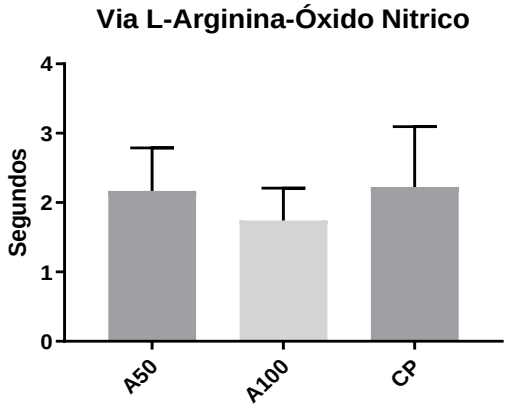
Footprint evaluation

Footprint evaluation following administration of Ruta graveolens aqueous extract, followed by the formalin test show that Ruta graveolens aqueous extract did not statistically affect footprint.

Analysis of the mechanism of antinociceptive action

Figure 4 shows that Ruta graveolens aqueous extract antinociceptive activity is exerted, at least partially, through activation of the L-arginine-NO pathway.

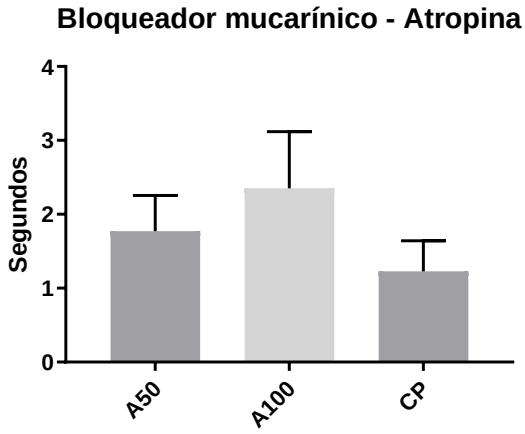
Legend for Figure 4 - Effect of Ruta graveolens aqueous extract in the tail immersion assay following administration of L-arginine (NO precursor, 600 mg/Kg, i.p.), 15 minutes before the administration of the agonist (Ruta graveolens aqueous extract). Each column represents the average of the 4 groups and the vertical lines indicate the S.E.M. (Kruskal-Wallis followed by Dunn's post-hoc test).



Possible influence on the Cholinergic System

Figure 6 demonstrates that Ruta graveolens aqueous extract antinociceptive activity is exerted, exerted, at least partially, through activation of the muscarinic system.

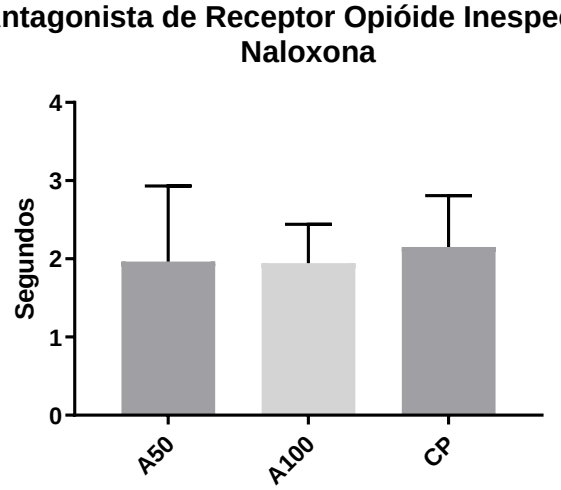
Legend for Figure 5 - Representation of the pain threshold of groups A50 (pretreated with rue and then treated with atropine), A100 (pretreated with quercetin and then treated with atropine) and CP (treated with atropine only) by the tail immersion method.



Possible influence on the Opioid System

Results depicted in Figure 6 indicate that Ruta graveolens aqueous extract antinociceptive activity is exerted, exerted, at least partially, through activation of the opioid system.

Legend for figure 6 - Effect of Ruta graveolens aqueous extract in the tail immersion assay on the opioid System. Representation of the pain threshold of groups A50 (pretreated with rue and then treated with naloxone), A100 (pretreated with rue and then treated with naloxone) and CP (treated only with naloxone) using the immersion assay tail



4 DISCUSSION

The roles of opioid, serotonergic and adrenergic receptors in regulating nociceptive modulation have been demonstrated in many studies. It is known that opioid receptors are involved in antinociception[11].

