Anti-inflammatory and anti-allodynic activities in mice of the essential oil and desmethyloencecalin from *Zinnia grandiflora* Nuttall

Valeria Reyes-Pérez, Vinicio Granados-Soto, Edelmira Linares, Robert Bye, Rachel Mata, and Myrna Deciga-Campos

*Facultad de Química, Universidad Nacional Autónoma de México, Mexico City, Mexico; Departamento de Farmacobiología, Cinvestav, South Campus, Mexico City, Mexico; Instituto de Biología, Universidad Nacional Autónoma de México, Mexico City, Mexico; Escuela Superior de Medicina, Instituto Politécnico Nacional, Mexico City, Mexico

**ABSTRACT**

*Zinnia grandiflora* (Asteraceae) is used to alleviate several painful disorders. The effects of an essential oil prepared by hydrodistillation from *Z. grandiflora* (EOZ) and its major compound on neuropathic pain, allodynia, and inflammation in mice were assessed. Headspace solid-phase microextraction analysis of an aqueous extract was also carried out. GC-MS analyses of all volatiles revealed that germacrene D (13.24%) and desmethyloencecalin (DME) (13.24%) were the most abundant compounds. EOZ was effective in reducing inflammation and allodynia in mice. EOZ (300 mg/paw) reverted tactile allodynia more effectively than gabapentin (100 μg/paw). DME (0.1–10 μg/paw) responses in the carrageenan model were concentration-dependent; the maximum effect was observed at 10 μg/paw, like that of diclofenac (30 μg/paw). In the spinal nerve ligation model, DME (0.1–10 μg/paw) exerted a concentration-dependent anti-allodynic action; its effect was better than that of gabapentin. A method to quantify zinagrandinolide E in the crude drug was developed and validated.

**Introduction**

*Zinnia grandiflora* Nuttall (Asteraceae) is a conspicuous perennial herb with striking yellowish flowers and prominent low mounded crowns from rhizomes (1). The ubiquitous geographic distribution in the foothills and adjacent prairie and desert margins of the southern Rocky Mountains and northern Sierra Madre Occidental (Fig. S1, Supplementary Information) were well documented since the early botanical explorations in the states of Colorado, Kansas, Oklahoma, Texas, New Mexico, and Arizona as well as Sonora, Chihuahua, Coahuila, Durango, and Zacatecas (2–4). All medical uses registered are included in Table 1S, Supplementary Information. The Navajo, Ramah Navajo, Acoma, Laguna, and Zuni cultures use the ‘Rocky Mountain zinnia’ to alleviate fever, strokes, stomachache, and several painful disorders (5–8). In Mexico, *Z. grandiflora* is regarded as ‘tepozana del burro’ or ‘tepozana del becerro’, where it is traditionally used for treating diarrhea and diabetes (9).

