Acta Veterinaria-Beograd 2025, 75 (2), 193-211
UDK: 636.09:615.212
DOI: 10.2478/acve-2025-0016

Research article

ANTINOCICEPTIVE EFFECT OF P-CYMENE AND CINNAMALDEHYDE: THEIR RELATIONSHIP WITH THE L-ARGININE-NITRIC OXIDE PATHWAY IN RATS

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(Received 12 March 2024, Accepted 16 May 2025)

Based on the previously known therapeutic properties of the active principles from essential plant oils, we investigated the antinociceptive effects of p-cymene (PC) and cinnamaldehyde (CNA) on carrageenan (CG)-induced inflammatory hyperalgesia in female Wistar rats, as well as their relationship with the L-arginine-nitric oxide pathway. Hyperalgesia was induced by intraplantar administration of CG (500 μg) into the rat hind paw, lasting 6 hours. The electronic von Frey apparatus measured the paw withdrawal threshold induced by pressure. Motor coordination in PC-treated animals was assessed using the Rota-rod test. PC and CNA (5, 25, 50 mg/kg BW), administered orally 50 minutes before CG, reduced CG-induced hyperalgesia, with PC showing a significantly dose-dependent ($p<0.05$, $p<0.01$, $p<0.001$) and stronger antinociceptive effect ($p<0.001$) than CNA. Compared to diclofenac (10 mg/kg), PC (50 mg/kg) demonstrated superior antinociceptive activity ($p<0.001$), while CNA (50 mg/kg) had a lower effect ($p<0.05$, $p<0.01$, $p<0.001$). Co-administration of PC (5 mg/kg) or CNA (5 mg/kg) with NOS inhibitors L-NAME (5 mg/kg) or AG (0.3 mg/kg) significantly enhanced the antinociceptive effect ($p<0.01$, $p<0.001$), with PC+L-NAME showing greater potentiation ($p<0.001$) than PC+AG. L-ARG (10 mg/kg), an NO donor, significantly reduced or reversed the antinociceptive effect of PC/CNA+NOS inhibitors ($p<0.05$, $p<0.01$, $p<0.001$). In the Rota-rod test, PC (100 mg/kg BW) did not impair motor coordination or cause CNS depression in female rats. Given their significant antinociceptive effects on CG-induced hyperalgesia, the close relationship between the L-arginine-NO system and their mechanism of action, and the fact that PC does not adversely affect the CNS, both PC and CNA are promising candidates for the development of new analgesic drugs in veterinary practice

Keywords: p-cymene, cinnamaldehyde, NOS inhibitors, L-arginine, carrageenan, inflammatory hyperalgesia, rat

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INTRODUCTION

Over the past several decades, the scientific and medical professional community has shown a pronounced interest in finding new possibilities for applying plant extracts and essential oils derived from plants in treating various diseases in animals and humans. This interest stems from their potential to achieve effective therapeutic outcomes in treating inflammation, pain, bacterial, fungal, and parasitic infections, and malignant diseases [1-5].

The practice of using drugs with active ingredients derived from natural plant products or their semi-synthetic derivatives for the treatment of pain is well-established. Opioid analgesics such as morphine, codeine, buprenorphine, and salicylates are known for their effectiveness in the alleviation and elimination of pain in humans and animals. However, these drugs also come with a range of side effects such as nausea, vomiting, constipation, gastritis, and gastro-duodenal ulcer, including potentially fatal outcomes due to respiratory depression caused by morphine. Additionally, the administration of conventional analgesics in veterinary medicine brings about the issue of residues, which often limits their use in farm animals due to the potential for drug residues in meat, milk, and other animal products intended for human consumption. The mentioned properties of these analgesics, associated with the pharmacotherapy of painful conditions in animals, open up the possibility for discovering new, alternative analgesics that will, on one hand, meet the standards in pain control, and on the other, reduce side effects and eliminate the presence of residues. Essential oils and their active components are promising candidates to fulfil these requirements, especially in the context of the inflammatory pain therapy in animals [6,7].

Recent literature sources confirmed the antinociceptive effects of monoterpenes, diterpenes, and sesquiterpenes from essential oils [1,8,9].

p-Cymene is an aromatic monocyclic monoterpene [1-methyl-4-(1-methylethyl) benzene] that is abundant in essential oils of many plants, including the genera of *Protium*, *Artemisia*, *Thymus*, and *Origanum*. It is also present in various foods, including orange juice, carrots, tangerines, butter, and spices like cardamom, turmeric, and oregano [10,11]. Cinnamaldehyde is a natural, aromatic compound and the most important constituent of cinnamon. It is a phenylpropanoid of authentic flavor and scent, by which cinnamon is recognised [12,13]. Previous research has shown that p-cymene exhibits antinociceptive activity in mice models of orofacial hypernociception induced by formalin, capsaicin, and glutamate [14], as well as in carrageenan-induced pleurisy and the tail-flick test [15]. In contrast, cinnamaldehyde has been used as a pronociceptive agent in various animal models of inflammatory hyperalgesia [7,16,17]. However, recent studies have shown that cinnamaldehyde has anti-inflammatory, antiproliferative, and anticancer effects [18,19], and it can be expected that this phenylpropanoid may aid in managing pain associated with these conditions.

It is known that inflammatory hyperalgesia results from the sensitization of primary afferent neurons, which is more accurately described as hypernociception (decrease in nociceptive threshold) in different animal models [20]. This effect is triggered by inflammatory mediators, including prostaglandins (PGs), which directly increase the sensitivity of the peripheral nociceptive neuronal network. A coordinated cascade of cytokines also occurs, leading to the subsequent release of other direct mediators [21]. In inflammation genesis, nitric oxide (NO) plays a significant role. Some literary sources highlight the importance of the NO/cGMP pathway in triggering a hypernociceptive response [22,23], while other evidence supports its antinociceptive activity [24,25].

Based on numerous scientific studies examining the antinociceptive effect of p-cymene and the dual antinociceptive/pronociceptive effect of cinnamaldehyde on inflammatory pain, our goal was to investigate the impact of these two aromatic compounds on carrageenan-induced hyperalgesia and their potential connection with the L-arginine-NO system.

