Canadian Journal of Physiology and Pharmacology

Citral inhibits the nociception in the rat formalin test: Effect of metformin and blockers of opioid receptor and the NO-cGMP-K⁺ channel pathway

Journal:	<i>Canadian Journal of Physiology and Pharmacology</i>
Manuscript ID	cjpp-2021-0458.R1
Manuscript Type:	Article
Date Submitted by the Author:	07-Oct-2021
Complete List of Authors:	Ortiz, Mario; Universidad Autonoma del Estado de Hidalgo, Área Académica de Medicina del Instituto de Ciencias de la Salud, Laboratorio de Farmacología Cariño-Cortés, Raquel; Área Académica de Medicina del Instituto de Ciencias de la Salud. Universidad Autónoma del Estado de Hidalgo, Pachuca, Hidalgo, Mexico, Muñoz Pérez, Victor; Autonomous University of Hidalgo State, Reproductive Biology; Medina-Solís, Carlo; Universidad Autónoma del Estado de Hidalgo Instituto de Ciencias de la Salud Castañeda-Hernández, Gilberto; Centro de Investigacion y de Estudios Avanzados del IPN, Department of Pharmacology
Is the invited manuscript for consideration in a Special Issue:	Not applicable (regular submission)
Keyword:	Antinociception; Citral; nociception; K ⁺ channels; Metformin; Formalin test; Rat; Peripheral effect.

SCHOLARONE™
Manuscripts

Citral inhibits the nociception in the rat formalin test: Effect of metformin and blockers of opioid receptor and the NO-cGMP-K⁺ channel pathway

Mario I. Ortiz^{1*}, Raquel Cariño-Cortés¹, Víctor M. Muñoz-Pérez¹, Carlo Eduardo Medina-Solís²
Gilberto Castañeda-Hernández³

1. Área Académica de Medicina del Instituto de Ciencias de la Salud. Universidad Autónoma del Estado de Hidalgo, Pachuca, Hidalgo, Mexico.

2. Área Académica de Odontología del Instituto de Ciencias de la Salud de la Universidad Autónoma del Estado de Hidalgo. Pachuca, Mexico.

3. Departamento de Farmacología, Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional, Ciudad de México, Mexico.

Running title: Activation of K⁺ channels by citral

*Corresponding author:

Mario I. Ortiz, Ph.D.

Laboratorio de Farmacología, Área Académica de Medicina del Instituto de Ciencias de la Salud
Universidad Autónoma del Estado de Hidalgo

Eliseo Ramírez Ulloa 400, Col. Doctores. Pachuca, Hgo. 42090, MEXICO

Phone Number: +52-77-1717-2000 Ext. 2361

Fax Number: +52-77-1717-2000 Ext. 2361

E-mail: mario_i_ortiz@hotmail.com

Abstract

The objective of the present study was to scrutinize the effect of nitric oxide (NO), cGMP, potassium channel blockers and metformin on the citral-produced peripheral antinociception. The rat paw 1% formalin test was used to assess nociception and antinociception. Rats were treated with local peripheral administration of citral (10-100 $\mu\text{g/paw}$). The antinociception of citral (100 $\mu\text{g/paw}$) was evaluated with and without the local pretreatment of naloxone, NG-L-nitro-arginine methyl ester (L-NAME, a NO synthesis inhibitor), 1H-(1,2,4)-oxadiazolo(4,2-a)quinoxalin-1-one (ODQ, a soluble guanylyl cyclase inhibitor), metformin, opioid receptors antagonists, and K^+ channel blockers. Injection of citral in the rat paw significantly decreased the nociceptive effect of formalin administration during the two phases of the test. Local pretreatment of the paws with L-NAME and ODQ did not reduced the citral-induced antinociception. Glipizide or glibenclamide ($\text{K}_{\text{ir}}6.1$ -2; ATP-sensitive K^+ channel blockers), tetraethylammonium or 4-aminopyridine (K_{v} ; voltage-gated K^+ channel blockers) or charybdotoxin ($\text{K}_{\text{Ca}}1.1$; big conductance calcium-activated K^+ channel blocker) or apamin ($\text{K}_{\text{Ca}}2.1$ -3; small conductance Ca^{2+} -activated K^+ channel antagonist), or metformin, but not the opioid antagonists, reduced the antinociception of citral. Citral produced peripheral antinociception during both phases of the formalin test. These effects were due to the activation of K^+ channels and a biguanide-dependent mechanism.

Key words: Antinociception; Citral; nociception; K^+ channels; Metformin; Formalin test; Rat; Peripheral effect.

