

ANTINOCICEPTIVE EFFECT OF 4-ALLYL-2-METHOXYPHENOL AND DICLOFENAC CO-ADMINISTRATION IN THE FORMALIN TEST IN RATS

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Abstract

Pain is one of the main ailments that affects humanity. To treat it, non-steroid anti-inflammatory analgesics have been widely used and also opioid analgesics, but to a lesser extent. The use of natural extracts has provided excellent results and represents a new treatment alternative. This study aimed to evaluate the antinociceptive effect of the co-administration of 4-allyl-2-methoxyphenol (eugenol; clove essential oil, *Syzygium aromaticum*) and diclofenac through the formalin test. Eugenol-diclofenac combinations were used at total doses of 725 µg/kg b.w. and 962.5 µg/kg b.w. to measure the antinociception through the formalin test in Wistar Kyoto rats. The combination eugenol-diclofenac in a dose of 962.5 µg/kg b.w. showed the highest effect in the behavioural nociceptive inhibition compared to the combination of 725 µg/kg b.w. and individual treatments (eugenol 1200 µg/kg b.w. and diclofenac 250 µg/kg b.w.). In this context, it was obtained a $92.29 \pm 0.64\%$ of antinociception in phase 1 accentuated up to $94.84 \pm 0.79\%$ in phase 2. The eugenol and diclofenac combination in the dose of 962.5 µg/kg b.w. showed a supra additive antinociceptive effect, higher than the dose of 725 µg/kg b.w. and the individual doses, which suggests a combination of the antinociceptive mechanisms of eugenol and diclofenac.

Rezumat

Scopul acestui studiu a fost evaluarea efectului antinociceptiv al administrării concomitente de 4-aliil-2-metoxifenol (eugenol; ulei esențial de cuișoare, *Syzygium aromaticum*) și diclofenac, utilizând testul formalinei. Combinațiile eugenol-diclofenac au fost utilizate în doze totale de 725 µg/kg corp și 962,5 µg/kg corp pentru a evalua antinocicepția prin testul formalinei la șobolani Wistar Kyoto. Combinația eugenol-diclofenac în doză de 962,5 µg/kg corp a arătat cel mai mare efect în inhibiția nociceptivă comportamentală comparativ cu doza de 725 µg/kg corp și tratamentele individuale (eugenol 1200 µg/kg corp și diclofenac 250 µg/kg corp). În acest context, s-a obținut un rezultat de $92,29 \pm 0,64\%$ antinocicepție în faza 1, accentuat până la $94,84 \pm 0,79\%$ în faza 2. Combinația eugenol și diclofenac în doză 962,5 µg/kg corp prezintă un efect antinociceptiv supraaditiv mai mare decât doza 725 µg/kg corp și dozele individuale, ceea ce sugerează o combinație de mecanisme antinociceptive ale eugenolului și diclofenacului.

Keywords: eugenol, diclofenac, anti-nociceptive effect, formalin test

Introduction

The International Association for the Study of Pain (IASP) describes pain as a non-pleasant sensorial and emotional experience. It is associated with real or potential tissue damage or described in equivalent terms [8, 39].

Pain sensation refers to a process of tissue damage or destruction with lancinating and deleterious characteristics in the organism. Emotionally, a person manifests anxiety, excitement and fear, with alterations of the physical, social, and psychological conditions that complicate its therapeutic approach.

During the evolution of pain, it might become chronic, because of biopsychosocial processes [5], which limited physical activity, and can lead to inability; therefore, it is a relevant topic of health. For the treatment of pain, diverse groups of analgesics are used: the ones known as non-steroidal anti-inflammatory drugs (NSAIDs) that act by inhibition of cyclooxygenases (COXs), as paracetamol, diclofenac (DFC) and ketorolac. The opioids that act at receptors level δ (δ), κ (κ) and μ (μ), are adjuvants like antidepressants, anxiolytics, neuroleptics, anti-epileptics, anticonvulsants, muscle relaxants, antidepressants, anti-emetics, anti-spastics,

anaesthetics and molecules of natural origin, among others [2, 6, 9, 15, 17].