MATERIALS AND METHODS

Animals

The adult female Wistar rats (180-220 g) used in the present study were obtained from the Military Academy Breeding Farm, Belgrade, Serbia. The animals were housed in groups of four in home cages (42.5 × 27 × 19 cm) under standard conditions: temperature of 21 ± 1 °C, relative humidity of 55-60 %, and a 12/12 h light/dark cycle. Food and water were provided ad libitum, except during the experimental procedure. The animals were habituated individually in a plexiglass chamber for 30 minutes before testing. All behavioral testing was done between 9:00 A.M. and 2:00 P.M. For each tested dose of the test substances/drug (p-cymene, cinnamaldehyde, diclofenac), combinations of the test substances (p-cymene, cinnamaldehyde) with NOS inhibitors and NO-donor (L-arginine), as well as for the controls (vehicle-control groups), groups of 6 rats each were formed.

Drugs and administration

Substances used in this study were: carrageenan (CG), *p*-cymene (PC), cinnamaldehyde (CNA), diclofenac (DC), N^G-nitro-L-arginine methyl ester (L-NAME), aminoguanidine (AG) and L-arginine (L-ARG), were purchased from Sigma Aldrich, Inc., as well as absolute ethanol (Zorka Pharma-HEMIJA), saline (Hemofarm AD), and purified water (Aq. purify.). *p*-Cymene (PC), CNA and DC were diluted or dissolved with ethanol-aqueous solvent, while L-NAME, L-ARG and AG were dissolved in saline (vehicle). The vehicle-control group was treated orally with an ethanol-aqueous solvent. The remaining rats were divided into groups and treated orally with PC, CNA, or DC. Oral administration was performed using a gastric tube. The volume of drugs or vehicles

for oral administration did not exceed 0.1ml/100g body weight (BW). N^G-nitro-L-arginine methyl ester (L-NAME), AG and L-ARG were administered intraperitoneally. Carrageenan was dissolved in saline (5 mg/ml) and administered intraplantary (i.pl.) in the hind paw at a final volume of 0.1ml/paw, using a 1 ml syringe and 26G (0.45 x 12 mm) needle.

EXPERIMENTAL METHODS

Measurement of hyperalgesia

Hyperalgesia was induced by subcutaneous injection of ¯carrageenan (CG, 500 μg/paw) into the plantar surface of the hind paw [23]. Hyperalgesia was measured via a nociceptive mechanical paw test described by Vivancos and others (2004). The Electronic von Frey apparatus (ElUnit, Belgrade, Serbia) measured the paw withdrawal threshold in grams. An increasing pressure, up to 80 g, was applied to the rat's hind paw until the animal presented a paw flexion as the nociceptive threshold. First, the basal (control) reaction was obtained, and then the hyperalgesia was induced by intraplantar (i.pl.) injection of CG. The intensity of hyperalgesia was quantified as the differences in pressures [*d*(g)] applied before and after the CG injection. Measurement was repeated three times at each time point with minimal intervals of 2 minutes, and the average *d* for each rat was used for further calculations. Force differences are expressed as a per cent antinociceptive activity (%AA) and calculated according to the following formula:

%AA = (control group average *d* – test group average *d*)/ (control group average *d*) x 100.

If the test group average *d* was greater than the control group average *d*, a value of 0 % AA was assigned [23].

The percentage inhibition (%I) of the antinociceptive effect induced by L-arginine was expressed as follows [23]:

%I = 100 – (*%AA* AC+N + L-ARG) / (*%AA* AC+N) x 100.

AC denotes aromatic compound (PC, CNA), and N denotes NOS inhibitors (L-NAME, AG).

Experimental protocol

All tested substances and NOS inhibitors were applied as a pre-treatment before CG in this experiment. p-Cymene (PC), CNA, and DC were administered orally 50 minutes before CG. NOS inhibitors were administered intraperitoneally 30 minutes before CG. The NO-donor L-arginine was administered intraperitoneally simultaneously with the CG injection (0 h) (Figure 1). Pre-drug *d* was obtained before the i.pl. CG injection. Post-drug *d* was measured at 1, 2, 3, 3.30, 4, 4.30, 5, 5.30 and 6 h after CG

administration. Protocols concerning the dose and time of administration of aromatic compounds (PC, CNA) and other substances were obtained from literature data and previously published research [23,26].

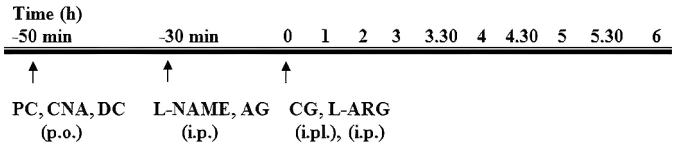

Figure 1. Timeline of the experimental protocol.

Rota-rod motor coordination test

In this study, the Rota-rod test was used to assess the effect of PC on motor coordination in rats. Motor performance was evaluated with an automated Rota-rod system, which incorporated software and a database developed by ElUnit, Belgrade, Serbia. The rats were trained for 5 days before the test and then divided into a vehicle-control group (n=6) and a PC-treated group (100 mg/kg, p.o.), (n=6). The test assessed the rats' ability to maintain balance on a rotating rod at speeds ranging from 4 to 15 rpm over 5 minutes. Measurements were taken at 0, 45, 90, and 180 minutes following drug administration. To assess the potential depressive effects of PC on the central nervous system, righting reflexes and tail-pinch tests were incorporated alongside the Rota-rod motor coordination test. A positive tail-pinch response was observed as any physical movement on a flat surface in reaction to a tail pinch with forceps. The righting reflex was deemed normal when a rat returned to an upright position from a prone posture. All tests were performed immediately after the application of PC or vehicle and before placing the rats on the rotating rod [26].