Introduction

Citral, also known as geranial or neral, is an aromatic terpene derivative that can be found in different plants and citrus fruits. Citral is found as a clear yellowish liquid, with a characteristic lemon or citrus odor (Api et al. 2020). Citral is found an additive in foods, cosmetics, detergents, and other industrial products (Sharma et al. 2021). Citral has several biological activities in the health area, such as hypoglycemic, antiviral, diuretic, anti-inflammatory, expectorant, spasmolytic, among others (Api et al. 2020; Sharma et al. 2021; Lin et al. 2008; Mishra et al. 2019; Long et al. 2019). There are reports about the action mechanism of citral *in vitro* and *in vivo*. Some researchers have suggested that citral could inhibit the nitric oxide production in lipopolysaccharide (LPS)-stimulated cells (Mishra et al. 2019; Long et al. 2019; Katsukawa et al. 2010) and to decrease cyclooxygenase-(COX)-2 LPS-induced expression (Lee et al. 2008). On another hand, citral inhibits several transient receptor potential (TRP) ion channels (TRPV1–4, TRPM8, and TRPA1) in dorsal root ganglion neurons (Stotz et al. 2008). In a recent study, citral inhibited the production of the pro-inflammatory cytokines, IL-1 β and TNF- α , and promoted the synthesis of the anti-inflammatory cytokine, IL-10 in human myometrial tissue (Muñoz-Pérez et al. 2021). In another study, glycine and GABA-derivative mechanisms were triggered by citral on neurons of the trigeminal subnucleus in mice (Nguyen et al., 2019). In another publication, the citral-induced antinociception in the formalin test involved the possible participation of 5-HT_{1A} and 5-HT₃ receptors, but not of nitric oxide or opioid receptors (Nishijima et al. 2014). A recent investigation found that the citral-produced antihyperalgesic effect involved the activation of the CB₂ cannabinoid receptor, but not the ATP-sensitive K⁺ channel (Gonçalves et al. 2020). Likewise, the activation of the TLR4, TLR2/Dectin-1, and CB₂ receptor/ATP-Sensitive K⁺ channel pathways by citral were involved in its anti-inflammatory effects (Gonçalves et al. 2020). There is evidence

about the participation of spinal serotonin in the antinociception of citral in an arthritic rat model (Mota et al. 2020).

Activation of the soluble guanylyl cyclase (sGC) by nitric oxide (NO) produces cGMP (Mónica et al. 2016; Xiao et al. 2019). This pathway is involved in platelet aggregation, immunity, inflammation, cell death, smooth muscle relaxation, neurotransmitter delivery and central and peripheral nociception and antinociception (Koesling et al. 2016). In diverse experimental models, the local administration of NO synthase and sGC antagonists were able to modify the antinociceptive effects of nalbuphine, gabapentin, fluoxetine, and diclofenac, but not of curcumin, indomethacin or acetaminophen (De Paz-Campos et al. 2012; Gil-Flores et al. 2010; Ghorbanzadeh et al. 2018; Ortiz et al. 2003, 2006, 2020). Otherwise, opening of K^+ channel is involved in the inhibition and excitability of cells and tissues. K^+ channels may be modulated by the NO-cGMP pathway, other second messengers and analgesic drugs (Dascal and Kahanovitch 2019; Alexander et al. 2019). In this sense, there is evidence about the activation of K^+ channels by some analgesic drugs, such as gabapentin, diclofenac and nalbuphine. However, discrepant results have been found with other analgesic drugs (Gil-Flores et al. 2010; Ortiz et al. 2003, 2020). Therefore, our study was carried out to investigate the possible activation of opioid receptors, a biguanide-dependent mechanism, the NO-cGMP pathway and K^+ channels in the antinociceptive peripheral effects of citral in the rat formalin test.

MATERIALS AND METHODS

Animals

Male Wistar rats (weight range: 180-220 g; aged 7-9 weeks) from the CINVESTAV, IPN (Ciudad de México, Mexico) were employed for all the experiments. The rats were housed with the care (temperature, light-dark cycles, and food) previously described in other studies (De Paz-Campos

et al. 2012; Gil-Flores et al. 2010; Ortiz et al. 2003, 2006, 2020). The suffering of the animals was avoided and the minimum amount of them was used. The rats were not reused and at the end of each experiment they were euthanized in a CO₂ chamber. A study protocol was evaluated and registered by the Institutional Committee for Animal Care and Use (CINVESTAV, IPN, Mexico City, Mexico. Register number: 0169-15) prior to the start of the study. During the study, animals were treated in accordance with Canadian Council on Animal Care (CCAC) (Olfert et al. 1993) and the Guiding principle on Ethical Standards for Investigation in Animals (Zimmermann 1983).

Drugs

Cital, metformin (a hypoglycemic drug), naltrexone and naloxone (both opioid receptors blockers), NG-L-nitro-arginine methyl ester (L-NAME)(a NO synthase inhibitor), 1 H-(1,2,4)-oxadiazolo (4,2-a) quinoxalin-1-one (ODQ)(a NO-sensitive soluble guanylyl cyclase inhibitor), glipizide and glibenclamide (both ATP-sensitive K⁺ channel blockers; K_{ir}6.1-2), 4-aminopyridine (4-AP) and tetraethylammonium chloride (TEA) (both voltage-gated K⁺ channel blockers; K_v), charybdotoxin (a big conductance Ca²⁺-activated K⁺ channel blocker; K_{Ca}1.1), and apamin (a small conductance Ca²⁺-activated K⁺ channel blocker; K_{Ca}2.1-3) were acquired from Sigma-Aldrich (Toluca, Mexico). Glibenclamide, glipizide and ODQ were dissolved in a 20% DMSO solution. L-NAME, TEA, 4-AP, apamin, charybdotoxin, naloxone, naltrexone and metformin were dissolved in saline solution.

