SUPPLEMENTARY MATERIAL

Analgesic properties of a food grade lecithin delivery system of *Zingiber officinale* and *Acmella oleracea* standardized extracts in rats

Carla Lobina¹, Roberta Sau¹, Federica Fara¹, Paola Maccioni¹, Mauro A.M. Carai² and Giancarlo Colombo¹

¹Neuroscience Institute, Section of Cagliari, National Research Council of Italy, I-09042 Monserrato (CA), Italy; ²Cagliari Pharmacological Research, I-09127 Cagliari (CA), Italy

Corresponding author:
Giancarlo Colombo
Neuroscience Institute, Section of Cagliari
National Research Council of Italy
S.S. 554, km. 4,500
I-09042 Monserrato (CA)
Italy
Phone: +39 070 675-4342
Fax: +39 070 675-4320
E-mail: colomb@unica.it

Abstract

This study investigated whether (i) the 5:1 combination of standardized extracts of *Zingiber officinale* and *Acmella oleracea* is endowed with analgesic effects and (ii) the phospholipid-based formulation of *Zingiber officinale* and *Acmella oleracea* extracts (ZAP) potentiated the analgesic effects of the plain extract combination (PEC). To this end, rats were exposed to acute pain (Tail Flick test) and chronic, inflammatory pain [Von Frey monofilament test and Randall-Selitto paw pressure test in rats treated intraplantarily with complete Freund's adjuvant (CFA)]. The plain combination of *per se* ineffective doses of the two extracts produced analgesic effects in healthy rats. ZAP was more potent and effective than the corresponding doses of PEC. ZAP also produced analgesic effects in CFA-treated rats. Studies are now warranted to assess whether the analgesic properties of ZAP may generalize to humans.

Experimental section

The experimental procedures employed in the present study fully complied with European Directive no. 2010/63/EU and subsequent Italian Legislative Decree no. 26, March 4, 2014, on the “Protection of animals used for scientific purposes”. The present study was approved by the Italian Ministry of Health (authorization no. 622/2016-PR).

Animals

Adult, male Wistar rats (Envigo, San Pietro al Natisone, Italy) were used. Rats were housed 3 per cage in standard plastic cages with wood chip bedding. Rat body weight averaged approximately 300 g at the time of each experiment. The animal facility was under a 12:12 hour light/dark cycle (lights on at 7:00 a.m.), at a constant temperature of 22±2°C and relative humidity of approximately 60%. Standard rat chow was always available in the homecage, except in the 3 hours preceding drug administration, so that rats had empty stomachs at the time of drug intragastric infusion. Tap water was always available in the homecage. Rats were extensively habituated to handling and intragastric infusions before the start of each experiment. Each experiment used an independent set of rats.

Extract preparation and administration
**Zingiber officinale** extract was a lipophilic CO₂ extract standardized to contain 25.0-33.0% of active compounds as sum of gingerols and shogaols. **Acmella oleracea** extract was an hydroalcoholic extract standardized in 24.6% total alkylamides by HPLC.

The phospholipid-based formulation of **Zingiber officinale** and **Acmella oleracea** extracts (ZAP) consisted in the association of the two standardized extracts in a 5:1 ratio; they were additionally combined with sunflower lecithin in a 1:1 weight ratio (expressed as sum of the botanical extracts and lecithin, respectively). Food additives such as microcrystalline cellulose (E460), sucrose esters of fatty acids (E473), hydroxypropyl cellulose (E463), and silicon dioxide (E551) were also included in order to improve physical and technological characteristics of the final product. ZAP contained 11.4% and 2.4% **Zingiber officinale** and **Acmella oleracea** extracts, respectively.

In Experiments 2-4, doses of ZAP were selected as to match, in terms of content of each single extract, those of **Zingiber officinale** and **Acmella oleracea** extracts tested in the plain extract combination (PEC). **Zingiber officinale** extract and ZAP were suspended in distilled water with a few drops of Tween 80 and administered intragastrically at the infusion volume of 5 ml/kg. **Acmella oleracea** extract was suspended in distilled water with a few drops of 1:1 mixture of Tween 80 and PEG 600 and administered intragastrically at the infusion volume of 1 ml/kg. The reference compound, diclofenac (Sigma-Aldrich, Milan, Italy) (e.g.: Wandji et al. 2018), was dissolved in distilled water and administered intragastrically at the infusion volume of 3 ml/kg.

**Experimental procedure**

As advisable when assessing pain-like responses in laboratory animals (see Deuis et al. 2017), in each experiment rats were tested in a random order by an investigator blinded to the treatment group to which each rat was allocated.