Statistical analysis

The data were statistically analysed using One-Way Analysis of Variance (ANOVA), the post hoc Tukey's HSD test for multiple comparisons, and the Z-test for proportions. A probability of less than 5 % ($p<0.05$) was considered statistically significant. Numerical and statistical processing of the results was performed using Microsoft Excel, IBM SPSS for Windows, and the Pharmacologic Calculation System.

RESULTS

Antinociceptive effect of p-Cymene and Cinnamaldehyde on Carrageenan-induced hyperalgesia

p-Cymene (PC) (5-50 mg/kg, p.o.), administered 50 minutes before CG injection into the rat hind paw, produced a significant dose-dependent antinociceptive effect in female rats (Figure 2A). At a dose of 5 mg/kg, PC caused a significant reduction

in hypernociception ($p<0.05$, $p<0.01$, $p<0.001$) at the 2 h time point (22.9 %), and from the 3.5 h (23 %) to the 5.5 h (34.9 %) time points after CG administration (not shown). This terpene, at the two higher tested doses (25 and 50 mg/kg), exhibited a pronounced antinociceptive effect ($p<0.001$) at all observation time points. At a dose of 25 mg/kg, PC achieved the maximum antinociceptive activity (%AA) at the 5.5 h time point (96.5 %), and the effect lasted until the final measurement at 6 h (95.9 %) (not shown). At the highest dose of 50 mg/kg, PC achieved maximum antinociceptive activity (%AA) at the 1 h and 2 h time points (100 %), with the effect lasting until the final measurement at 6 h (90.2 %) (Table 1).

Table 1. Antinociceptive activity (% AA) for PC (50 mg/kg, p.o.) and CNA (50 mg/kg, p.o.) on CG-induced hyperalgesia

Aromatic compounds (AC)	Time (h)								
	1	2	3	3.5	4	4.5	5	5.5	6
PC	100.0$^{///}$	100.0$^{///}$	97.7$^{///}$	95.7$^{///}$	97.1$^{///}$	95.4$^{///}$	98.3$^{///}$	95.0$^{///}$	90.2$^{///}$
CNA	79.0	70.5	70.7	80.5	75.9	61.7	60.3	58.3	71.5

$^{///}$ $p<0.001$ PC vs. CNA, Z-test for proportions.

The oral ED50 value of PC for the antinociceptive effect on carrageenan-induced hyperalgesia in female rats for the observation period of 3.5 to 5.5 hours was 9.34 mg/kg, 10.45 mg/kg, 8.54 mg/kg, 19.12 mg/kg, and 6.33 mg/kg, respectively, with a confidence interval of 95 %. Cinnamaldehyde (CNA) (5-50 mg/kg, p.o.), administered 50 minutes before CG injection into the hind paw of rats, produced a significant antinociceptive effect ($p<0.001$) only at the highest tested dose of 50 mg/kg (Figure 2B).

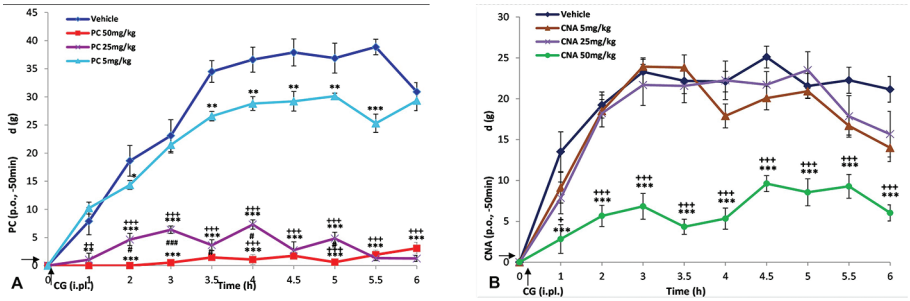

Figure 2A. Antinociceptive effect of PC (5-50 mg/kg; p.o.) on CG-induced hyperalgesia. PC was administered 50 min before CG (500 µg/paw). The symbols denote mean ± SE of 6 rats/group. *$p<0.05$, **$p<0.01$, ***$p<0.001$ vs. vehicle; $^{++}p<0.01$, $^{+++}p<0.001$ vs. PC 25 and 50 mg/kg; $^{\#}p<0.05$, $^{\#\#\#}p<0.001$ PC 25 mg/kg vs. PC 50 mg/kg, one-way ANOVA followed by post hoc Tukey`s HSD test.

Figure 2B. Antinociceptive effect of CNA (5-50 mg/kg; p.o.) on CG-induced hyperalgesia. CNA was administered 50 min before CG (500 µg/paw). The symbols denote mean ± SE of 6 rats/group. ***$p<0.001$ CNA 50 mg/kg vs. vehicle; $^{+}p<0.05$, $^{+++}p<0.001$ CNA 50 mg/kg vs. CNA 5 and 25 mg/kg, one-way ANOVA followed by post hoc Tukey`s HSD test.

A high level of hypernociception reduction was achieved at all observation time points, starting at 1 h (79 %), with the highest value at 3.5 h (80.5 %) and the final observation time point at 6 h (71.5 %) (Table 1).

Comparative study of the antinociceptive effects of p-Cymene, Cinnamaldehyde and Diclofenac in female rats

Diclofenac (DC), administered at a recommended dose of 10 mg/kg, p.o., 50 minutes before the application of CG in female rats, causes a significant reduction in hypernociception ($p<0.001$) induced by CG. Comparing the antinociceptive effects of PC (50 mg/kg) and DC (10 mg/kg) in female rats, it is observed that PC achieves a greater antinociceptive effect than the NSAID diclofenac on carrageenan-induced hyperalgesia, with statistical significance at time points of 1 h ($p<0.001$), 3.5 h ($p<0.001$), and 5.5 h ($p<0.01$) after CG administration (Figure 3). In contrast, the antinociceptive effect of CNA (50 mg/kg) was significantly lower at time points of 2 h ($p<0.001$), 3 h ($p<0.01$), 4.5 h ($p<0.001$), 5 h ($p<0.001$), and 6 h ($p<0.05$) compared to the antinociceptive effect of DC (10 mg/kg) on carrageenan-induced hyperalgesia (Figure 3).

