Artículo Original | Original Article

Complete essential oils of *Laurus nobilis* inducing antinociceptive action by opioid mechanism in C-Reflex and spinal Wind-Up model in rat

[Aceites esenciales totales de Laurus nobilis inducen acción antinociceptiva mediante mecanismo opioide en el modelo de Reflejo-C y Wind-Up espinal en ratas]

This article is written in tribute to Dr. Claudio Laurido (RIP) died on October 2017, on the Third Anniversary of his Death

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Abstract: The essential oil of *Laurus nobilis* L. was used to test their antinociceptive efficacy. It was applied intraperitoneally (i.p.) to rats subjected to a nociception test (C reflex and spinal wind-up). The results showed that the essential oil applied at higher doses (0.06 mg/Kg) causes a complete abolition of the spinal wind-up, while the C reflex was unchanged, indicating a clear antinociceptive effect. At lower concentrations (0.012 mg/Kg), there was a lowering in the wind-up by 85% within ten minutes of the essential i.p. oil application. Interestingly, there was an effect of naloxone (0.08 mg/Kg i.p.) When applied, a change occurs that almost entirely reversed the antinociception caused by the essential oil from *Laurus nobilis*. We conclude that there is a significant antinociceptive effect of the essential oil of *Laurus nobilis* subjected to electric nociception. In addition, it was observed that naloxone reversed the antinociceptive effect (wind-up) produced by *Laurus nobilis*.

Keywords: *Laurus nobilis* L.; Pain; C reflex; Spinal Wind-up

RESUMEN: El aceite esencial de *Laurus nobilis* L. se usó para probar su eficacia antinociceptiva. Se aplicó por vía intraperitoneal (i.p.) a ratas sometidas a una prueba de nocicepción (reflejo-C y wind-up espinal). Los resultados mostraron que el aceite esencial aplicado a dosis más altas (0.06 mg/Kg) abolió completamente el wind-up espinal, mientras que el reflejo-C no cambió, lo que indica un claro efecto antinociceptivo. A concentraciones más bajas (0.012 mg/Kg), hubo una disminución en el wind-up en un 85% dentro de los diez minutos del i.p. la aplicación del aceite esencial. Curiosamente, hubo un efecto de la naloxona (0.08 mg/Kg i.p.) la cual revierte casi por completo la antinocicepción causada por el aceite esencial de *Laurus nobilis*. Concluimos que existe un efecto antinociceptivo significativo del aceite esencial de *Laurus nobilis* sometido a nocicepción eléctrica. Además, se observó que la naloxona revirtió el efecto antinociceptivo (wind-up) producido por *Laurus nobilis*.

Palabras clave: *Laurus nobilis* L.; Dolor; Reflejo-C; Wind-up espinal

INTRODUCTION

The study of medicinal plants is becoming increasingly important, either from the popular knowledge (Barraza *et al*., 2014) and from the point of view of knowledge of the pharmacological properties of plants (de Almeida *et al*., 2003; Kumar, 2006; Wiart, 2007; Giogetti *et al*., 2011). Regarding *Laurus nobilis*, many studies have been conducted on other biological activities, among others, antioxidant protection (Kaurinovic *et al*., 2010; Ozcan *et al*., 2010; Al-Hashimi & Mahmood, 2016; Akcan *et al*., 2017; Alejo-Armijo *et al*., 2017); insecticidal effect (Chahal *et al*., 2016; Jemaa *et al*., 2011; Salehi *et al*., 2014); antimicrobial effect (Fratianni *et al*., 2007; Ozcan *et al*., 2010; Fukuyama *et al*., 2011; El *et al*., 2014; Videla *et al*., 2016; Aliberti *et al*., 2016; Fidan *et al*., 2019); antibacterial effects (Moghtader & Farahmand, 2013; Ouibrahim *et al*., 2013; Chahal *et al*., 2017; Mansour *et al*., 2018); antifungal effects (Marrufo *et al*., 2013; Rosello *et al*., 2015; Chahal *et al*., 2017); acaricidal effect (Macchioni *et al*., 2006); analgesic and anti-inflammatory activity (Sayyah *et al*., 2003; Esra *et al*., 2007; Kaileh *et al*., 2007; Alejo-Armijo *et al*., 2017; Maajida *et al*., 2019).