Previous studies on *Z. grandiflora* resulted in the isolation of a few elemanolides, including zinagrandinolides A-C, which were moderately cytotoxic against several cancer cell lines (NCI-H460, MCF-7, SF-268, and MIA Pa Ca-2) and a normal human fibroblast cell type (WI-38) (10). In addition, we analyzed an aqueous extract from the aerial parts of *Zinnia grandiflora*, collected in Mexico, which was not toxic to mice (LD<sub>50</sub> > 5 g/kg) when tested by the Lorke method. This extract showed significant antinociceptive and anti-inflammatory activities when evaluated by the formalin (ED<sub>50</sub> = 224.62 ± 38.17 mg/kg, p.o.) and carrageenan-induced paw edema models in mice, respectively. However, the contribution of the plant volatilome to these pharmacological actions has not been assessed. Chemical investigation of the active extract led to the isolation of zinagrandinolides D-F (11). Chemical investigation of the active extract led to the isolation of zinagrandinolides D-F (11). The major compound, zinagrandinolide E (ZGE), exerted prominent antinociceptive, anti-inflammatory, and anti-allodynic actions in mice using the formalin test, carrageenan-induced paw edema, and an STZ-induced diabetic neuropathy model (11,12). Local peripheral administration of ZGE (1–30 μg/paw) provoked concentration-dependent acute anti-inflammatory and anti-allodynic effects. The anti-inflammatory was comparable to diclofenac (30 μg/paw).
Local peripheral (3–30 μg/paw) or intrathecal (3–30 μg) administration of ZGE partially reversed tactile allodynia in hyperglycemic mice in a concentration fashion; these effects were better or comparable with those of pregabalin (30 μg/paw or 30 μg intrathecal). The anti-inflammatory and anti-allodynic activities of ZGE are multitarget, involving opioidergic, serotoninergic, GABAergic, and the NO- cGMP-ATP-sensitive K+ channels systems (12). Continuing with our scientific validation of the potential anti-inflammatory and antinociceptive effects of Z. grandiflora, this work aimed to: (i) Establish the anti-inflammatory and anti-allodynic action of essential oil (EOZ) of the crude drug using the carrageenan-induced inflammation model and a neuropathic pain model in mice; (ii) analyze the plant volatile (i.e. plant volatile organic compounds and essential oil) which might contribute to the antinociceptive and anti-inflammatory effects of Z. grandiflora; (iii) finally, to validate an UPLC analytical procedure to quantify at least one of the active compounds in the infusion for crude drug standardization.

### Materials and methods

#### Plant material

The aerial parts of Z. grandiflora were collected in Chihuahua, Mexico and identified by Drs. R. Bye and E. Linares. An authenticated voucher specimen (Bye 39042 & Linares) has been deposited at the National Herbarium (MEXU), Instituto de Biología, UNAM, Mexico City. They also provided the picture of Z. grandiflora presented in the TOC graphic.
Extraction of the essential oil of Zinnia grandiflora

The essential oil of *Z. grandiflora* (EOZ) was obtained by hydrodistillation of 150 g of air-dried and ground aerial parts of the plant material in 2 l of distilled water using a modified Clevenger-type apparatus. The system was operated at atmospheric pressure, and at maximum temperature of water boiling point (13). The aqueous hydrodistilled mixture was partitioned with dichloromethane (3 × 300 ml) and dried over anhydrous sodium sulfate. The resulting organic phase was concentrated *in vacuo* to yield 120 mg of a yellowish oil. This process was repeated 15 times to obtain enough oil for evaluating and separating the major compounds.

Isolation of the major compounds from EOZ: germacrene D, desmethylisoencecalin, D-limonene, β-caryophyllene, and caryophyllene oxide

GC-MS identified major components in EOZ. In addition, the major compounds’ separation, and identification (NMR, IR, and MS) were carried out to confirm their structures (Table 1) further. Separation of compounds was performed by preparative thin-layer chromatography. Three hundred mg of EOZ were chromatographed in a plate (20 × 20 cm) covered with silica gel. Elution was carried out with toluene: hexane: ethyl acetate (85:10:5). After elution, those bands observed under a UV-Vis lamp were carefully scraped and macerated during 2 h with CH₂Cl₂ for desorption of compounds. After maceration, the silica was filtered using Whatman filter paper (grade 1). Then, the organic solvent was evaporated *in vacuo* and the pure compounds stored in amber vials in refrigeration. This procedure yielded 2.7 mg of D-limonene (4), 1.2 mg of β-caryophyllene (20), 5.8 mg germacrene D (24), 4.1 mg of desmethylisoencecalin (25), and 2.6 mg of caryophyllene oxide (32), per plate. Compounds 4, 20, 24 and 32 were characterized by comparing their IR, NMR, and MS spectral data with those of authentic samples from Sigma-Aldrich, while compound 25 (Figure 7) by comparing with those previously described (Figs. S2 – S6, Supplementary Information) (14).