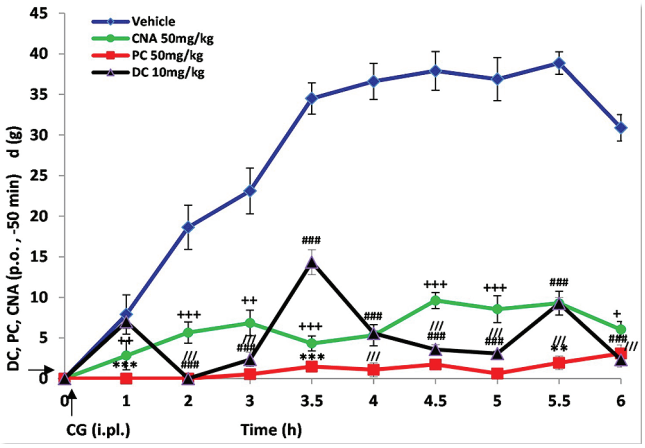

Figure 3. Comparison of the antinociceptive effects of PC (50 mg/kg; p.o.), CNA (50 mg/kg; p.o.) and DC (10 mg/kg; p.o.) on hyperalgesia induced by CG in male rats. Aromatic compounds and DC were administered p.o., 50 min before CG. The symbols denote mean ± SE of 6 rats/group. ** $p<0.01$, ***$p<0.001$ PC 50 mg/kg vs. DC 10 mg/kg; $^{+}p<0.05$, $^{++}p<0.01$, $^{+++}p<0.001$ CNA 50m/kg vs. DC 10 mg/kg; $^{\#\#\#}p<0.001$ DC 10 mg/kg vs. vehicle; $^{/}p<0.05$, $^{///}p<0.001$ PC 50m/kg vs. CNA 50m/kg, one-way ANOVA followed by post hoc Tukey`s HSD test.

The antinociceptive effect of PC (50 mg/kg) was significantly more pronounced ($p<0.05$, $p<0.01$, $p<0.001$) compared to CNA (50 mg/kg) at all observation time points (1-6 h) (Figure 3). The antinociceptive activity (% AA) of PC (50 mg/kg) compared to the same parameter for CNA (50 mg/kg) is consistently higher, with a significance level of 99.9 % (Table 1).

The influence of NOS inhibitors on the antinociceptive effect of p-Cymene and Cinnamaldehyde on Carrageenan-induced hyperalgesia

Co-administration of PC (the lowest effective dose of 5 mg/kg, p.o.) and L-NAME (5 mg/kg, i.p.) or AG (0.3 mg/kg, i.p.) significantly increased the antinociceptive effect ($p<0.001$) compared to the same effect of PC at the lowest effective dose (5 mg/kg) (Figure 4).

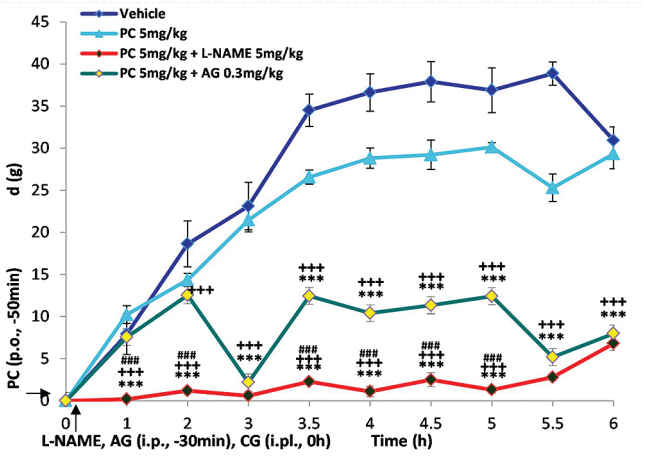

Figure 4. Antinociceptive effect of PC (5 mg/kg; p.o.) + L-NAME (5 mg/kg; i.p.) or AG (0.3 mg/kg; i.p.) combination on hyperalgesia induced by CG (500 μg/paw). PC was administered 50 min, and L-NAME or AG 30 min before CG. The symbols denote mean ± SE of 6 rats/group. ***$p<0.001$ PC 5 mg/kg vs. PC+NOS inhibitors; $^{+++}p<0.001$ PC+NOS inhibitors vs. vehicle; $^{###}p<0.001$ PC+L-NAME vs. PC+AG, one-way ANOVA followed by post hoc Tukey`s HSD test.

p-Cymene and L-NAME administered together caused a significant reduction ($p<0.001$) of carrageenan-induced hyperalgesia at all observed time points (1-6 h). Similarly, PC and AG administered together caused a significant reduction in hypernociception ($p<0.001$) from 3 h to 6 h after CG administration (Table 2).

Table 2. Antinociceptive activity (%AA) for co-administration of aromatic compounds (AC) (5 mg/kg, p.o.) and L-NAME (5 mg/kg, i.p.) or AG (0.3 mg/kg, i.p.) on CG-induced hyperalgesia

AC + NOS inhibitors	Time (h)								
	1	2	3	3.5	4	4.5	5	5.5	6
PC	0	23.0	7.1	23.0	21.3	22.9	18.3	34.9	5.1
PC+L-NAME	97.4^{***+++} ##	93.6*** +++ #	97.4*** + ###	93.4^{***+++} ###	97.0^{***+++} ###	93.4*** +++ ###	96.4^{***+++} ###	92.8*** ###	77.9*** ###
PC+AG	4.3	32.7	90.5$^{***###}$	63.9$^{***##}$	71.6$^{***###}$	70.08$^{***###}$	66.3$^{***#}$	86,67*** ###	74.1*** ###
CAN	32.5	3.6	0	0	19.0	20.0	3.2	25.2	34.0
CNA+L-NAME	54.3**	71.3***	49.2^{***+++}	39.9***	18.9	38.3^{**+++}	9.0	25.3	50.3^{*+++}
CNA+AG	84.0^{**+++}	71.3 ***	24.6**	28.2***	12.5	15.9	40.1*** $^{+++}$	20.0	13.8

** $p<0.01$, *** $p<0.001$ PC/CNA+NOS inhibitor vs. PC/CNA; $^{+}$ $p<0.05$, $^{+++}$ $p<0.001$ PC/CNA+L-NAME vs. PC/CNA+AG; $^{\#}$ $p<0.05$, $^{\#\#}$ $p<0.01$, $^{\#\#\#}$ $p<0.001$ PC+NOS inhibitors vs. CNA+NOS inhibitors, Z-test for proportions.