Numerous investigations have been carried out on the chemical composition of *Laurus nobilis* L. (Novak, 1985; Kilic *et al*., 2004; Fang *et al*., 2005; Dellacqua *et al*., 2006; Fukuyama *et al*., 2011; Patrakar *et al*., 2012; Alejo-Armijos *et al*., 2017; Caputo *et al*., 2017; Mansour *et al*., 2018; Fidan *et al*., 2019)

The definition of pain, according to the International Association for the Study of Pain states that: "Pain is an unpleasant sensory and emotional experience associated with actual or potential tissue damage or described by the patient as related to that injury" According to the above, pain is a sensory experience that should be expressed through language. If we wished to study pain in animals is a requirement that animals can talk. Therefore, M. Zimmerman (1986), adapted the definition of pain from the International Association for the Study of Pain, so it could be applied to animals: "An aversive sensory experience caused by actual or potential injury producing progressive motor and vegetative reactions triggers a behavior learned avoidance and may modify species-specific behaviors, including social".

Nociception and animal testing

Using the so-called argument from analogy, we can say that if a particular animal behavior is also present in humans, we can associate and say that apparently, the animals should feel the same and this justifies our experiments in pain studies, which in this case will call it nociception (Latin *nocere*, damage). The human babies have not language. This is an excellent reference to view their nociceptive reactions like those seen in animals. If you click baby foot with a sharp object, their reaction will be to immediately remove the affected limb. On the other hand, if you throw an object toward a person, it tends to avoid it, etc. Both reactions are observed in animals, the first called withdrawal reflex, and the ultimate, avoidance reflex. Both have the purpose of preventing nociception. Based on these behaviors (and other unnamed) we have formulated various tests nociception in animals such as Randall-Selitto test (Randall & Selitto, 1957), Von Frey filaments (Pitcher *et al*., 1999), Thermal Gradient Test (Hargreaves *et al*., 1988), Electric Tail flick (Gardmark *et al*., 1998), nociception by stimulation of the trigeminal nerve (Laurido, 2011). They measure different aspects of nociception and are used in assays such as the test Randall-Selitto, used usually with animals with inflamed paw by injection, and normal paw, to evaluate drugs for analgesia; Tail Flick Apparatus for rapid and sensitive sampling analgesic effects, etc.

The main aim of this work was to test the anti-inflammatory effect of i.p. injections of *Laurus nobilis* challenged against the C reflex and wind-up paradigm.

MATERIALS AND METHODS
Plant material

Leaves of *Laurus nobilis* were collected at the University of Santiago of Chile campus. In order to corroborate the plant material corresponds to Laurus, leaves and flowers were compared with the repository located at the Department of Botany and Herbarium of the Natural History National Museum, Santiago, Chile.

Extraction of essential oil from Laurus

Laurus nobilis leaves dried in darkness and at room temperature were subjected to hydrodistillation for 3 hours. Then the essential oil was stored at 4-5ºC.

Animals

Thirty rats (*Rattus norvegicus*) of the Sprague-Dawley strain, normal male weighing between 250 and 350 g were used. The animals were obtained from the animal facilities of the Faculty of Medicine of the University of Chile and were maintained under a light-dark cycle of 12/12 h., with food and water ad libitum. Animals were translated to our campus in aerated plastic boxes in an air conditioned car and then they were adapted for 24 hours after the travel before the experiments. All experiments were performed in accordance with the ethical guidelines of the International Association for the Study of Pain (Zimmermann, 1986) and the Committee on Bioethics of the University of Santiago of Chile.

The animals were divided into five experimental groups:
1. Six rats for control saline.
2. Six rats for injections of control Tween 80.
3. Six rats for experiments with different concentrations of the essential oil of *Laurus nobilis* (high dose)
4. Six rats for experiments with different concentrations of the essential oil of *Laurus nobilis* (low dose)
5. Six rats for experiments using the essential oil of *Laurus nobilis* and naloxone.