Measurement of antinociceptive activity

The rat paw 1% formalin test was used to assess nociceptive and antinociceptive effects (Ortiz et al 2003, 2006, 2020). The right hind paw of the rat was chosen for the administration of formalin and to determine its nociceptive effects. The rat was placed for 30 minutes in an acrylic cylinder for adaptation. After that, 50 microliters of a 1% formalin solution were injected subcutaneously (s.c.) in the mentioned paw. Immediately after the injection of formalin, the rat was returned to the

cylinder to quantify nociceptive behavior. In the present study, the number of flinches of the injected paw was quantified for one minute, every five minutes and up to 60 minutes. The number of flinches in the rat produced a biphasic curve. The area under the curve was determined for both phases of the test. To determine the peripheral antinociception of citral, groups of rats were administered with citral (10-100 $\mu\text{g/paw}$) subcutaneously in the right paw, 20 minutes before the administration of 50 μL of formalin in the same paw. To demonstrate that the effect of citral was local at the injection site, a 100 $\mu\text{g/paw}$ dose of citral was administered in the contralateral (left) paw 20 minutes before formalin was administered to the right paw and the responses were evaluated.

Study design

In order to demonstrate the possible participation of opioid receptors, the production of NO and cGMP and a biguanide-dependent mechanism in the citral-induced antinociception, groups of independent rats were administered with the corresponding vehicles or naloxone (50 $\mu\text{g/paw}$), naltrexone (50 $\mu\text{g/paw}$), L-NAME (100 $\mu\text{g/paw}$), ODQ (100 $\mu\text{g/paw}$), or metformin (100-400 $\mu\text{g/paw}$) 10 minutes after the administration of citral (100 $\mu\text{g/paw}$), and then the responses were evaluated as previously mentioned.

To determine the possible participation of K^+ channels in the effects of citral, other groups of independent rats underwent local peripheral administration of the corresponding vehicles or the different K^+ channel blockers (glibenclamide: 25-100 $\mu\text{g/paw}$, glipizide: 25-100 $\mu\text{g/paw}$, apamin: 0.1-2 $\mu\text{g/paw}$, charybdotoxin: 0.1-2 $\mu\text{g/paw}$, 4-AP: 25-100 $\mu\text{g/paw}$, or TEA: 25-100 $\mu\text{g/paw}$) 10 minutes after the local peripheral administration of citral and then the responses were determined. All dose of citral and blockers were administered in a volume of 50 μL . Each of the rats in all experimental groups received three injections, and the appropriate controls for multiple injections

and vehicles were performed before starting the formal study. The doses and administration schedules of the antagonists and citral for peripheral administration were selected based on previous pilot experiments carried out in our research center and on previous reports (Ambriz-Tututi et al. 2009; Ortiz et al. 2005, 2006, 2010, 2020, 2021). Rats in all experimental groups were evaluated for possible side reaction.

Data analysis and statistics

All experimental results are given as the means $\pm$ S.E.M. for 5-6 animals per group. The formalin test curves were constructed by plotting the number of flinches during 60 minutes. The number of flinches for both phases of the test are reported. The results of the effects of citral without blockers and with the blockers were examined by analysis of variance (ANOVA) with SPSS version 20 software for Windows (SPSS Inc., Chicago, IL, USA). After that, the Dunnett's test was used to compare differences between the different groups. Differences were considered to reach statistical significance when $P < 0.05$.

Results

Antinociceptive activity produced by citral

The local peripheral injection of formalin produced a flinching behavior indicative of nociception. Subcutaneous injection of citral in the right paw of the rats reduced the number of flinches produced by formalin administration. The citral-induced antinociception was statistically significant during both phases of the 1% rat formalin test ($P < 0.001$ on both phases; Figure 1).

To demonstrate that the effect of citral was local at the injection site, the administration of citral (100 μ g/paw) was carried out in the left paw, formalin in the right paw and the number of flinches

was determined. In this research strategy, citral in the contralateral paw did not significantly modify the flinching behavior after formalin injection ($P > 0.05$ in both phases; Figure 1).

Effect of the administration of ATP-sensitive ($K_{ir}6.1-2$) and voltage-gated (K_v) K^+ channel blockers on the antinociception of citral

Subcutaneous peripheral injection of glipizide and glibenclamide significantly decreased the antinociception induced by citral during both phases of the test ($P < 0.05$; Figure 2). Subcutaneous local administration of TEA and 4-AP decreased the citral-induced antinociceptive action in the two phases of the test ($P < 0.05$; Figure 3).

Effect of the administration of charybdotoxin, apamin and metformin on the antinociception of citral

Subcutaneous local administration of apamin and charybdotoxin in the rat paw significantly diminished the citral-produced antinociception during the phase one ($P < 0.05$; Figure 4), but not during the phase two of the test ($P > 0.05$; Figure 4). On the other hand, metformin administered locally significantly decreased the antinociception produced by citral during the phase two of the formalin test ($P < 0.05$; Figure 4), but not during the phase one ($P > 0.05$; Figure 4).

Effect of the NO-cGMP pathway inhibitors and opioid receptor blockers on the citral-induced antinociception

Subcutaneous injection of L-NAME and ODQ did not decrease the antinociceptive effects produced by citral during the two phases of the test ($P > 0.05$; Figure 5). Subcutaneous peripheral injection of naloxone and naltrexone did not reverse the citral-produced antinociception during the two phases of the formalin test ($P > 0.05$; Figure 5).