From the NSAIDs class, DFC is derived from the phenylacetic acid and is used highly in clinical practice inhibiting COX-1 and COX-2 enzymes with the subsequent inability of prostanoid synthesis [3]. It is used to ease the pain in conditions like osteoarthritis, mainly in oral formulations [30]. In the context of pain, there are other therapeutic options which come from natural extracts, for example, *Calendula officinalis* [38], *Zingiber officinale* [24], *Arnica montana L.*, eugenol derived from *Syzygium aromaticum*, and a few others, which have anti-inflammatory and analgesic properties [13]. Eugenol (4-allyl-2-methoxyphenol; EGL) is an essential oil isolated from cloves, utilized in odontology dentistry as an analgesic. It has anti-inflammatory, antibacterial, anaesthetic and neuroprotective properties [20]. Besides, it suppresses the cyclooxygenase 2 in macrophages of rats treated with lipopolysaccharides [26]. Other mechanisms related to eugenol have been explored [29, 23, 49] due to its diverse pharmacological properties. In pain cases where conventional treatments do not eradicate or reduce it, the alternative seeks to diminish the nociception using small doses of a combination of drugs or through different administration methods. This strategy was better compared to conventional analgesics in postoperative pain monotherapy [48]. Also, drug combinations may lead to additive or synergistic effects when drugs are associated with different action mechanisms inferred from analysis and scientific deduction [43]. This research evaluates the antinociceptive effect of the combination of EGL with DFC, using the formalin test in rats as a model.

Materials and Methods

Drugs and reagents: Diclofenac sodium for injection (15 mg/3 mL) (Novartis Laboratories) and eugenol (Sigma-Aldrich, 99% pure, 1.067 g/mL) were used. The nociception test used dilutions of drugs made in sterile saline solution.

Experimental animals: There were used Wistar Kyoto rats of four to five weeks of age, with a weight of

250 to 350 g. They were allowed to accommodate to a regulated temperature of 25°C, a cycle of light-darkness of 12/12 h, with free access to food and water. Each experimental group was constituted of five rats. The experimental models were accustomed to bioterium conditions. They were sacrificed under the recommendations of the ethic guides for pain research in experimental animals from the IASP (International Association for the Research of Pain), and the attachment to the Principles of Laboratory Animal Care [31].

Nociception Test: The rat-formalin model (FMN) [14] was used. Three days and one hour before the experiment, the rats were acclimatized in the cylinder for 60 min. Subsequently, it was injected a volume of 50 µL of 5% formaldehyde solution (FMN 5%) into the paw and it was observed the rat's behaviour (flinches).

Nociceptive behavioural response: The measurement of flinches was performed for one hour, at intervals of 5 min and the observations were divided into two phases: phase 1 (F1), the flinches were checked between 0 min and 15 min and the phase 2 (F2) was recorded between 15 min to 60 min [46].

Antinociceptive evaluation: The antinociceptive effect after administering a dose of 1200 µg/kg b.w. of EGL applied in the paw was evaluated, likewise for the DFC in doses of 250 µg/kg b.w. paw [33].

In two groups it was applied the EGL + DFC combination: group 1 administered 50% of the total dose for EGL and DFC (600 µg/kg b.w. and 125 µg/kg b.w. respectively) with a total dose of the combination of 725 µg/kg b.w.; group 2 was given 75% of the total dose for EGL (900 µg/kg b.w.) and 25% for DFC (62.5 µg/kg b.w.) with a total dose of 962.5 µg/kg b.w. for the combination.

Statistical analyses: The experimental groups were distributed according to Table I. The standard error (SEM) was calculated for each group for the short-courses with n = 5 experimental units per group. Shapiro Wilks' test of normality to each result, as well as the analysis of variance (ANOVA) and a Tukey post-test (*p ≤ 0.05), were applied. All data were modelled by Origin software version 8.0.

Table I

Experimental design, five treated and one control group, each with 5 test animals

Groups	Treatments ^a (µL)	FMN 5% (µL); *10	Total administered volume (µL)
Formalin (control)	-	50	50
Saline solution (0.9%)	50	-	50
DFC (250 µg/kg b.w.)	25	25*	50
EGL (1200 µg/kg b.w.)	25	25*	50
EGL + DFC (725 µg/kg b.w.)	25	25*	50
EGL + DFC (962.5 µg/kg b.w.)	25	25*	50

^a groups solution, - means without, * 10% formalin solution to maintain the same concentration as the Control.

Results and Discussion

Administering DFC (250 µg/kg b.w.) and EGL (1200 µg/kg b.w.) 20 min before the 5% FMN solution, caused a decrease in the rat's flinches. DFC administration

reduced the 45 flinches produced to 28, recorded in the group with 5% FMN, during the first 15 min in phase 1 of the test. In phase 2, a diminution from 30 to 10 flinches starting from minute 15, was registered

up-to-the-minute 60, reaching a significant decrease $*p \leq 0.05$ vs. 5% FMN, Figure 1.