Co-administration of CNA (the lowest effective dose of 5 mg/kg, p.o.) and L-NAME (5 mg/kg, i.p.) or AG (0.3 mg/kg, i.p.) significantly increased the antinociceptive effect ($p<0.05$, $p<0.01$, $p<0.001$) compared to the same effect of CNA at the lowest effective dose (5 mg/kg), but not at all observation time points (Figure 5). Cinnamaldehyde and L-NAME administered together caused a significant reduction of carrageenan-induced hyperalgesia from 2 h to 3.5 h ($p<0.001$), 4.5 h ($p<0.001$), and 6 h ($p<0.05$) after CG administration. Cinnamaldehyde and AG administered together caused a significant reduction of carrageenan-induced hyperalgesia from 1 h to 3.5 h ($p<0.01$, $p<0.001$) and 5 h ($p<0.001$) after CG administration (Table 2). The selected sub-effective NOS inhibitor doses (L-NAME, AG) used in these experiments were based on data from our previous research [23].

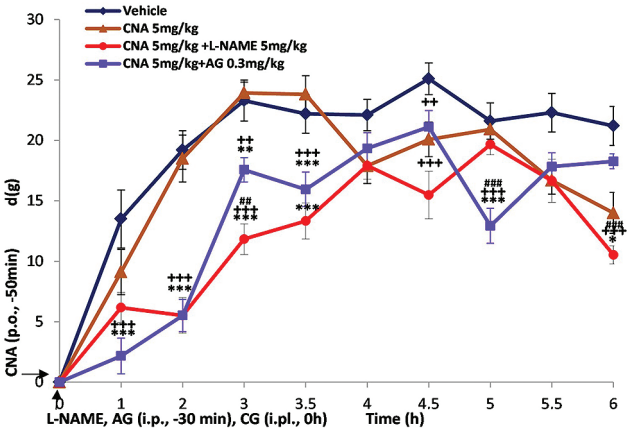

Figure 5. Antinociceptive effect of CNA (5 mg/kg; p.o.) + L-NAME (5 mg/kg; i.p.) or AG (0.3 mg/kg; i.p.) combination on hyperalgesia induced by CG (500 μg/paw). CNA was administered for 50 min, and L-NAME or AG was administered for 30 min before CG. The symbols denote mean ± SE of 6 rats/group. *$p<0.05$, **$p<0.01$, ***$p<0.001$ CNA 5 mg/kg vs. CNA+NOS inhibitors; $^{++}p<0.01$, $^{+++}p<0.001$ CNA+NOS inhibitors vs. vehicle; $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ CNA+L-NAME vs. CNA+AG, one-way ANOVA followed by post hoc Tukey`s HSD test.

The influence of L-Arginine on the antinociceptive effects of p-Cymene or Cinnamaldehyde combined with NOS inhibitors in Carrageenan-induced hyperalgesia

In this part of the experiment, L-ARG (10 mg/kg, i.p.) significantly decreased the antinociceptive effect ($p<0.001$) of the combination of PC (5 mg/kg, p.o.) and L-NAME (5 mg/kg, i.p.) (Figure 6A) or AG (0.3 mg/kg, i.p.) (Figure 6B). L-ARG showed a pronounced inhibitory effect and suppressed antinociception at all observed time points (1-6 h) for the combination of PC and L-NAME or AG, respectively (Table 3).

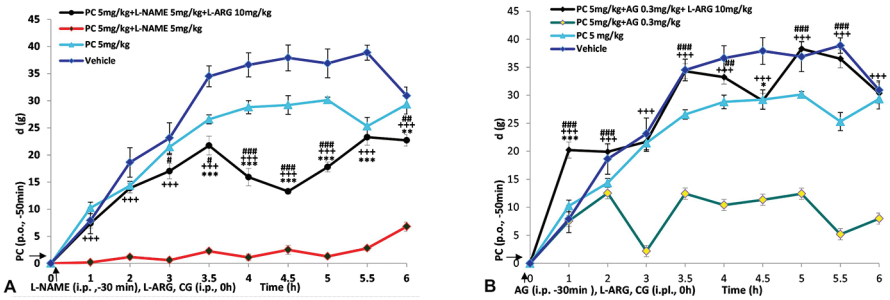

Figure 6A. Influence of L-ARG (10 mg/kg; i.p.) on the antinociceptive effect of PC (5 mg/kg; p.o.) + L-NAME (5 mg/kg; i.p.) combination. PC was administered 50 min, L-NAME 30 min before and L-ARG was administered at the same time as CG (500 μg/paw). The symbols denote the mean ± SE of 6 rats/group.**p<0.01, ***p<0.001 vs. vehicle; $^{+++}$p<0.001 PC+L-NAME+L-ARG combination vs. PC+L-NAME; $^{\#}$p<0.05, $^{\#\#}$p<0.01, $^{\#\#\#}$p<0.001 PC+L-NAME+L-ARG vs. PC 5 mg/kg, one-way ANOVA followed by post hoc Tukey`s HSD test.
Figure 6B. Influence of L-ARG (10 mg/kg; i.p.) on the antinociceptive effect of PC (5 mg/kg; p.o.) + AG (0.3 mg/kg; i.p.) combination. PC was administered 50 min, AG 30 min before and L-ARG was administered at the same time as CG (500 μg/paw). The symbols denote the mean ± SE of 6 rats/group. *p<0.05, ***p<0.001 vs. vehicle; $^{+++}$p<0.001 PC+AG+L-ARG combination vs. PC+AG; $^{\#\#}$p<0.01, $^{\#\#\#}$p<0.001 PC+AG+L-ARG vs. PC 5 mg/kg, one-way ANOVA followed by post hoc Tukey`s HSD test.