In the present study, the mechanisms for opioid receptors were evaluated, through the application of the opioid antagonist naloxone, where it can be observed in some doses that the pain threshold did not change, and it can be seen that the chemical components of Ruta graveolens continue to act on the receptors.

Asgarpanah[13] in their studies report a positive effect of Ruta Graveolens L. on antinociceptive action showing attenuation of numbers in the contortion of the acetic acid test. Showing significant activity in opiodergic and α2-adrenergic receptors, but without results in serotonergic receptors.

In our study, the contortions by acetic acid were not analyzed, but the study corroborates with Asgarpanah[13] in relation to the tail test, and in some doses the nociceptive threshold increased. In the analysis of graph 2 for formalin test there is a considerable increase in the group A100, while for graph 3 in the capsaicin test it is possible to analyze the increase in the threshold in groups A100 and A500.

Capsaicinoids consist of a class of chemical compounds present in plants of the genus Capsicum. The most common compound is N-vanyl-8-methyl-6 (E) -nonenamide, known as capsaicin. It is an alkaloid that appears in the form of crystalline, lipophilic, colorless and odorless powder, with the molecular formula C18H27NO3[14].

However, capsaicin is an exogenous agonist for the TRPV1 receptor (Transient receptor potential cation channel, subfamily V, member 1), which provides more potent responses than those triggered by factors such as temperature, pH and endogenous lipids[15,16].

Thus, when activated by heat, acidosis or by groups of exogenous agonists (capsaicin), TRPV1 transiently opens sodium and calcium channels, depolarization initiated mediated by the influx of sodium and calcium, resulting in action potentials that propagate to the spinal cord and brain, generating a feeling of warmth, burning or itching[17].

In the present study using the tail test, water at 50 ° C, after administration of rue and capsaicin, it was observed in the concentrations of 100mg/Kg and 500mg/Kg (figure 3) a greater resistance by parts of the animals, can it can be concluded that based on the analyzed results, rue and capsaicin had positive results for analgesia.

In their studies Garcia[18] on the influence of 5-HT3 Receptors on nociceptive processing in rats submitted to the fomalin test, had positive results in which nociceptive thresholds were reduced. The formalin-induced nociception model allows the assessment of two types of nociception as well as the action of analgesic drugs, thus being considered a reliable model for assessing pain.

In the study mediated by Garcia[18] and the present study enabled the use of the same technique, in these experiments the injection of an irritating substance (formalin) in the subcutaneous space of the animals' right posterior paw determined the appearance of behavioral changes, translated by characteristic motor responses that allowed assessing the intensity of the nociceptive response to the chemical stimulus.

In view of the experimental models, Sousa[19] ,evidenced a biphasic response: the first is related to the neurogenic origin that is characterized by a short period (0-15 minutes) that is generally attributed to the direct activation of nociceptors and the second phase, of inflammatory origin that consists of a longer period (15-60 minutes) and associated with the release of local endogenous mediators that generate inflammatory responses responsible for the activation and sensitization of primary afferents.

In the present study, toe spreading was not observed in the first minutes (0-5), which corroborates the studies by Sousa[19], which allowed only the analysis of the first response.

Cury[20] describes in his article that nitric oxide (NO) is involved in many physiological processes and several lines of evidence have indicated that nitric oxide plays a complex and diverse role in modulating pain. Nitric oxide is an important neurotransmitter involved in the nociceptive process.

Experimental data have also shown that nitric oxide inhibits nociception in the peripheral nervous system and also in the central nervous system. Furthermore, it is recognized that nitric oxide mediates the analgesic effect of opioids and other analgesic substances.

In the study by Kawabata[21] showed that the L-arginine substrate of the enzyme nitrate oxide synthase (NOS), significantly increases the nociceptive behavior induced by formalin, suggesting that nitric oxide has a pro-nociceptive activity, since it is the final product of the interaction between L-arginine and the NOS enzyme.

In the present study, the results covered in figures 2 and 5 show that at the concentrations of 50 mg/Kg and 100 mg/Kg Ruta graveolens aqueous extract induces

antinociceptive activity, while 500 mg/kg concentration induced pro-nociceptive activity with activation of the L-arginine-oxide-nitric system.

5 CONCLUSION

The results suggest that Ruta graveolens aqueous extract presents antinociceptive activity (50 mg/Kg and 100 mg/Kg) and from pharmacological test is was observed that it can exert this effect through the opioid, via nitric oxide and cholinergic pathway.

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