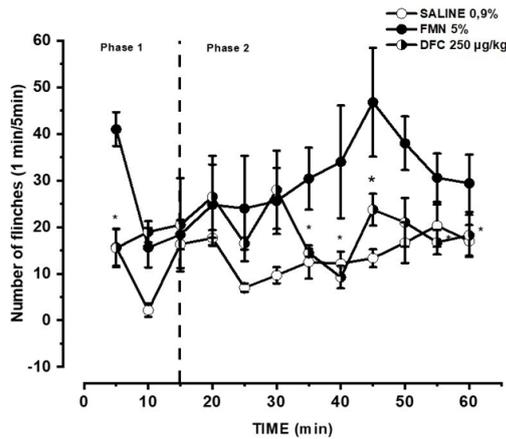


Figure 1.

Time course of the formalin test with DFC (250 µg/kg b.w.)

Each symbol represents the mean ± S.M.E. of 5 Wistar Kyoto rats ($*p \leq 0.05$ vs. 5% FMN) over the 60 min post-injection

EGL administration, compared to 5% FMN, decreases the number of flinches from 31 to 10 in F1, and in F2 it reduced from 20 to 10 flinches ($*p \leq 0.05$), Figure 2.

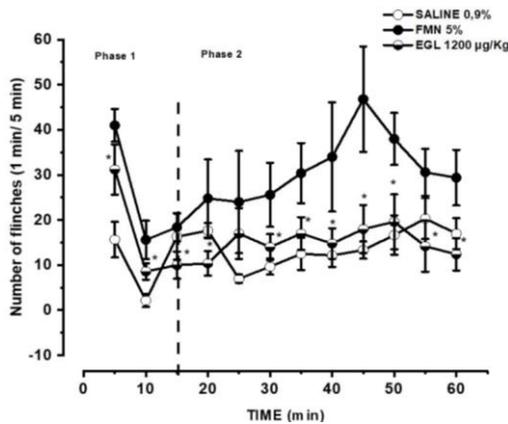


Figure 2.

Vi Time course of the formalin test with EGL (1200 µg/kg b.w.)

Each symbol represents the mean ± S.M.E. of 5 Wistar Kyoto rats ($*p \leq 0.05$ vs. 5% FMN) over the 60 min post-injection

When applying the combination EGL + DFC (725 µg/kg b.w.) starting from the initial doses, it was observed that from the minute 5 up-to-minute 60 (F1 and F2) there were only 13 flinches, which represents 31 fewer flinches vs. 5% FMN ($*p \leq 0.05$). Likewise, the combination of EGL + DFC (962.5 µg/kg b.w.) favours the diminution of the number of flinches done by FMN 5% up to 5-3 flinches as a maximum, since the minute 5 up-to-minute 60 (F1 and F2; $*p \leq 0.05$), Figure 3.

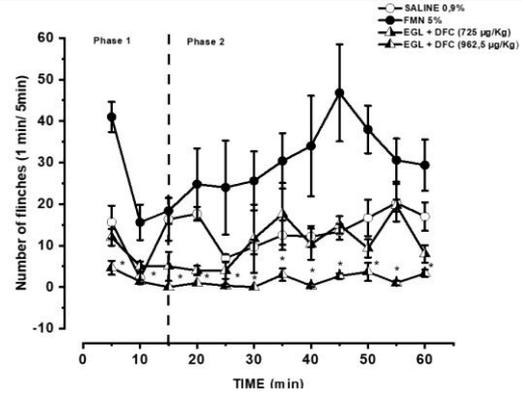


Figure 3.

The dose-response curve for EGL+DFC in doses used (725 µg/kg b.w. and 962.5 µg/kg b.w.)

Each symbol represents the $x \pm$ S.M.E in 5 Wistar Kyoto rats ($*p \leq 0.05$ vs. 5% FMN) over the 60 min post-injection

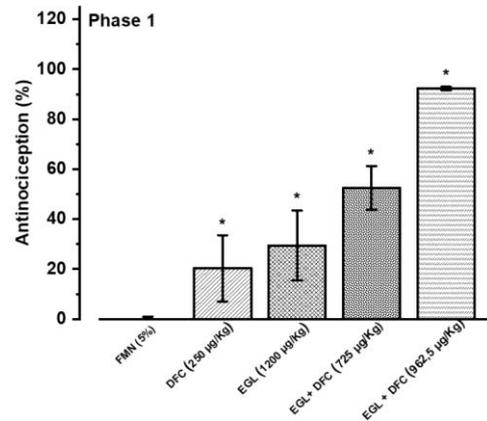


Figure 4.