Table 3. The inhibition (*%I*) of the antinociceptive effect of co-administration of aromatic compounds (AC) (5 mg/kg, p.o.) and L-NAME (5 mg/kg, i.p.) or AG (0.3 mg/kg, i.p.) by L-ARG (10 mg/kg, i.p.) on CG-induced hyperalgesia

AC + NOS inhibitors + L-ARG	Time (h)								
	1	2	3	3.5	4	4.5	5	5.5	6
PC+ L-NAME+L-ARG	97.3	91.5	96.5	89.6	93.1	81.2	92.7	88	70
PC+ AG+L-ARG	62.6	37	90	63.7	68.7	60.9	67.6	85.8	74
CNA+ L-NAME+L-ARG	0	0	14.6	50.6	31.3	37.4	17.9	13.9	35
CNA+ AG+L-ARG	66.7	68	16	31.3	26.5	26.4	52.2	0.8	18

L-ARG (10 mg/kg, i.p.) significantly decreased the antinociceptive effect (p<0.05, p<0.01, p<0.001) of the combination of CNA (5 mg/kg, p.o.) and L-NAME (5 mg/kg, i.p.) (Figure 7A) or AG (0.3 mg/kg, i.p.) (Figure 7B). L-ARG showed a pronounced inhibitory effect and suppressed antinociception from 3.5 h to 5 h, and at 6 h for the combination of CNA and L-NAME, and from 1 h to 5 h, and at 6 h for the combination of CNA and AG (Table 3).

The dose of the NO-precursor L-ARG (10 mg/kg, i.p.) used in these experiments was selected as a sub-effective dose for nociception based on our previous research [23].

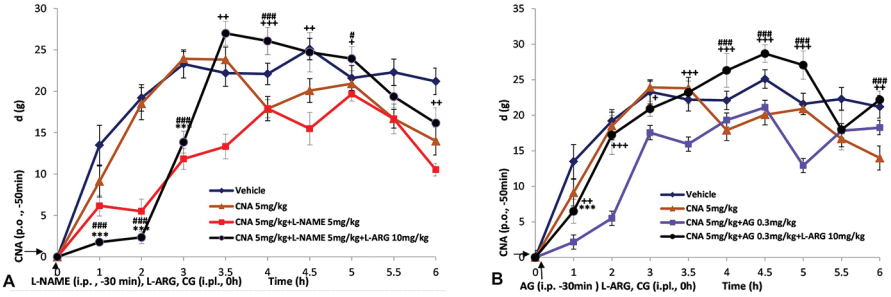

Figure 7A. Influence of L-ARG (10 mg/kg; i.p.) on the antinociceptive effect of CNA (5 mg/kg; p.o.) + L-NAME (5 mg/kg; i.p.) combination. CNA was administered 50 min, L-NAME 30 min before and L-ARG was administered at the same time as CG (500 μg/paw). The symbols denote the mean ± SE of 6 rats/group. ***$p<0.001$ vs. vehicle; $^{+}p<0.05$, $^{++}p<0.01$, $^{+++}p<0.001$ CNA+L-NAME+L-ARG combination vs. CNA+L-NAME; $^{\#}p<0.05$, $^{\#\#\#}p<0.001$ CNA+L-NAME+L-ARG vs. CNA 5 mg/kg, one-way ANOVA followed by post hoc Tukey`s HSD test.

Figure 7B. Influence of L-ARG (10 mg/kg; i.p.) on the antinociceptive effect of CNA (5 mg/kg; p.o.) +AG (0.3 mg/kg; i.p.) combination. CNA was administered 50 min, AG 30 min before and L-ARG was administered at the same time as CG (500 μg/paw). The symbols denote the mean ± SEM of 6 rats /group. ***$p<0.001$ vs. vehicle; $^{+}p<0.05$, $^{++}p<0.01$, $^{+++}p<0.001$ CNA+AG+L-ARG combination vs. CNA+AG; $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ CNA+AG+L-ARG vs. CNA 5 mg/kg, one-way ANOVA followed by post hoc Tukey`s HSD test.

Effect of p-Cymene on motor coordination in female rats

Female rats treated with an ethanol-aqueous solvent (0.1ml/100g BW) (vehicle-control group, n=6) did not fall off the rotating rod at 0, 45, 90, and 180 minutes after vehicle administration. Additionally, the female rats treated with PC at the double-maximal dose of 100 mg/kg did not show disturbances in motor coordination at any time point of measurement. All tested rats displayed no signs of CNS depression; the righting reflex was fully preserved, and walking on a flat static surface (after the tail pinch test) was normal.

DISCUSSION

The antinociceptive effect of p-Cymene and Cinnamaldehyde on inflammatory hyperalgesia and comparison with a conventional analgesic

In this study performed on female Wistar rats, oral administration of PC in the pre-treatment of carrageenan-induced hyperalgesia inhibited nociceptive behavior, lasting 6 hours for the two higher doses (25 and 50 mg/kg). The antinociceptive activity of this monoterpene was demonstrated in earlier experiments by other researchers using acetic acid-induced abdominal contractions and hot plate tests [28], and formalin-induced hypernociception tests in mice [29]. The duration of the antinociceptive effect of PC in our experiment is particularly noteworthy, lasting for 6 hours when

administered in the pre-treatment phase of CG hyperalgesia (50 minutes before CG application). This confirmed that the long-lasting antinociceptive effect of PC ensures effective management of inflammatory pain under experimental conditions.