Headspace-Solid Phase Microextraction (HS-SPME)

An amount of dried plant material (200 mg), sodium chloride (100 mg) and HPLC water (15 ml) were placed and hermetically sealed in a 30 ml vial with a polypropylene hole-cap and PTFE/coated silicone septa and heated during 30 min simulating the preparation of an infusion. Four fibers of different polarities were used for the analyses (Supelco Technology): a polydimethylsiloxane coated fiber (PDMS; 100 μm, for non-polar compounds), a polydimethylsiloxane/divinylbenzene coated fiber (PDMS/DVB; 65 μm, for medium polar compounds), a divinylbenzene/carboxen/polydimethylsiloxane coated fiber (DVB/Carboxen/PDMS; 50/30 μm, for a broad range of compounds) and a carboxen/polydimethylsiloxane coated fiber (Carboxen/PDMS; 75 μm, for non-polar compounds). All fibers were introduced to the closed vials to collect the headspace (HS). The experimental conditions were: Extraction temperature, 60°C; equilibrium time, 5 min; extraction time, 30 min; desorption time of fibers in GC injector, 2 min at 250°C. Conditioning of fibers before the GC-MS analyses was carried out according to the manufacturer’s instructions. Relative proportions of individual components adsorbed in the fibers were calculated based on the total ion chromatogram (TIC) peak areas as a percentage of the sum of all peak areas. All samples were analyzed in triplicate, and the relative standard deviation was less than 2%.

Gas chromatography analyses

GC-MS analyses were performed in an Agilent 6890 N series gas chromatograph equipped with an LECO PEGASUS 4D time-of-flight mass spectrometer detector (MS-TOF; Agilent Technology), using a 5% diphenyl-95% dimethyl polysiloxane capillary column (DB-5) (20 m × 0.18 mm × 0.18 μm) (Bellefonte). The GC-MS conditions were flow rate of the carrier gas (He): 1.0 ml/min; diluted sample with CH₂Cl₂; splitless; injection volume: 1 μl; injector and MS transfer line temperatures: 300°C and 250°C, respectively; oven temperature ramp: 40°C (held 3 min), then rise to 300°C at 20°C/min (15 min, isothermally); ionization mode: electronic impact at 70 eV; and mass range scanning: 33–500 m/z. The identification of the components was carried out by co-injection of the sample with a solution containing the homologous series of n-alkanes (C₁₀–C₅₀) to establish the retention indexes (equation of Van den Dool), and by comparison of the mass spectral fragmentation patterns with those stored in the National Institute of Standards and Technology (NIST) MS database. All samples were analyzed in triplicate, and the relative standard deviation was less than 2%.

UPLC analysis and method validation

Standards and chemicals

Acetonitrile and water of LC-MS grade and formic acid (FA) of HPLC-grade, were purchased from J.T. Baker. Zinagrandinolide E (purity ≥ 98%) was used as reference compound (Figure 7) and was isolated from *Z. grandiflora* as previously described (11). The identity and purity of the compound was confirmed by
chromatographic (TLC, UPLC) and by spectral means (NMR, ESI-MS) (Figs. S7 and S8, Supplementary Information).

ZGE is soluble in methanol and have limited solubility in water. Hence, binary mixture of dioxane-methanol (1:1) was selected for the solubility test of the standard. The stock standard solution of ZGE, was prepared at the final concentration of 1 mg/ml. Working solutions of the standard were prepared by diluting the stock solution in dioxane-methanol (1:1). The dichloromethane fraction, obtained by partitioning the infusion (11), was used as biological matrix. Sample solutions of the biological matrix (75 μg/ml) were prepared following the same procedure as the stock standard solution. Before analysis, all solutions were filtrated using 0.20 μm GHP membranes.