Antinociception percent in the formalin test, phase 1 (0 - 15 min)

The data represents the $x \pm$ S.M.E of 5 Wistar Kyoto rats ($*p \leq 0.05$ vs. 5% FMN)

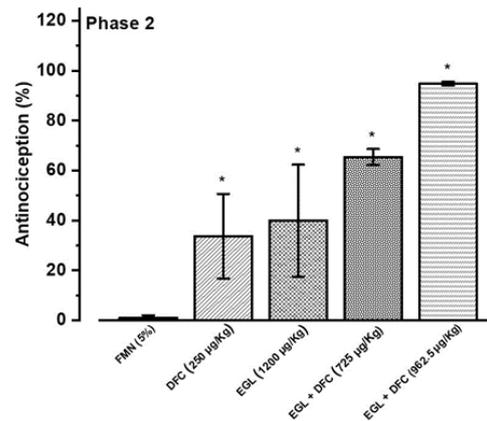


Figure 5.

Antinociception percent in the formalin test, phase 2 (15 - 60 min)

The data represents the $x \pm$ S.M.E of 5 Wistar Kyoto rats ($*p \leq 0.05$ vs. 5% FMN)

The treatment effects are expressed as a percentage of antinociception, calculated from the area under

the curve of antinociception. The group EGL + DFC in the dose 962.5 $\mu\text{g}/\text{kg}$ b.w. produced $92.29 \pm 0.649\%$ in F1 and $94.84 \pm 0.79\%$ in F2, respectively. Also, EGL + DFC in the dose 725 $\mu\text{g}/\text{kg}$ b.w. produced $52 \pm 8.81\%$ (F1) and $65 \pm 3.22\%$ (F2), respectively. On the other hand, the DFC dose resulted in $20.36 \pm 13.26\%$ (F1) and $33.66 \pm 16.9\%$ (F2) and EGL showed $29.41 \pm 14\%$ (F1) and $39.92 \pm 22.41\%$ (F2), Figure 4 and Figure 5.

When co-administered, the active molecules may favour pharmacological interactions, such as pharmacokinetics or pharmacodynamics. The last ones are due to the effect on receptors or organs in which they act, modifying the mechanism of action and potency of the response expected [19]. In some pathologies such as pain, drug interactions can be a benefit to the patient. Also, attacking it in different ways it optimizes the therapeutic efficacy; thus, in case of a synergism, it lowers the dose and favours the safety lines [32].

The chosen combination EGL + DFC (725 $\mu\text{g}/\text{kg}$ b.w. and 962.5 $\mu\text{g}/\text{kg}$ b.w.) started from the premise pointed out by Tallarida *et al.* 2006 [45], who argued to lower the effective dose of a combination of drugs, there must be combined compounds with complementary mechanisms of action in constant proportions from effective individual doses.

In this research, DFC (250 $\mu\text{g}/\text{kg}$ b.w.) and EGL (1200 $\mu\text{g}/\text{kg}$ b.w.) significantly decreased the number of flinches produced by 5% FMN ($*p \leq 0.05$) in F1 (Figure 4) and F2, but with a higher antinociceptive effect in F2 (Figure 5). The result for EGL and DFC coincides with previous studies where it is shown that the antinociceptive effect corresponds to the doses for EGL and DFC of 1200 $\mu\text{g}/\text{kg}$ b.w. and 250 $\mu\text{g}/\text{kg}$ b.w. respectively, Lugo-Lugo *et al.* [33]. This is consistent with the data reported by Picazo *et al.* [43], where they observed a dose-slope effect in phase 2 of the formalin test when administering DFC at 100 and 1000 $\mu\text{g}/\text{paw}$. Also, De Paz-Campos *et al.* [11], observed an antinociceptive effect of DFC (131 mg/kg b.w., O.A.) in F2, applying the formalin model. Hasani *et al.* in 2011 [22] demonstrated that the intraperitoneal administration of DFC (10 mg/kg b.w.) favours a decrease in the nociceptive behaviour and preventive analgesia in the thermal model of inflammation-induced, 10 minutes after administration. Aparecido *et al.* in 2009 [4], confirmed that EGL (400 mg/kg b.w. O.A.) shows effective antinociception in the carrageenan model, reducing the oedema two to four hours after delivering the carrageenan at 41.1% in comparison to that produced by indomethacin and celecoxib. Further, Fonseca *et al.*, in 2016 [18], reported an ortho-eugenol pre-treatment (100 mg/kg b.w.), which lessens the number of twists in 92.4% using acetic acid model; meanwhile, in the glutamate model, the reduction in the time of licking was 53.3%. Likewise, Aguilar-González and López-Malo [1], show that *Syzygium aromaticum* extract inhibits both acute and chronic

inflammation in the carrageenan model. The described studies support the given effect reported in our work, both for EGL and DFC, decreasing the number of flinches when applying an irritant (5% FMN).