**Induction of chronic pain**

Inflammatory, chronic pain was induced by an intraplantar injection of 50 μl complete Freund's adjuvant (CFA; Sigma-Aldrich, Milan, Italy) into the center of the left hind paw of each rat (see Muley et al. 2016). CFA injection was carried out under light (isoflurane inhalation) anesthesia.

**Tail Flick test**

Over the 5 days preceding the test session, rats were habituated to stay on the surface of the Tail Flick apparatus (TSE, Bad Homburg, Germany). To this end, rats were gently hand-restrained by the same investigator who subsequently conducted the experiment. Habituation was intended to reduce movements to a minimum once the rat was positioned on the apparatus surface. Each daily habituation session lasted 10 min.

On the test day (at the time of food pellet removal), the tail of each rat was marked (by red ink) with a circular spot, of 1-cm in diameter, located 6 cm from the tail tip. Two hours before extract administration, rats were exposed to the Tail Flick test for assessment of baseline values. Rats were re-exposed to the Tail Flick test 20, 60, 120, and 180 min after extract administration. At each exposure to the Tail Flick test, each rat was gently held on the apparatus surface corresponding with the time of projection of a 56°C beam of light on the red spot. A cut-off time of 20 s was used to prevent tissue damage. At each exposure to the Tail Flick test, two recordings were performed (60 s apart); the value assigned to each rat was the average of these two recordings. The measured variable was the latency (expressed in s) of tail withdrawal (threshold of the nocifensive response).

**Von Frey monofilament test**

Over the 5 days preceding the test session, rats were habituated to stay inside the small Plexiglas cages of the electronic “Dynamic Plantar Aesthesiometer” (Ugo Basile, Comerio, Italy) used to perform the Von Frey monofilament test. Habituation was intended to reduce ambulation and exploratory behaviors (possible confounding factors) to a minimum. Each daily habituation session lasted 10 min. The analgesia test was performed 24 hours after CFA injection; this time period was selected on the basis of literature data (e.g.: Fang et al. 2013; Li et al. 2014; Thorn et al. 2015; De Rantere et al. 2016) as it
corresponds to the time of maximal hyperalgesia at the Von Frey monofilament test. On the test day, two hours before extract administration, rats were exposed to the Von Frey monofilament test for assessment of baseline values. Rats were re-exposed to the Von Frey monofilament test 20, 60, 120, and 180 min after extract administration. At each exposure to the Von Frey monofilament test, each rat was initially loosely restrained inside the Plexiglas cage (the floor of which was made of wire mesh) and allowed to become accustomed to this environment for approximately 10 min; subsequently, when immobile, the rat was exposed to the noxious stimulus produced by the application, from beneath, of the monofilament to the plantar surface of the left hind paw. The filament was applied with a 50-g force, reached progressively in 20 s. At each exposure to the Von Frey monofilament test, two recordings were performed (60 s apart); the value assigned to each rat was the average of these two recordings. The measured variables were (a) the lowest force (expressed in g) at which the paw was withdrawn and (b) the latency (expressed in s) of paw withdrawal (threshold of the nocifensive response).

Randall–Selitto paw pressure test

Over the 5 days preceding the test session, rats were habituated to firm handling and having both hind paws exposed to the conical tips of the Randall–Selitto apparatus (Ugo Basile, Comerio, Italy). Habituation was performed by the same investigator who subsequently conducted the experiment. Each daily habituation session lasted 10 min.

The analgesia test was performed 7 days after CFA injection; this time period was selected on the basis of literature data (e.g.: Stein et al. 1988; Bianchi et al. 2007; Arora et al. 2015) as it corresponds to the time of maximal hyperalgesia at the Randall–Selitto paw pressure test. On the test day, two hours before extract administration, rats were exposed to the Randall–Selitto paw pressure test for assessment of baseline values. Rats were re-exposed to the Randall–Selitto paw pressure test 20, 60, 120, and 180 min after extract administration. At each exposure to the Randall–Selitto paw pressure test, each rat was gently and firmly handled and its left hind paw was placed onto the tip of the lower cone; an increasing mechanical force (ranging from 0 to 250 g; the latter reached in 15 s) was applied, so that the tip of the device pressed onto the medial portion of the dorsal surface of the paw until paw withdrawal. A cut-off of 170 g was applied. At each exposure to the Randall–Selitto paw pressure test, two recordings were performed (60 s apart); the value assigned to each rat was the average of these two recordings. The measured variable was the lowest force (expressed in 10-g steps, according to the scale calibration of the apparatus) at which the paw was withdrawn (threshold of the nocifensive response).