**Instrumentation and chromatographic conditions**

Liquid chromatographic system consisted in an Acquity UHPLC equipment (H class) from Waters Corporation. The instrument is equipped with a quaternary pump, auto-sampler, and a photodiode array (PDA) detector. An Acquity UPLC BEH Phenyl column (1.7 μm, 2.1 × 100 mm) equipped with a guard column (Vanguard 2.1 × 5.0 mm) was used for sample processing. The column temperature was controlled with a column oven at 40°C. A binary mobile phase consisting of acetonitrile (A) and water with 0.1% formic acid (B) was found the most appropriate for running samples. Flow rate and injection volume were established at 0.3 mL/min and 3 μL, respectively. Elution was carried out according to the following gradient program: 0 min → 15% A; 3.0 min → 45% A; 10 min → 80% A; 11 min → 100% A; 12 min → 100% A; 12.5 min → 15% A; 15 min → 15% A. PDA detection was carried out at 254 nm. Under the conditions described above, the retention time \(T_R\) of ZGE was 8.3 min. The concentrations were calculated using peak area ratios and the linearity of the calibration curve was determined using least squares regression analysis. All statistical calculations relative to quantitative analysis were performed using Origin 8.0 software (Origin Labs).

**Method validation**

The proposed UPLC method for quantitative analysis was validated based on linearity, LOD, LOQ, intra-day and inter-day precisions, and accuracy. The stock solution of ZGE with a final concentration of 1 mg/ml was prepared dissolving the standard in dioxane-methanol (1:1). Six working solutions in the range of 5–100 μg/ml were prepared in sextuplicate from serial dilutions from the stock solution. All dilutions were made in dioxane-methanol (1:1). The linearity was assessed estimating the slope, y-intercept, and coefficient of determination \(R^2\) using the least-squares method.

Limits of quantitation (LOQ) were determined through the analysis of solutions containing decreasing concentrations of the analyte ranging from 10 to 0.1 μg/ml, to achieve the lowest determinable level with acceptable precision, and accuracy under the established conditions. The limit of detection (LOD) was estimated based on the relation between standard deviation \(S_{y/x}\) of the standard intercept \(b_0\) and the slope \(b_1\) of the analytical curve, according with Equation (1) (15).

\[
LD = S_{y/x} \times 3.3(b_1)
\]

The method’s precision was analyzed preparing six identical replicates for ZGE on two different days and by two different analysts. Variations of the peak area were taken as the measures of precision and are expressed as standard deviations (SDs). The standard deviation (SD) and coefficient of variation (CV) were calculated for each day.

Finally, method accuracy was tested by recovery experiments, assaying different three samples of different concentrations of ZGE (10, 50, and 75 μg/ml) by triplicate. All compounds were added simultaneously to dichloride methane soluble fraction and analyzed according to the method previously described. The mean percentage recovery for ZGE was found between 98% and 102% by means of Fisher’s F-test (15).

**Experimental animals**

Female Swiss Webster mice (body weight range 25–30 g), obtained from Cinvestav, South Campus (Mexico City), were used in this study. The animals were maintained with free access to water and food, in a controlled environment at 22°C with 12-h light/dark cycles. All experiments were conducted according to the ethical guidelines for investigations of experimental pain in conscious animals (16). The protocol was approved by the local ethics committee (Cinvestav, protocol 042–13). The scientific procedures established by the Mexican Official Norm for Animal Care and Handling (NOM-062-ZOO-1999) and the Directive 2010/63/EU for animal experiments were followed. Animals were randomized in each treatment group, which contained eight animals. All efforts were made to minimize animal suffering and to reduce the number of animals used. The animals were sacrificed in a CO₂ chamber after experiments were ended.
Carrageenan-Induced paw edema and tactile allodynia

Before any treatment, animals were placed in open Plexiglas observation chambers for 40 min in a controlled temperature (25°C) for conditioning to their surroundings. The inflammation and mechanical allodynia were determined in the carrageenan-induced edema test (17). Here, we evaluated the possible anti-inflammatory and anti-allodynic responses of EOZ and DME. Mice were injected (20 μl) into the paw with increasing doses of EOZ (30–300 μg/paw in mineral oil) or DME (0.1–10 μg/paw in 30% DMSO in saline) 15 min before administration of 1% carrageenan. Diclofenac (DIC, 30 μg/paw in saline) was used as positive control. At the same time, two separated groups were administered with vehicle (VEH): mineral oil or 30% DMSO in saline, according to each group of experiments. Then, the time course of the anti-inflammatory and anti-allodynic effects of EOZ, DME, or control groups were carried out for 6 h. The inflammatory response was measured in a plethysmometer (model 7150, Ugo Basile). This equipment determines the volume of the mouse paw recorded every hour during 6 h after carrageenan administration. Tactile allodynia was evaluated with a known method (18). This procedure determines the 50% paw withdrawal threshold according to the following Equation (2):

\[
50\% \text{Withdrawal threshold (g) } = \frac{(10^6|Xf + kδ|)}{10,000}
\]