C Reflex and Wind-up

Spinal nociception was evaluated through nociceptive C reflex and wind-up. The C Reflex is a portion of an electromyographic recording evoked by electrical stimulation, which is the response of type C nerve fibers, corresponding to the range between 150 to 450 ms post-stimulus. This procedure is described briefly below. Once anesthetized the animals (injected i.p. urethane corresponding to a concentration of 1 g/kg of body weight). Two platinum stimulating electrodes were inserted subdermally in the toes four and five in the right hind paw. Stimulation was performed with a Grass stimulator (S11) with pulses of 2 ms duration, usually 6–7 mA for normal animals and 3-5 mA for monoarthritic rats, and a frequency of 0.1 Hz for the C reflex, or 1.0 Hz for the wind-up. The stimulator is connected to a stimulus isolator unit (Grass SIU5) and then to a constant current unit (Grass CCU1) to prevent animal electrocution and regulate the applied current. The EMG activity was recorded in the ipsilateral biceps femoris, using two recording electrodes connected to an amplifier (DAM-80, WPI Instruments) and digitized by a PowerLab (2/20). Once digitized, the signal was displayed and recorded using Chart v4.2.3 software on a PC computer. The software rectified full wave signal and integrates the electromyographic recording between 150 and 450 ms post-stimulus. The data were stored for later analysis.

Once anesthetized, the animals with the stimulation and register electrodes was stimulated with supra-threshold current for 20 min, to stabilize the preparation. Then, the stimulation threshold, which corresponds to the lowest current that is capable of generating a response is sought. The animals were stimulated for 15 minutes with twice the threshold 0.1 Hz to stabilize the response. Once stable C-reflex responses were obtained, the stimulus strength was lowered and the current required for threshold activation of the C reflex determined. Then we proceeded to record the stimuli at 0.1 Hz (C reflex) and 1 Hz with intensity 2.0 times the threshold for the wind-up. In the case of C reflex, the values of registers 10 to 20 are averaged. In the case of wind-up, the slope of the first 7-8 records with increasing intensity was obtained. The results were expressed in arbitrary units.

Statistical analysis

Results were expressed as means ± standard error of the mean (SEM). All the statistical analysis was done using one-way ANOVA, with a $p < 0.01$ being significant. The calculations were done with the SigmaPlot, v12 for Windows (®2011, Systat Software, Inc.). In order to define the minimum number of rats, we made experiments increasing the number of animals and calculating for each n, the standard deviation obtained and performing the statistical analysis in each case. The minimum number of animal that reaches a statistically significative result, then this result is considered. If the increase in the number of animals is greater than 5-6 without being statistically significant, then the experiment is not considered into the results.

RESULTS

We use the essential oil of *Laurus nobilis* L (Sayyah *et al.*, 2003) extracted by hydrodistillation and tested with the C reflex and spinal wind-up. The essential oil was prepared with Tween 80 at 5% v/v applied in

rats intraperitoneally at time zero. Results shows (Table No. 1) that at a high concentration of essential oil, wind-up is completely abolished, indicating a possible analgesic effect, whereas the C reflex remains unchanged.

Table No. 2 shows the application of a low concentration of the essential oil and the effect of naloxone. It can be seen that the application of 0.012 mg/Kg of essential oil at time zero, produces a lowering in the wind-up by 85% at time 30 minutes, compared to the control. It is also interesting to note that there was an effect of naloxone (0.08 mg/Kg, i.p.) (Sayyah *et al*., 2003; You *et al*., 2003), which

tends to reverse the analgesic effects of the essential oil in the wind-up. This would indicate a possible effect of opioid receptor level, the result described by the first time.

DISCUSSION
Our group has been dedicated to studying pain through different methods, being Dr. Claudio Laurido who directed these works (Laurido *et al*., 2001; Laurido, 2011; Laurido *et al*., 2012; Laurido *et al*., 2013; Valdes *et al*., 2015; Bustos *et al*., 2016; Valdes *et al*., 2018). This article contains the last results by the obtained one.

Table No. 1				
Time (min)	Control		Laurus (0.06 mg/Kg)	
	C Reflex	Wind-up	C reflex	Wind-up
0	4.6 ± 0.02	5.5 ± 0.01	0.34 ± 0.01	5.5 ± 0.01
10	4.8 ± 0.01	5.3 ± 0.02	0.30 ± 0.02	0
20	4.7 ± 0.03	5.4 ± 0.10	0.32 ± 0.01	0

Table No. 1
The table shows the effect on the C reflex and wind-up of the application of a high dose of *Laurus* essential oil. Results are expressed as mean ± SE, n = 6.