Study design

**Experiment 1** – Healthy rats were divided into 10 groups, matched for baseline levels, and treated acutely with the following extract combinations: 0 mg/kg *Zingiber officinale* extract + 0 mg/kg *Acmella oleracea* extract (n=10); 25 mg/kg *Zingiber officinale* extract + 0 mg/kg *Acmella oleracea* extract (n=10); 50 mg/kg *Zingiber officinale* extract + 0 mg/kg *Acmella oleracea* extract (n=10); 100 mg/kg *Zingiber officinale* extract + 0 mg/kg *Acmella oleracea* extract (n=10); 0 mg/kg *Zingiber officinale* extract + 10 mg/kg *Acmella oleracea* extract (n=10); 0 mg/kg *Zingiber officinale* extract + 20 mg/kg *Acmella oleracea* extract (n=10); 25 mg/kg *Zingiber officinale* extract + 5 mg/kg *Acmella oleracea* extract (n=10); 0 mg/kg *Zingiber officinale* extract + 10 mg/kg *Acmella oleracea* extract (n=10); 0 mg/kg *Zingiber officinale* extract + 20 mg/kg *Acmella oleracea* extract (n=10); 25 mg/kg *Zingiber officinale* extract + 5 mg/kg *Acmella oleracea* extract (n=10); 50 mg/kg *Zingiber officinale* extract + 10 mg/kg *Acmella oleracea* extract (n=11); 100 mg/kg *Zingiber officinale* extract + 20 mg/kg *Acmella oleracea* extract (n=11). *Zingiber officinale* and *Acmella oleracea* extracts were administered via two separate administrations, occurring in rapid succession. Rats were then exposed to the Tail Flick test.

**Experiment 2** – Healthy rats were divided into 5 groups (n=11), matched for baseline levels, and treated acutely with 0, 125, 250, 500, and 1000 mg/kg ZAP. Comparison of the effect of ZAP with that of PEC was performed using part of the data collected in Experiment 1. Specifically, data on the corresponding doses of PEC were extrapolated from Experiment-1 dataset. This resulted in a considerable reduction of the number of rats (an advisable option particularly when the experimental procedure produces distress to the animals). An independent set of healthy rats was divided into two groups (n=12), matched for baseline levels, and treated acutely with 0 and 5 mg/kg diclofenac. Rats were then exposed to the Tail Flick test.

**Experiment 3** – CFA-treated rats were divided into 5 groups (n=12), matched for baseline levels, and treated acutely with 0, 125, 250, 500, and 1000 mg/kg ZAP. An independent set of CFA-treated rats was divided
into two groups \((n=12)\), matched for baseline levels, and treated acutely with 0 and 5 mg/kg diclofenac. Rats were then exposed to the Von Frey monofilament test.

**Experiment 4** – CFA-treated rats were divided into 5 groups, matched for baseline levels, and treated acutely with 0 \((n=12)\), 125 \((n=12)\), 250 \((n=13)\), 500 \((n=12)\), and 1000 \((n=13)\) mg/kg ZAP. An independent set of CFA-treated rats was divided into two groups \((n=12)\), matched for baseline levels, and treated acutely with 0 and 5 mg/kg diclofenac. Rats were then exposed to the Randall-Selitto paw pressure test.

**Statistical analysis**

Data from baseline recording were analyzed by 1-way ANOVA (experiments with PEC and ZAP) or Student \(t\) test (experiments with diclofenac).

In each experiment, data on drug effect at the different recording times were analyzed by 2-way (treatment, time) ANOVA for repeated measures on the factor time, followed by the Tukey’s test for post hoc comparisons.

**Results**

**Experiment 1**

Latency of tail removal – Baseline
1-way ANOVA: \(F(9,92)=0.36, P>0.05\)

Latency of tail removal – Time course
2-way ANOVA: \(F_{\text{treatment}}(9,92)=6.59, P<0.0001\); \(F_{\text{time}}(3,276)=15.28, P<0.0001\); \(F_{\text{interaction}}(27,276)=6.39, P<0.0001\)

![Image](image-url)

**Experiment 2**

Latency of tail removal – Baseline (ZAP)
1-way ANOVA: \(F(4,50)=0.30, P>0.05\)
Latency of tail removal – Time course (ZAP)
2-way ANOVA: $F_{\text{treatment}}(4,50)=11.16$, $P<0.0001$; $F_{\text{time}}(3,150)=32.61$, $P<0.0001$; $F_{\text{interaction}}(12,150)=6.05$, $P<0.0001$