Discussion

In the formalin test, two phases of nociceptive behavior are observed in the animals. According to previous reports, transient receptor potential channels (TRPV1) in primary afferent neurons are activated in phase one or early phase of the test (0-10 min). On the other hand, there is a combination of peripheral nerve depolarizations and central sensitization during the phase two or late phase (Muley et al. 2016). The formalin test is widely used to evaluate analgesic, anti-inflammatory and neuromodulator drugs (Muley et al. 2016). Opioids, such as morphine and fentanyl are able to decrease the two phases of the test (Ortiz et al., 2003; Muley et al. 2016). Nevertheless, nonsteroidal anti-inflammatory drugs suppress only the phase late (Ortiz et al., 2003; Muley et al. 2016). In the actual study, peripheral subcutaneous administration of citral significantly diminished the two phases of the 1% rat formalin test. These antinociceptive effects were of local peripheral origin, since the administration of citral (100µg/paw) in the contralateral paw did not produce any significant modification of the nociceptive effect produced by formalin. Our results agree with the systemic antinociceptive effects of citral (Nishijima et al., 2014; Ortiz et al. 2010) or the essential oil of leaves of *Myrcia ovata* (which is rich in citral) (Dos Santos et al., 2014) in the formalin trial in rodents. Likewise, the antinociceptive effect produced by citral agrees with its anti-inflammatory and antihyperalgesic actions (Gonçalves et al., 2020). In general, the evidence of antinociception of pure citral in animal models is limited. Therefore, to our knowledge this is the first finding about the ability of citral to produce local peripheral antinociception in a rodent test. In agreement with the local peripheral effect of citral, a recent study demonstrated that the application of citral to the skin prevents UVB-induced skin carcinogenesis by reducing levels of oxidative stress and pro-inflammatory cytokines, and increasing apoptotic rate in the skin (Kremer et al 2019).

Potassium (K^+) is the main intracellular cation of the organism; about 98% of potassium is found inside cells, mainly in muscle and nerve cells. Potassium channels are proteins embedded in the cell membrane that control the movement of K^+ . This movement is determined by two forces, the concentration gradient and the electrical gradient. After the opening of potassium channels, K^+ would diffuse out of the cell following its concentration gradient, causing hyperpolarization (increased electronegativity inside the cell) (Alexander et al. 2019). These channels are highly selective and are grouped according to the standardized nomenclature for K^+ channels published by the International Union of Basic and Clinical Pharmacology (IUPHAR) in voltage-gated (K_v), calcium and sodium-activated (K_{Ca} and K_{Na}), two P domain (K_{2P}), and finally inwardly rectifying (K_{ir}) K^+ channels (Alexander et al. 2019). Potassium channels can be modulated by changes in cell voltage, second messengers and many drugs that are capable of activating or inhibiting their opening or closing (Alexander et al. 2019; Dascal and Kahanovitch 2015). Analgesic, anti-inflammatory or neuromodulator drugs evaluated in different nociceptive models were able to modulate K^+ channels in peripheral tissues to produce their antinociceptive effects (De Paz-Campos et al. 2012; Ortiz et al. 2003, 2005, 2006, 2020; Ghorbanzadeh et al. 2019). In the present study, local peripheral administrations of glipizide and glibenclamide (ATP-sensitive K^+ channel blockers ($K_{ir}6.1-2$) reverted the citral-produced antinociception in the two phases of the 1% formalin test; suggesting that citral could activate these channels to produce its antinociceptive action in the periphery. These results with sulfonylureas do not agree with the inactivity of systemically administered glibenclamide on the systemic antihyperalgesic effect of citral in mice (Gonçalves et al. 2020). This inconsistency has been reported with other drugs (Ghorbanzadeh et al. 2018, 2019; Ortiz et al. 2006; Kaster et al. 2007; Ostadhadi et al. 2017; Zhou et al. 2014). For example, the local peripheral antinociceptive action of gabapentin was mediated by the activation

of ATP-sensitive K^+ -channel (Ortiz et al. 2006). In contrast, the antidepressant-like effect of gabapentin in mice was mediated by blocking the ATP-sensitive K^+ -channel (Kaster et al. 2007). However, the activation of ATP-sensitive K^+ -channel by citral observed in our study, agrees with activation of ATP-Sensitive K^+ channel by citral in its anti-inflammatory effects (Gonçalves et al. 2020). Various phytochemicals such as flavonoids, terpenoids, polyphenols and alkaloids have beneficial effects on a few metabolic diseases such as diabetes mellitus and hyperlipidemia. Some of these phytochemicals can activate or inactivate enzymes, channels or membrane receptors, thereby improving homeostasis in the body. In this sense, citral showed anti-dyslipidemic and anti-diabetic activities in diverse experimental models (Sharma et al. 2021; Mishra et al. 2019). In a recent study, similar to glibenclamide, citral was able to control carbohydrate metabolism in the liver and stimulated insulin release in dyslipidemic diabetic rats (Mishra et al. 2019). Therefore, attention should be paid to the combination of terpenoids and hypoglycemic agents such as glibenclamide in diabetic patients or those with any pain syndrome.

Alternatively, in other experiments the subcutaneous administration in the rat paw of TEA, 4-AP (Figure 3), apamin and charybdotoxin (Figure 4) were able to reverse the effects of citral; suggesting the activation of voltage-sensitive and calcium-activated K^+ channels by citral, in order to produce antinociception in the periphery. Pharmacological interactions of non-sulfonylureas K^+ channels blockers and citral in inflammation, nociception or hyperalgesia animal models are missing. In whole-cell recordings on HEK293T cells overexpressing $K_v1.3$ channels, citral exhibited inhibitory effects on these channels (Ye et al. 2019).