Table No. 2					
Time (min)	Control		Laurus (0.012 mg/Kg)		Naloxone (mg/Kg)
	C Reflex	Wind-up	C Reflex	Wind-up	
0	4.7 ± 0.01	5.5 ± 0.10	4.87 ± 0.010	5.6 ± 0.12	
10	4.8 ± 0.01	5.3 ± 0.01	4.71 ± 0.020	2.2 ± 0.1 *	
30	4.6 ± 0.01	5.4 ± 0.02	4.94 ± 0.015	0.81 ± 0.12 *	0.08
50	4.7 ± 0.02	5.3 ± 0.10	5.22 ± 0.012	5.62 ± 0.02	
60	4.7 ± 0.02	5.5 ± 0.12	5.03 ± 0.020	7.36 ± 0.8	

Table No. 2
The table shows the application of a lower concentration of the *Laurus* essential oil and the effect of naloxone. Results are expressed as mean ± SE, *= $p < 0.01$ indicate the values are statistically different from the wind-up control, n = 6

In this study, the results of the anti-inflammation effect of the essential oil of *Laurus nobilis* occurred in normal rats are shown. There is only one previous work in which the effect of *Laurus nobilis* in the tail flick test and the formalin test (Sayyah *et al*., 2003) was studied. A significant analgesic effect of the extract, which came to completely suppress the wind-up cord (Table No. 1) was found. NMDA receptors play an important role

in the initiation and maintenance of chronic pain, and so the study of this type of pain in animal models of chronic pain (monoarthritis, for example) could give interesting results. Another effect found what the possible existence of actions of the essential oil of *Laurus nobilis* on spinal opioid receptors because when naloxone was applied, an increase in the wind-up occurs; reaching values approximate those of the controls (Table No. 2).

In attempting to establish a correlation between the observed effects and properties of some components of *Laurus nobilis* essential oil can be seen in Table No. 3. Components 1,8-cineole, alpha-terpinyl acetate, and sabinene are described as antagonists TRPA1 receptors, anti-inflammatory, and analgesic, and anti-inflammatory, respectively. Eugenol, as a modulator of the opioid system and the glutamatergic receptors (AMPA and kainate) and inhibition of TNF-α. However, when analyzing the percentage of these compounds present in the essential oil of *Laurus nobilis* and pretend to correlate with antinociception, it becomes very difficult. This is because there may be synergistic or antagonistic effects among the remaining components (81 compounds) of the complete essential oil of *Laurus nobilis*. There are other extraction methods different from hydrodistillation, by ultrasound application (Muñiz-Marquez *et al*., 2013), or enzyme-assisted (Boulila *et al*., 2015), but whether these extractions can make differences on the observed antinociception, remains to be elucidated.

Table No. 3			
Sample %	**Compound Isolated**	**Pharmacological action**	**Bibliographic reference**
58.59	**1,8-cineole**	**TRPM8 receptor antagonist**	**(Takaishi *et al*., 2012)**
8.82	**Alpha terpinyl acetate**	**Antimicrobial and anti-inflammatory. Possible peripheral analgesic.**	**(Peana *et al*., 1999)**
3.32	**Sabinene**	**Anti-inflammatory**	**(Valente *et al.,* 2013)**
0.16	**Eugenol**	**Modulator of the opioid system and glutamatergic receptors (Kainate and AMPA), TNF-α inhibitor.**	**(Bo *et al*., 2013)**

Table No. 3
Some compounds isolated from the essential oil of Laurus presenting pharmacological actions relevant to this work. (Taken from Sayyah *et al*., 2003)

CONCLUSION
We conclude that the essential oil of *Laurus nobilis* has a significant analgesic activity in the C reflex model. The pro-nociceptive effect of naloxone was also observed, possibly due to the modulatory effect of the spinal opioid system that presents the essential oil of *Laurus nobilis*. Whether the essential oil presents an anti-inflammatory activity, remains to be elucidated through the use of animal models of chronic pain.

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