(2)

Where, Xf is the value of the last von Frey filament used (in logarithmic units); k is the correction factor based on the response patterns of a calibration table and the tabulated value based on the pattern of positive and negative responses, and δ indicates the average differences between stimuli in logarithmic units (18,19). In normal mice a value 1–1.6 g is considered normal, while the presence of allodynia is considered when the 50% withdrawal threshold of the limb is less than 0.2 g.

Spinal nerve L4/L5 ligation

Surgical procedure for spinal nerve ligation was carried out for the induction of neuropathic pain (20). Mice were anesthetized with a mixture of ketamine (50 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.). After surgical preparation, a skin incision was made to the left of the dorsal midline guided by the iliac crests as the midpoint of incision. The left L4 and L5 spinal nerves were exposed, isolated, and tightly ligated with 6–0 silk suture distal to the dorsal root ganglion. Mice were monitored until complete recovery from anesthesia. After surgical recovery, animals with motor deficiencies, such as paw dragging were excluded from the experiments.

As previously described, tactile allodynia was determined using the Von Frey filaments (18). Experiments were carried out 14 days after surgery when animals were wholly recovered from surgery, and produced repeatable allodynic values less than 0.2 g.

Before experimentation, mice were placed over a mesh grid floor, covered by acrylic cages to restrict the displacement area of the animals. The acclimation process lasted 30 min before registration of the basal value of tactile allodynia. After this, mice were administrated intraplantarily (20 μl, i.pl.) in the left paw with different doses of EOZ (30–300 μg/paw in mineral oil), DME (0.1–10 μg/paw in 30% DMSO in saline), gabapentin (GBP, 100 μg/paw in saline) or diclofenac (30 μg/paw in saline).

Statistical analysis

Results are expressed as mean ± SEM of the percentage of inflammation (%) or 50% of paw withdrawal threshold (g) in time courses, or as the mean ± SEM of the analysis of area under the curve of parameters previously mentioned against time (arbitrary units, AUC). All experiments were performed in groups of eight mice (n = 8). Statistical differences were evaluated in the GraphPad Prism software (version 6.01 for Windows), using either two-way ANOVA followed by a Bonferroni’s test, or one-way ANOVA followed by a Dunnett’s or a Bonferroni’s test.

Results and discussion

Volatile composition of the infusion of Zinnia grandiflora and its essential oil

According to the ethnobotanical background, Z. grandiflora is prepared in infusion or poultice for pain relief (5,6). So far, we have demonstrated the anti-inflammatory and antinociceptive effect of the infusion and the major active principle, zinagrandinolide E (11,12). However, the contribution of the plant volatileome to the pharmacological action has not been established. Further, in recent years, essential oils’ therapeu- tic potential has gained interest since some of their components meet drug lead optimization or fragment-based drug discovery (21).

The volatile organic compounds of the active infusion were first analyzed using HS-SPME, followed by GC-MS. Four types of SPME-fibers were utilized to detect a more significant number of compounds in the headspace, according to their affinity with each fiber coat. Each fiber is coated with specific materials with
different adsorption properties related to the molecular weight and polarity of the compounds in the vegetal material (22). According to the total ionic chromatograms (TICs) in Figure 1, differential extractions were observed with the four fibers employed; altogether, 34 volatile components were identified in the infusion of Z. grandiflora (Table 1). The PDMS/DVB fiber, which mostly adsorbs medium polar compounds, extracted a larger number of molecules, predominating germacrene D (24) and DME (25).