The combination of EGL + DFC 725 $\mu\text{g}/\text{kg}$ b.w. reduce up to $52.48 \pm 8.81\%$ nociception in F1, and $65.38 \pm 3.22\%$ during F2, using the formalin test. This effect is higher than the individual ones after administering of EGL and DFC. Likewise, the percentage of antinociception for the combination EGL + DFC 962.5 $\mu\text{g}/\text{kg}$ b.w. was $92.29 \pm 0.64\%$ in F1, increasing up to $94.84 \pm 0.79\%$ in F2 ($*p < 0.05$ vs. 5% FMN). EGL + DFC (962.5 $\mu\text{g}/\text{kg}$ b.w.), showed increased efficacy considering a supra additive effect by complementary mechanisms in order to optimize efficiency. EGL was reported to act on Na^+ [44], Ca^{++} [40, 47], TRP [34, 37] channels and cannabinoid receptors [36], which complements the mechanism of action of DFC on COX. In this regard, some authors agreed that the combination of biomedical pain relievers and natural products contributes to positive interactions of antinociception since one of the basic principles of pharmacological interactions is to produce the same effect through different routes of action. For example, it has been suggested for EGL, that the opiate pathway [10, 21, 41] is responsible for the analgesic effect, knowing that CFD acts through cyclooxygenases [7]. The consequence of this association is the maximum effect, as established by Tallarida *et al.* [45]. For that reason, De Paz-Campos [11] proposed a synergistic interaction between DFC and curcumin, a plant-based product, which gave an antinociceptive effect in the rat formalin model. On the other hand, the treatment with yohimbine reversed the antinociceptive effect of ortho-eugenol, which suggests an adrenergic system contribution. Also, Halder *et al.* [21] demonstrated an antinociceptive behaviour after administering a dose of 0.1 mL/kg b.w. of *Syzygium aromaticum* extract in mice, in both phases of the formalin model.

Previously reported by other authors, in the tail-flick test, an increase in latencies indicates the participation of the opioid pathway in the nociceptive behaviour. Dal Bó *et al.* [10] confirmed that EGL promoted the antinociceptive opioid route and glutamatergic receptors (AMPA/Kainate) and reported TNF- α inhibition, a critical component in the severe pain. Otherwise, Guenette *et al.* [20] demonstrated that the supply of EGL decreases the thermal sensibility, evaluated with the Hargreaves test in a neuropathic pain model supported by the antagonist effect of EGL over the receptors TRVP1. Kim and Park [27] showed that preconditioning with EGL results in the recovery of the periodontal ligament *via* oxidative stress modulation. Besides, Khalilzadeh *et al.* [25] comment that EGL has an analgesic and anaesthetic effect when administered topically due to the inhibitory effect on the voltage-gated sodium channels, high voltage-dependent calcium channels and weak activation of the receptors TRVP1.

In this context and considering the action mechanisms proposed for EGL and DFC, its combination contributed to the antinociceptive effect with a lower dose of both components in the proportions established EGL + DFC 725 µg/kg b.w. and 962.5 µg/kg b.w. EGL has also been combined with other NSAIDs [28, 35, 50] and with an ester of aspirin with excellent results and low toxicity at cellular and genomic levels. Li *et al.* [28] mixed EGL, zinc oxide, and poly-methyl-methacrylate in stomatology, obtaining good effects and lower irritability in pulp tissues.

Furthermore, Martínez-Herrera *et al.* [35] reported that EGL toxicity at the cellular level showed variability in response, depending on the cell line used [16, 42, 45]. In summary, it is expected that the combination of DFC and EGL represents a safe profile for use as an alternative in the treatment of localized pain. Besides, the data accomplished the expectancy of the OMS strategy (2012) [39], which favours and supports the use of traditional medicine or its combination with drugs (biomedical origin), always with the prospect to obtain the best results considering high therapeutic efficiency and the decrease in resistance to treatments.

Conclusions

The co-administration of eugenol and diclofenac in the dose of 962.5 µg/kg b.w. presents a higher supra-additive antinociceptive effect than the doses of 725 µg/kg b.w. and the individual effect of each component. The effect obtained is probably due to the combination of antinociceptive mechanisms of action of EGL and DFC.

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Conflict of interest

The authors declare no conflict of interest.

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