Metformin is an oral hypoglycemic drug used to treat patients with diabetes or polycystic ovary syndrome. Metformin is an AMP-activated protein kinase (AMPK) activator in the liver, which leads to decrease of fatty acid synthesis and gluconeogenesis. Recently, it was showed that

metformin decreased the nociceptive response in the hot-plate model and inhibited the mechanical allodynia induced by chronic constriction injury in mice (Augusto et al. 2019). This last result does not agree with the lack of antinociceptive activity of metformin alone in our study (Figure 4). However, metformin was able to decrease the citral-induced antinociceptive action during the phase two of the formalin test (Figure 4). This last result comes to an agreement with the ability of metformin to block the action of citral against rhabdomyosarcoma cells *in vitro* (Duan et al. 2021). Likewise, our result about metformin agrees with previous observations of the ability of metformin to block the antinociceptive actions of several analgesic drugs (Ortiz 2011; Guzmán-Priego et al. 2017). Therefore, preclinical and clinical studies assessing the possible interaction of terpenoids and metformin are encouraged.

NO and cGMP are produced in diverse physiological and pathological conditions. The participation of the NO-cGMP pathway in peripheral antinociception induced by analgesic, anti-inflammatory and neuromodulator drugs has been previously defined (Ghorbanzadeh et al. 2018, 2019; Ortiz et al. 2003, 2006, 2020). Previous studies demonstrated the inhibition of NO production by citral in cell cultures stimulated with lipopolysaccharides (Lin et al. 2008; Katsukawa et al. 2010; Lee et al. 2008). In our study, local subcutaneous administration in the rat paw of L-NAME and ODQ did not reverse the citral-induced antinociceptive effect in the 1% formalin test (Figure 5). With these findings, the possible participation of the NO-cGMP pathway in the peripheral antinociception produced by citral in the two phases of the test is ruled out. Our findings do not agree with published previous results in other experimental models. In this sense, citral activated the production of NO in the citral-induced relaxation of isolated rat duodenum, ileum, and aorta (da Silva et al. 2018; Mangprayool et al. 2013). The discrepancies between these last results and the absence of participation of the NO-cGMP pathway in our study are probably due to the difference in the

experimental models. The tangible participation of the NO-cGMP pathway in the citral-induced antinociception should be evaluated in other nociceptive models.

Local peripheral administration of naloxone and naltrexone (opioid receptor antagonists) did not block the antinociceptive effects of citral. Then, the probable participation of opioid receptors in the peripheral antinociception by citral is ruled out. These effects, are consistent with the lack of participation of opioid receptors in the systemic antinociceptive action produce by citral in the formalin test (Nishijima et al. 2014). However, our result is different to the significant reduction of the antinociception of the essential oil from *Myrcia ovata* (which is rich in citral) by naloxone (Dos Santos et al. 2014). This discrepancy is due to the fact that the antagonistic effect of naloxone was on the antinociception of a combination of neral and geranial isomers and other chemical substances contained in the essential oil, and not only of citral.

Conclusions

Citral produced peripheral antinociception on both phases of the 1% rat formalin test. The mechanism of action of the peripheral antinociceptive activity of citral includes the activation of the K⁺ channels and a biguanide-dependent mechanism. Citral was not able to activate opioid receptors and the NO-cGMP pathway in its peripheral antinociceptive effect.

Acknowledgements

This work was supported by the grant “Red Temática: Farmacología de la Reproducción” from REDES-PRODEP-SEP, Mexico (2017-2022). Authors acknowledge to Q.F.B. Martha Patricia González García from Pharmacology Department-Cinvestav, M.V.Z. Ricardo Gaxiola Centeno, M.V.Z. Rafael Leyva Muñoz, M.V.Z. Benjamín Chávez Álvarez, M.V.Z. Carlos Giovanni Sam Miranda and René Pánfilo Morales from Cinvestav-UPEAL for their technical assistance in this

project.

Conflict of Interest statement

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

References

- Alexander, S.P.H., Mathie, A., Peters, J.A., Veale, E.L., Striessnig, J., Kelly, E., et al. 2019. The Concise Guide to Pharmacology 2019/20: Ion channels. *Br. J. Pharmacol.* **176**: S142–S228. doi:10.1111/bph.14749.
- Ambriz-Tututi, M., Rocha-González, H.I., Castañeda-Corral, G., Araiza-Saldaña, C.I., Caram-Salas, N.L., Cruz, S.L., et al. 2009. Role of opioid receptors in the reduction of formalin-induced secondary allodynia and hyperalgesia in rats. *Eur. J. Pharmacol.* **619**: 25-32. doi:10.1016/j.ejphar.2009.08.001.
- Api, A.M., Belsito, D., Biserta, S., Botelho, D., Bruze, M., Burton, G.A. Jr, et al. 2020. RIFM fragrance ingredient safety assessment, citral, CAS Registry Number 5392-40-5. *Food Chem. Toxicol.* **141** Suppl 1: 111339. doi: 10.1016/j.fct.2020.111696.
- Augusto, P.S.A., Braga, A.V., Rodrigues, F.F., Moraes, M.I., Dutra, M.M.G.B., Batista, C.R.A., et al. 2019. Metformin antinociceptive effect in models of nociceptive and neuropathic pain is partially mediated by activation of opioidergic mechanisms. *Eur. J. Pharmacol.* **858**: 172497. doi: 10.1016/j.ejphar.2019.172497.