Latency of tail removal – Baseline (PEC – data extrapolated from Experiment 1)
1-way ANOVA: $F(3,38)=0.16$, $P>0.05$

Latency of tail removal – Time course (PEC – data extrapolated from Experiment 1)
2-way ANOVA: $F_{\text{treatment}}(3,38)=8.47$, $P<0.0005$; $F_{\text{time}}(3,114)=22.26$, $P<0.0001$; $F_{\text{interaction}}(9,114)=6.87$, $P<0.0001$

Figure S2 (Experiment 2) – Effect of the acute administration of ZAP in healthy rats exposed to the Tail Flick test (top panel). The bottom panel reproduces Figure-1 data. Each bar is the mean ± SEM of $n=11$ (top panel) and $n=10-11$ (bottom panel) rats. χ: $P<0.05$, *: $P<0.005$, §: $P<0.0005$, and #: $P<0.0001$ compared to 0 mg/kg ZAP (top panel) or 0 mg/kg Zingiber officinalis extract + 0 mg/kg Acmella oleracea extract (bottom panel) (Tukey’s test).

Experiment 3

Force applied at time of paw withdrawal – Baseline (ZAP)
1-way ANOVA: $F(4,55)=0.20$, $P>0.05$

Force applied at time of paw withdrawal – Time course (ZAP)
2-way ANOVA: $F_{\text{treatment}}(4,55)=6.95$, $P<0.0001$; $F_{\text{time}}(3,165)=6.59$, $P<0.0005$; $F_{\text{interaction}}(12,165)=2.93$, $P<0.0001$

Force applied at time of paw withdrawal – Baseline (diclofenac)
Student t test: $P>0.05$

Force applied at time of paw withdrawal – Time course (diclofenac)
2-way ANOVA: F_{treatment}(1,22)=83.84, P<0.0001; F_{time}(3,66)=3.64, P<0.05; F_{interaction}(3,66)=2.13, P>0.05

Latency of tail removal – Baseline (ZAP)
1-way ANOVA: F(4,55)=0.28, P>0.05

Latency of tail removal – Time course (ZAP)
2-way ANOVA: F_{treatment}(4,55)=7.31, P<0.0001; F_{time}(3,165)=6.35, P<0.0005; F_{interaction}(12,165)=3.45, P<0.0005

Latency of tail removal – Baseline (diclofenac)
Student t test: P>0.05

Latency of tail removal – Time course (diclofenac)
2-way ANOVA: F_{treatment}(1,22)=77.61, P<0.0001; F_{time}(3,66)=3.13, P<0.05; F_{interaction}(3,66)=1.84, P>0.05

Figure S3 (Experiment 3) – Effect of the acute administration of ZAP in CFA-treated rats and exposed to the Von Frey monofilament test. Each bar is the mean ± SEM of n=12 rats. *: P<0.005 and #: P<0.0001 compared to 0 mg/kg ZAP (Tukey’s test). Insets: Effect of acute administration of diclofenac. Each bar is the mean ± SEM of n=12 rats. #: P<0.0001 compared to 0 mg/kg diclofenac (Tukey’s test).

Experiment 4

Force applied at time of paw withdrawal – Baseline (ZAP)
1-way ANOVA: $F(4,57)=0.39$, $P>0.05$

Force applied at time of paw withdrawal – Time course (ZAP)
2-way ANOVA: $F_{\text{treatment}}(4,57)=72.63$, $P<0.0001$; $F_{\text{time}}(3,171)=94.59$, $P<0.0001$; $F_{\text{interaction}}(12,171)=21.45$, $P<0.0001$

Force applied at time of paw withdrawal – Baseline (diclofenac)
Student $t$ test: $P>0.05$

Force applied at time of paw withdrawal – Time course (diclofenac)
2-way ANOVA: $F_{\text{treatment}}(1,22)=279.90$, $P<0.0001$; $F_{\text{time}}(3,66)=215.70$, $P<0.0001$; $F_{\text{interaction}}(3,66)=190.30$, $P<0.0001$

Figure S4 (Experiment 4) – Effect of the acute administration of ZAP in CFA-treated rats and exposed to the Randall-Selitto paw pressure test. Each bar is the mean ± SEM of $n=12$-13 rats. $\chi$: $P<0.05$ and #: $P<0.0001$ compared to with 0 mg/kg ZAP (Tukey’s test). Inset: Effect of acute administration of diclofenac. Each bar is the mean ± SEM of $n=12$ rats. $\star$: $P<0.005$ and #: $P<0.0001$ compared to 0 mg/kg diclofenac (Tukey’s test).

References