To complete the study of the volatile organic compounds of the plant, analysis of the Eoz by GC-MS revealed the presence of 24 compounds (Figure 2). Germacrene D (13.24%) and DME (13.24%) were in higher proportion, followed by caryophyllene oxide (9.77%), D-limonene (6.05%), α-cadinene (5.1%), and eucalyptol (4.67%) (Table 1). These results are consistent with the volatile compounds identified in the infusion. However, with the HS-SPME technique more molecules were identified as this type of extraction is softer, handier, and more sensitive than hydrodistillation (22). α-Tolualdehyde, phenylethyl alcohol, l-verbene, dihydroactinolide, trans-α-bisabolene, n-hexadecanoic acid, and phytol were not extracted by the fibers used, raising the question if some of them are generated during hydrodistillation (23). The major components of the oil (germacrene D, DME, caryophyllene oxide, and D-limonene) were isolated and characterized as described in the experimental section. Furthermore, the presence of DME in the infusion was corroborated by GC-MS examination, using the co-elution technique, of the infusion’s hexane fraction (Fig. 9S, Supplementary Information).

Figure 1. Total ion chromatograms of the volatile compounds of the infusion of Zinnia grandiflora using phase solid microextraction (HS-SPME). Each chromatogram represents a sample obtained with a different fiber of SPME: PDMS, 100 µm (red); PDMS/DVB, 65 µm (blue); DVB/Carboxen/PDMS, 50/30 µm (gray); Carboxen/PDMS, 75µm (black). Each sample was analyzed in triplicate (only one replica per fiber represented on graphic).

Figure 2. Total ion chromatograms of the essential oil of Zinnia grandiflora (Eoz). Analyzed by triplicate.
Anti-Inflammatory and anti-allodynic effects of the essential oil

Carrageenan-induced inflammation has been quite helpful for predicting anti-inflammatory drug activity. However, this model also offers the possibility of exploring the effects on localized inflammatory pain, as several studies have shown that the initial edema development caused by carrageenan injection is followed by a period of allodynia (11,12,24).

Carrageenan induced a maximal inflammatory response between 5 and 6 h, whereas the maximal tactile allodynia was observed after 3 h (Figure 3a,c). Local peripheral injection of EOZ (30, 100, and 300 µg/paw) slightly reduced the percentage of inflammation, compared with the VEH group. Significant differences were observed at the concentration of 300 µg/paw, 5 and 6 h after carrageenan injection (Figure 3a). The preventive anti-inflammatory effect observed with all treatments were independent of the concentration (Figure 3b) and comparable with the effect of diclofenac (30 µg/paw), which is a reference compound in this model (24).

Also, tactile allodynia was partially reverted (Figure 3c) by all treatments of EOZ in a concentration-dependent manner (Figure 3d). However, this effect was not more significant than that produced by diclofenac. Although EOZ was slightly effective in preventing carrageenan-induced changes, it had greater antinociceptive than anti-inflammatory potential. The results are consistent with the previously reported antinociceptive effect of the infusion in both phases of the formalin test and the anti-inflammatory response in the carrageenan-induced paw edema (11).

Neuropathic pain occurs after damage or dysfunction of the peripheral or central nervous systems, characterized by hyperalgesia, allodynia, and spontaneous pain. On this issue, there are limited studies on the effectiveness of EOs or their components against chronic pain, including neuropathy. The SNL model has become the standard procedure for mechanistic studies of peripheral neuropathy and screening for novel analgesics (20).

EOZ partially reverted tactile allodynia in a concentration-dependent manner in the SNL model (Figure 4). The best effect was observed at the...
concentration of 300 μg, 3 h after the administration (Figure 4a). It was more significant than that produced by the positive control, gabapentin (GBP, 100 μg), which is effective in the relief of neuropathic pain in humans, rats, and mice (25,26). Diclofenac (DIC, 30 μg) was also administered as a control aiming to compare its effect in the carrageenan model. It exhibited an anti-allodynic poor action, consistent with previous studies where diclofenac (3, 10, and 30 mg/kg, i.p.) is ineffective in a model of neuropathic pain in mice (26).