- 321 da Silva, R.E.R., de Moraes, L.P., Silva, A.A., Bastos, C.M.S., Pereira-Gonçalves, Á., Kerntopf
322 M.R., et al. 2018. Vasorelaxant effect of the *Lippia alba* essential oil and its major
323 constituent, citral, on the contractility of isolated rat aorta. *Biomed. Pharmacother.* **108**:
324 792-798. doi: 10.1016/j.biopha.2018.09.073.
- 325 Dascal, N., and Kahanovitch, U. 2015. The Roles of G $\beta\gamma$ and G α in Gating and Regulation of GIRK
326 Channels. *Int. Rev. Neurobiol.* **123**: 27–85. doi:10.1016/bs.irn.2015.06.001.
- 327 De Paz-Campos, M.A., Chávez-Piña, A.E., Ortiz, M.I., and Castañeda-Hernández, G. 2012.
328 Evidence for the Participation of ATP-sensitive Potassium Channels in the
329 Antinociceptive Effect of Curcumin. *Korean J. Pain.* **25**: 221-227. doi:
330 10.3344/kjp.2012.25.4.221.
- 331 Dos Santos, G.C., Gomes, G.A., Gonçalves, G.M., de Sousa, L.M., Santiago, G.M., de Carvalho,
332 M.G., et al. 2014. Essential oil from *Myrcia ovata*: chemical composition, antinociceptive
333 and anti-inflammatory properties in mice. *Planta Med.* **80**: 1588-1596. doi: 10.1055/s-
334 0034-1383120.
- 335 Duan, C., Evison, A., Taylor, L., Onur, S., Morten, K., and Townley, H. 2021. The common
336 diabetes drug metformin can diminish the action of citral against Rhabdomyosarcoma cells
337 in vitro. *Phytother. Res.* **35**: 1378-1388. doi: 10.1002/ptr.6898.
- 338 Ghorbanzadeh, B., Kheirandish, V., and Mansouri, M.T. 2019. Involvement of the L-
339 arginine/Nitric Oxide/Cyclic GMP/KATP Channel Pathway and PPAR γ Receptors
340 in the Peripheral Antinociceptive Effect of Carbamazepine. *Drug Res. (Stuttg).* **69**: 650-
341 657. doi:10.1055/a-0959-5896.
- 342 Ghorbanzadeh, B., Mansouri, M.T., Naghizadeh, B., and Alboghobeish, S. 2018. Local
343 antinociceptive action of fluoxetine in the rat formalin assay: role of l-arginine/nitric

oxide/cGMP/K(ATP) channel pathway. *Can. J. Physiol. Pharmacol.* **96**: 165-172.
doi:10.1139/cjpp-2017-0003.

Gil-Flores, M., Ortiz, M.I., Castañeda-Hernández, G., and Chávez-Piña, A.E. 2010. Acemetacin antinociceptive mechanism is not related to NO or K⁺ channel pathways. *Methods Find. Exp. Clin. Pharmacol.* **32**: 101-105. doi: 10.1358/mf.2010.32.2.1423883.

Gonçalves, E.C.D., Assis, P.M., Junqueira, L.A., Cola, M., Santos, A.R.S., Raposo, N.R.B., et al. 2020. Citral Inhibits the Inflammatory Response and Hyperalgesia in Mice: The Role of TLR4, TLR2/Dectin-1, and CB2 Cannabinoid Receptor/ATP-Sensitive K(+) Channel Pathways. *J. Nat. Prod.* **83**: 1190-1200. doi: 10.1021/acs.jnatprod.9b01134.

Guzmán-Priego, C.G., Méndez-Mena, R., Baños-González, M.A., Araiza-Saldaña, C.I., Castañeda-Corral, G., and Torres-López, J.E. 2017. Antihyperalgesic Effects of Indomethacin, Ketorolac, and Metamizole in Rats: Effects of Metformin. *Drug Dev. Res.* **78**: 98-104. doi: 10.1002/ddr.21379.

Kaster, M.P., Budni, J., Binfaré, R.W., Santos, A.R., and Rodrigues, A.L. 2007. The inhibition of different types of potassium channels underlies the antidepressant-like effect of adenosine in the mouse forced swimming test. *Prog. Neuropsychopharmacol. Biol. Psychiatry.* **31**: 690-6. doi: 10.1016/j.pnpbp.2006.12.018.

Katsukawa, M., Nakata, R., Takizawa, Y., Hori, K., Takahashi, S., and Inoue, H. 2010. Citral, a component of lemongrass oil, activates PPARalpha and gamma and suppresses COX-2 expression. *Biochim. Biophys. Acta.* **1801**: 1214–1220. doi: 10.1016/j.bbalip.2010.07.004.