Cinnamaldehyde (CNA), administered at the same doses (5 – 50 mg/kg; p.o.) and in the same way as PC, showed significant antinociceptive activity ($p<0.001$), but only at the highest tested dose (50 mg/kg). Similarly, the antinociceptive effect of CNA lasted for 6 hours, just as it did with PC. Molania et al. (2022) recorded the antinociceptive effect of CNA in treating aphthous stomatitis in humans. In this clinical study, CNA was applied topically at a dose of 10 mg via a mucoadhesive patch to buccal lesions three times a day for seven days, during which pain and lesion size were significantly reduced. Additionally, the authors reported no side effects of topically applied CNA in humans [30]. The described clinical study on humans was preceded by a preclinical investigation demonstrating the anti-inflammatory and antioxidant effects of CNA (50 mg/kg, i.p.) administered systemically once daily for seven days in a rat model of gamma irradiation-induced stomatitis [31]. Therefore, CNA, when administered in various ways (topically/systemically) and dosing regimens (pre-treatment/treatment) across a range of doses (10-50 mg) in different mammalian species (rats, humans), demonstrates significant antinociceptive effects. However, we should not overlook that CNA has been used in some experiments as a pronociceptive agent to induce inflammation and inflammatory pain through topical administration in mice and zebrafish [16,17]. This dual antinociceptive/pronociceptive effect of CNA suggests that natural aromatic compounds face certain challenges in pharmacotherapeutic use. Therefore, it is necessary to investigate optimal pharmaceutical formulations, doses, and routes of administration to ensure the desired therapeutic effect and minimize potential adverse effects in the use of CNA as an analgesic and anti-inflammatory drug.

Comparing the antinociceptive effect of the tested aromatic compounds with DC as a conventional analgesic for inflammatory pain, PC shows the same or significantly better analgesic activity ($p<0.01$; $p<0.001$) at all observation time points, while CNA exhibits a significantly stronger antinociceptive effect ($p<0.01$; $p<0.001$) only at specific observation time points (1 h and 3.5 h) (Figure 3). The presented results of this comparison, using DC as a reference drug, allow for the evaluation of the tested aromatic compounds. With comparable and even superior antinociceptive effects compared to DC, this confirms the potential of PC and CNA as effective alternatives for managing inflammatory pain. Additionally, the similarity in the intensity and duration of the antinociceptive effects of PC, CNA, and DC suggests that their mechanisms of analgesic action are, to the same extent, inflammatory hyperalgesia. Likewise, comparing the antinociceptive effects of PC and CNA on inflammatory hyperalgesia in this segment of the experiment provides a notable advantage to PC regarding the intensity of its impact (Table 1).

The relationship between the L-arginine-NO system and aromatic compounds (PC and CNA) in the modulation of carrageenan-induced hyperalgesia

As part of our research, we emphasised the potential relationship between the antinociceptive effects of PC and CNA and the L-arginine-NO system. The role of NO in developing and transmitting the pain signal has been proven for more than three decades [32]. Previous research has shown that inhibiting NO production achieves an antinociceptive effect when pain is caused by stimulating peripheral nerves with chemical agents (p-benzoquinone, formalin) and measuring hyperalgesia through mechanical or thermal stimuli [33,34], as well as in the visceral pain model [35]. In these studies, non-selective and selective inhibitors of NO-synthase (NOS) were used to inhibit NO production. The aim was to investigate the role of NO production in the genesis of inflammatory pain and the involvement of the L-arginine-NO system in the mechanism of action of some potential analgesics. In our earlier research, the inhibition of NO production was used as a model to demonstrate the mechanism of the analgesic effect of the nonsteroidal anti-inflammatory drug (NSAID) flunixin meglumine in inflammatory pain [23].

In this study, the antinociceptive effect of PC was enhanced in the presence of L-NAME, a nonselective inhibitor of NO-synthase (NOS), and AG, a selective inhibitor of inducible NO-synthase (iNOS), at a significance level of 99.9 %. Considering the duration, the potentiation of the antinociceptive effect of PC was longer in the presence of L-NAME (1-6 h) compared to AG (3-6 h) (see Figure 4). Moreover, it can be observed that the potentiation of the antinociceptive effect of PC is significantly better ($p<0.05$, $p<0.001$) and longer expressed in the presence of the nonselective NOS inhibitor L-NAME compared to the selective NOS inhibitor AG (see Table 2). The clearly expressed potentiation of the antinociceptive effect of the PC+NOS inhibitor combination, along with the distinct difference in the intensity of the observed effect depending on the selectivity of the applied NOS inhibitor, suggests a connection between the antinociceptive mechanism of action of PC and NOS inhibitors. Similarly, an increase in the antinociceptive effect of CNA was observed in the presence of NOS inhibitors, L-NAME and AG (Figure 5); however, this effect was significantly less pronounced ($p<0.05$, $p<0.01$, $p<0.001$) compared to the antinociceptive effect of co-administration of PC and NOS inhibitors (Table 2). The duration of the potentiation of the antinociceptive activity of the combination of CNA and the selective/nonselective NOS inhibitors L-NAME or AG showed no difference being observed within the first 3.5 hours of the experiment (Figure 5). Measured by intensity, the potentiation of antinociception of CNA with NOS inhibitors was similar, with minor deviations at specific observation time points (Table 2). Otherwise, the modulating role of NOS inhibitors in hypernociception has been known for some time. Thus, in our earlier research, it was shown that L-NAME (10 mg/kg, i.p.) administered alone as a pretreatment for carrageenan-induced hyperalgesia achieves an antinociceptive effect and that when administered in a sub-

effective dose of L-NAME (5 mg/kg, i.p.) in combination with flunixin meglumine, it potentiates the antinociceptive effect of this NSAID [23]. Similarly, L-NAME (30 mg/kg, i.p.) administered as a pretreatment potentiated the antinociceptive effect of the antidepressant venlafaxine or the combination of venlafaxine with morphine in the tail-flick test on mice [36]. On the other hand, studies have been published in which the NOS inhibitor L-NAME (30 mg/kg; 20 mg/kg) administered parenterally in the pretreatment of formalin-induced hyperalgesia on the rodent hind paw, reversed or attenuated the antinociceptive effect of α-terpineol and ß-caryophyllene, respectively [37,38]. Based on the literature data and our previous research on the possible dual antinociceptive/pronociceptive effect of NOS inhibitors, we applied confirmed sub-effective doses of L-NAME (5 mg/kg) and AG (0.3 mg/kg), respectively. Thus, we excluded the possibility of a direct effect of NOS inhibitors on carrageenan-induced hyperalgesia in female rats.