- 365 Koesling, D., Mergia, E., and Russwurm, M. 2016. Physiological Functions of NO-Sensitive
366 Guanylyl Cyclase Isoforms. *Curr. Med. Chem.* **23**: 2653–2665.
367 doi:10.2174/0929867323666160812145050.
- 368 Kremer, J.L., Melo, G.P., Marinello, P.C., Bordini, H.P., Rossaneis, A.C., Sábio, L.R., et al. 2019.
369 Citral prevents UVB-induced skin carcinogenesis in hairless mice. *J. Photochem.*
370 *Photobiol. B.* **198**: 111565. doi: 10.1016/j.jphotobiol.2019.111565.
- 371 Lee, H.J., Jeong, H.S., Kim, D.J., Noh, Y.H., Yuk, D.Y., and Hong, J.T. 2008. Inhibitory effect of
372 citral on NO production by suppression of iNOS expression and NF-kappa B activation in
373 RAW264.7 cells. *Arch. Pharm. Res.* **31**: 342–349. doi: 10.1007/s12272-001-1162-0.
- 374 Lin, C.T., Chen, C.J., Lin, T.Y., Tung, J.C., and Wang, S.Y. 2008. Anti-inflammation activity of
375 fruit essential oil from *Cinnamomum insularimontanum* Hayata. *Bioresour. Technol.* **99**:
376 8783-8787. doi: 10.1016/j.biortech.2008.04.041.
- 377 Long, N., Tang, H., Sun, F., Lin, L., and Dai, M. 2019. Effect and mechanism of citral against
378 methicillin-resistant *Staphylococcus aureus* in vivo. *J. Sci. Food. Agric.* **99**: 4423-4429.
379 doi: 10.1002/jsfa.9677.
- 380 Mangprayool, T., Kupittayanant, S., and Chudapongse, N. 2013. Participation of citral in the
381 bronchodilatory effect of ginger oil and possible mechanism of action. *Fitoterapia.* **89**: 68-
382 73. doi: 10.1016/j.fitote.2013.05.012.
- 383 Mishra, C., Khalid, M.A., Fatima, N., Singh, B., Tripathi, D., Waseem, M., et al. 2019. Effects of
384 citral on oxidative stress and hepatic key enzymes of glucose metabolism in
385 streptozotocin/high-fat-diet induced diabetic dyslipidemic rats. *Iran J. Basic Med. Sci.* **22**:
386 49-57. doi: 10.22038/ijbms.2018.26889.6574.

- Mónica, F.Z., Bian, K., and Murad, F. 2016. The Endothelium-Dependent Nitric Oxide-cGMP Pathway. *Adv. Pharmacol.* **77**: 1–27. doi:10.1016/bs.apha.2016.05.001.
- Mota, C.M.D., Rodrigues-Santos, C., Carolino, R.O.G., Anselmo-Franci, J.A., and Branco, L.G.S. 2020. Citral-induced analgesia is associated with increased spinal serotonin, reduced spinal nociceptive signaling, and reduced systemic oxidative stress in arthritis. *J. Ethnopharmacol.* **250**: 112486. doi: 10.1016/j.jep.2019.112486.
- Muley, M.M., Krustev, E., and McDougall, J.J. 2016. Preclinical Assessment of Inflammatory Pain. *CNS Neurosci. Ther.* **22**: 88–101. doi:10.1111/cns.12486.
- Muñoz-Pérez, V.M., Ortiz, M.I., Salas-Casa, A., Pérez-Guerrero, J., Castillo-Pacheco, N., Barragán-Ramírez, G., et al. 2021. In vitro effects of citral on the human myometrium: Potential adjunct therapy to prevent preterm births. *Birth Defects Res.* **113**: 613-622. doi: 10.1002/bdr2.1873.
- Nguyen, T.T.P., Jang, S.H., Park, S.J., Cho, D.H., and Han, S.K. 2019. Action of citral on the substantia gelatinosa neurons of the trigeminal subnucleus caudalis in juvenile mice. *Chin. J. Physiol.* **62**: 175-181. doi: 10.4103/CJP.CJP_32_19.
- Nishijima, C.M., Ganev, E.G., Mazzardo-Martins, L., Martins, D.F., Rocha, L.R., Santos, A.R., et al. 2014. Citral: a monoterpene with prophylactic and therapeutic anti-nociceptive effects in experimental models of acute and chronic pain. *Eur. J. Pharmacol.* **736**: 16-25. doi: 10.1016/j.ejphar.2014.04.029.
- Olfert, E.D., Cross, B.M., and McWilliam, A.A. 1993. 2nd Edition. Canadian Council on Animal Care (CCAC). Guide to the Care and Use of Experimental Animals. [online]. Available at: https://www.ccac.ca/Documents/Standards/Guidelines/Experimental_Animals_Vol1.pdf (Accessed: 22 December 2020)

- 410 Ortiz, M.I., Cariño-Cortés, R., and Castañeda-Hernández, G. 2020. Participation of the opioid
411 receptor - nitric oxide - cGMP - K(+) channel pathway in the peripheral antinociceptive
412 effect of nalbuphine and buprenorphine in rats. *Can. J. Physiol. Pharmacol.* **98**: 753-762.
413 doi:10.1139/cjpp-2020-0104.
- 414 Ortiz, M.I., Cariño-Cortés, R., Muñoz-Pérez, V.M., Salas-Casas, A., and Castañeda-Hernández, G.
415 2021. Role of the NO-cGMP-K(+) channels pathway in the peripheral antinociception
416 induced by alpha-bisabolol. *Can. J. Physiol. Pharmacol.* **99**: 1048-1056. doi: 10.1139/cjpp-
417 2020-0744.
- 418 Ortiz, M.I., Castro-Olgún, J., Peña-Samaniego, N., and Castañeda-Hernández, G. 2005. Probable
419 activation of the opioid receptor-nitric oxide-cyclic GMP-K⁺ channels pathway by
420 codeine. *Pharmacol. Biochem. Behav.* **82**: 695-703. doi:10.1016/j.pbb.2005.11.011.
- 421 Ortiz, M.I., Granados-Soto, V., and Castañeda-Hernández, G. 2003. The NO-cGMP-K⁺ channel
422 pathway participates in the antinociceptive effect of diclofenac, but not of indomethacin.
423 *Pharmacol. Biochem. Behav.* **76**: 187-195. doi:10.1016/s0091-3057(03)00214-4.
- 424 Ortiz, M.I., Medina-Tato, D.A., Sarmiento-Heredia, D., Palma-Martínez, J., and Granados-Soto,
425 V. 2006. Possible activation of the NO-cyclic GMP-protein kinase G-K⁺ channels
426 pathway by gabapentin on the formalin test. *Pharmacol. Biochem. Behav.* **83**: 420-427.
427 doi:10.1016/j.pbb.2006.03.002.
- 428 Ortiz, M.I., Ramírez-Montiel, M.L., González-García, M.P., Ponce-Monter, H.A., Castañeda-
429 Hernández, G., and Cariño-Cortés, R. 2010. The combination of naproxen and citral
430 reduces nociception and gastric damage in rats. *Arch. Pharm. Res.* **33**: 1691-1697. doi:
431 10.1007/s12272-010-1020-9.