To further confirm the connection between NO production inhibition and the mechanism of the antinociceptive effect of the aromatic compounds PS and CNA on inflammatory hyperalgesia, we included L-arginine (L-ARG) in the final phase of this part of the experiment.. As an NO donor, this amino acid, administered with CG, alters the antinociceptive effects of the aromatic compounds (PC, CNA) and NOS inhibitors administered together. This indicates that the increased presence of NO significantly reduced or even reversed the antinociceptive effect of PC and NOS inhibitors at all observed time points (Figures 6A, B). The inhibition values *(%I)* of the antinociceptive effect of the combination of PC and NOS inhibitors in the presence of L-ARG on carrageenan-induced hyperalgesia are high, as shown in Table 3. Similarly, L-ARG, acting as an NO donor, reduced or reversed the antinociceptive effect of the combination of CNA and NOS inhibitors, resulting in hypernociception that matched carrageenan-induced hyperalgesia at specific observation time points (Figures 7A,B, Table 3). The similar effects of co-administration of L-ARG, NOS inhibitors, and flunixin meglumine or venlafaxine on inflammatory and thermal hyperalgesia were demonstrated in our previous experiment [23] and by Mansouri et al. [36] However, in the experiments conducted by the mentioned research teams, the involvement of the L-arginine-NO system was additionally confirmed through the combined administration of flunixin meglumine or venlafaxine with L-ARG, without the presence of NOS inhibitors.

Another significant aspect of our research focused on evaluating the effect of PC on the motor coordination of female rats and visible signs of CNS depression. PC was chosen because of its superior, dose-dependent analgesic activity compared to CNA. The preservation of motor coordination and the absence of CNS depression in female rats exposed to PC at 2xEDmax for this experiment further support the safety of PC's potential use as an analgesic in clinical practice.

CONCLUSION

The study data show that PC and CNA possess significant antinociceptive properties in the pretreatment of inflammatory pain. The antinociceptive effect of PC exhibits a clear dose-dependence and is superior in potency compared to CNA. In terms of duration, their antinociceptive activity does not differ and fully covers the monitored duration of carrageenan-induced hyperalgesia. The antinociceptive effects of PC and CNA are closely related to excessive NO production in inflammatory pain and have a clear connection with the L-arginine-NO system in the mechanism of analgesic action. At a high dose, PC shows no harmful effects on the CNS in female rats. Considering the study's conclusions and the fact that PC and CNA are plant-derived aromatic compounds with presumably low side effects and no residues, we can regard them as strong candidates for developing new analgesic drugs for veterinary practice.

Acknowledgements

This research received support from the Ministry of Education, SAntcience and Technological Development of the Republic of Serbia, Grant No.31087, and Contract No. 451-03-66/2024-03/200143.

The publication of the manuscript was supported by INVENT TRADE d.o.o., Belgrade, Serbia.

Ethical approval

All experimental procedures were performed with the permission of the Ministry of Agriculture, Forestry and Water Management of the Republic of Serbia–Veterinary Directorate (permit N°323-07-1026/2016 – 05/7).

Authors' contributions

MM was the lead researcher and authored the article. ĐSM analyzed and interpreted the results of potential CNS harm. SMT assessed the dose-response of the examined substances and contributed to the article. DS reviewed and refined the article.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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ANTINOCICEPTIVNI EFEKAT P-CIMENA I CINAMALDEHIDA: POVEZANOST SA L-ARGININ-NO PUTEM KOD PACOVA

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Na osnovu prethodno poznatih terapeutskih svojstava aktivnih principa iz esencijalnih biljnih ulja, ispitivali smo antinociceptivne efekte p-cimena (PC) i cinamaldehida (CNA) na karagenanom (CG)-indukovanu inflamatornu hiperalgeziju kod ženki pacova soja Wistar, kao i njihov odnos sa L-arginin-NO putem. Hiperalgezija je izazvana intraplantarnom aplikacijom CG (500μg) u zadnju šapu pacova, u trajanju od 6 sati. Prag povlačenja šape, usled pritiska, merio se elektronskim von Frey aparatom. Motorička koordinacija kod pacova tretiranih PC-om procenjivana je Rota-rod testom. PC i CNA (5, 25, 50mg/kg TM), primenjeni oralno, 50 minuta pre CG, smanjili su CG-indukovanu hiperalgeziju, pri čemu je PC pokazao značajan dozno-zavisan ($p<0.05$, $p<0.01$, $p<0.001$) i jači antinociceptivni efekat ($p<0,001$) u poređenju sa CNA. U poređenju sa diklofenakom (10mg/kg), PC (50mg/kg) je pokazao superiornu antinociceptivnu aktivnost ($p<0,001$), dok je CNA (50mg/kg) imao slabiji efekat ($p<0,05$, $p<0,01$, $p<0,001$). Istovremena primena PC (5mg/kg) ili CNA (5mg/kg) sa NOS inhibitorima

L-NAME (5mg/kg) ili AG (0,3mg/kg) značajno je pojačala antinociceptivni efekat ($p<0,01$, $p<0,001$), pri čemu je PC+L-NAME pokazao veće pojačanje ($p<0,001$) u poređenju sa PC+AG. L-ARG (10mg/kg), NO donor, značajno je smanjio ili poništio antinociceptivni efekat PC/CNA+NOS inhibitora ($p<0,05$, $p<0,01$, $p<0,001$). U Rota-rod testu, PC (100mg/kg TM) nije narušio motoričku koordinaciju niti izazvao depresiju CNS-a kod ženki pacova. S obzirom na njihove značajne antinociceptivne efekte na CG-indukovanu hiperalgeziju, blisku povezanost njihovog mehanizma delovanja sa L-arginin-NO sistemom, kao i činjenicu da PC nema štetne efekte na CNS, PC i CNA su obećavajući kandidati za razvoj novih analgetika u veterinarskoj praksi.