- Ortiz, M.I. 2011. Blockade of the antinociception induced by diclofenac, but not of indomethacin, by sulfonylureas and biguanides. *Pharmacol. Biochem. Behav.* **99**: 1-6. doi: 10.1016/j.pbb.2011.03.008.
- Ostadhadi, S., Akbarian, R., Norouzi-Javidan, A., Nikoui, V., Zolfaghari, S., Chamanara, M., et al. 2017. Possible involvement of ATP-sensitive potassium channels in the antidepressant-like effects of gabapentin in mouse forced swimming test. *Can. J. Physiol. Pharmacol.* **95**: 795-802. doi:10.1139/cjpp-2016-0292.
- Sharma, S., Habib, S., Sahu, D., and Gupta, J. 2021. Chemical Properties and Therapeutic Potential of Citral, a Monoterpene Isolated from Lemongrass. *Med. Chem.* **17**: 2-12. doi: 10.2174/1573406416666191227111106.
- Stotz, S.C., Vriens, J., Martyn, D., Clardy, J., and Clapham, D.E. 2008. Citral sensing by TRANSient receptor potential channels in dorsal root ganglion neurons. *PLoS ONE*. **3**: e2082. doi: 10.1371/journal.pone.0002082.
- Xiao, S., Li, Q., Hu, L., Yu, Z., Yang, J., Chang, Q., et al. 2019. Soluble Guanylate Cyclase Stimulators and Activators: Where are We and Where to Go? *Mini Rev. Med. Chem.* **19**: 1544–1557. doi:10.2174/1389557519666190730110600.
- Ye, C.J., Li, S.A., Zhang, Y., and Lee, W.H. 2019. Geraniol targets K(V)1.3 ion channel and exhibits anti-inflammatory activity in vitro and in vivo. *Fitoterapia*. **139**: 104394. doi: 10.1016/j.fitote.2019.104394.
- Zhou, Q., Chen, P.C., Devaraneni, P.K., Martin, G.M., Olson, E.M., and Shyng, S.L. 2014. Carbamazepine inhibits ATP-sensitive potassium channel activity by disrupting channel response to MgADP. *Channels (Austin)*. **8**: 376-382. doi:10.4161/chan.29117.

Zimmermann, M. 1983. Ethical guidelines for investigations of experimental pain in conscious animals. *Pain* 16: 109–110. doi:10.1016/0304-3959(83)90201-4.

Figure captions.

Figure 1. Citral-induced local antinociception on the 1% formalin test. Rats receive a s.c. injection of vehicle (VEH) or citral into either the left (contralateral, CL) or right (ipsilateral, IL) paw before the formalin injection. Results are presented as the number of flinches for the two phases of the test. Every point of effect is the mean $\pm$ S.E.M. of 5-6 animals. *Significantly distinct from the vehicle group ($P < 0.05$), as determined by an ANOVA, accompany by a Dunnett's test.

Figure 2. Effect of glibenclamide and glipizide on the citral-induced antinociception during the formalin test. Rats receive a local injection of citral (-20 min) and then the inhibitors (-10 min) into the right paw. Results are presented as the number of flinches for the two phases of the test. Bars are the mean $\pm$ S.E.M. for 5-6 animals. *Significantly distinct from the vehicle group ($P < 0.05$) and #significantly distinct from the citral group ($P < 0.05$), as determined by an ANOVA test, followed by a Dunnett's test.

Figure 3. Consequence of the local injection of 4-AP and TEA on the citral-induced antinociception during the formalin test. Rats receive a local injection of citral (-20 min) and then the inhibitors (-10 min) into the right paw. Results are presented as the number of flinches for the two phases of the test. Every point of effect is the mean $\pm$ S.E.M. of 5-6 animals. *Significantly different from the vehicle group ($P < 0.05$) and #significantly different from the citral group ($P < 0.05$), as determined by an ANOVA test, followed by a Dunnett's test.

Figure 4. Effect of apamin, charybdotoxin (CHAR), and metformin (MET) on the citral-induced antinociception during the formalin test. Rats receive a local injection of citral (-20 min) and then

the inhibitors (-10 min) into the right paw. Results are presented as the number of flinches for the two phases of the test. Bars are the mean $\pm$ S.E.M. for 5-6 animals. *Significantly different from the vehicle group ($P<0.05$) and #significantly different from the citral group ($P<0.05$), as determined by ANOVA followed by a Dunnett's test.

Figure 5. Outcome of the subcutaneous administration of L-NAME, ODQ, naloxone (NLX), and naltrexone (NTX) on the citral-induced antinociception during the formalin test. Rats receive a local injection of citral (-20 min) and then the inhibitors (-10 min) into the right paw. Results are presented as the number of flinches for the two phases of the test. Bars are the mean $\pm$ S.E.M. for 5-6 animals. *Significantly different from the vehicle group ($P<0.05$) as determined by ANOVA followed by a Dunnett's test

Draft

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5