**Anti-Inflammatory and anti-allodynic effects of DME**

Usually, the major constituents of the essential oils reproduce fairly the biophysical and biological properties of the oil from which they were isolated; the oil's overall effect depends on the concentrations of its main constituents (27). DME was considered for further evaluation considering its high yield and stability. Previous investigations have shown that germacrene D readily converts to cadinenes, muurolenes, and elemene-type sesquiterpene hydrocarbons (28), although a few essences containing high amounts of germacrene D showed noted antinociceptive action in several pharmacological models (29). The poor stability of germacrene D might explain why the compound has not been evaluated as an antinociceptive or anti-inflammatory agent.

In the carrageenan-induced paw edema test, DME (0.1, 1, 3, and 10 μg/paw) significantly reduced inflammation in a concentration-dependent manner (Figure 5b). At the highest concentration tested, DME showed the maximal reduction in the first hour after carrageenan injection, lasting for the 6 h of the experiment (Figure 5a). These results indicate that DME acts in both, non-phagocytic and phagocytic phases of the carrageenan response (30). DME also showed a concentration-dependent anti-allodynic effect in this model (Figure 5d) with a significant reversion of tactile allodynia at concentrations of 1, 3, and 10 μg. The time course of DME’s anti-allodynic and anti-inflammatory responses at 10 μg/paw showed a similar pattern to diclofenac (30 μg/paw) (Figures 5a,c).

In the SNL model, DME (0.1, 1, 3, and 10 μg/paw) also exerted a concentration-dependent anti-allodynic effect (Figure 6b). DME (10 μg) exerted its maximal effect 2 h after administration, reverting tactile allodynia to a mean value of 0.67 g of the withdrawal threshold (Figure 6a). In contrast, the maximal effect of EOZ was 0.35 g (Figure 4a). In this set of experiments, the effect of GBP (100 μg) was comparable to the lowest dose of DME tested (0.1 μg) (Figure 6b). The greater effectiveness of DME over EOZ suggests that it is an important active principle of the complex mixture.

Evidence shows that inflammatory and immune mechanisms, both in periphery and central nervous systems, play an important role in neuropathic pain. Infiltration of inflammatory cells, in response to nerve damage, with subsequent release of inflammatory mediators, could sensitize primary afferent neurons contributing to pain hypersensitivity (31). Thus, the effect of DME in the carrageenan model suggests a partial decrease of the inflammation process, which in turn, helps to the reversal of allodynia in the peripheral nervous system. The same rationale could explain, at least in part, the reversion of tactile allodynia in the SNL model, which involved a nerve damage; however, more experiments are needed to confirm these hypotheses.
Figure 5. Anti-inflammatory and anti-allodynic effect of the intraplantar administration of desmethylisoencecalin in the carrageenan-induced paw edema test. (a) Time course of the inflammation percentage. (b) AUC of the time course (Inflammation percentage against time, AU). (c) Time course of the 50% withdrawal threshold (g). (d) AUC of the time course (50% withdrawal threshold against time, AU). VEH: mineral oil, DME: desmethylisoencecalin, DIC: diclofenac; Carr: Carrageenan. Each measurement represented as the mean ± SEM with 8 mice per group. Significantly different from vehicle values (VEH) (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001), as determined by two-way ANOVA, followed by Bonferroni’s post hoc test (panel a and c) or one-way ANOVA, followed by Dunnnett’s post hoc test (panel b and d).

Figure 6. Anti-allodynic effects of desmethylisoencecalin in the SNL model. (a) Time course of the 50% of withdrawal threshold (g). (b) AUC of the time course (50% withdrawal threshold against time, AU). VEH: mineral oil, DME: desmethylisoencecalin, GBP: gabapentin, DIC: diclofenac. Each measurement is the mean ± SEM with 8 mice per group. Significantly different from vehicle values (VEH) (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001), as determined by two-way ANOVA, followed by Bonferroni’s post hoc test (panel a) or one-way ANOVA, followed by Dunnnett’s post hoc test (panel b).
Finally, is worth mentioning that there is plenty information regarding the antinociceptive and/or anti-inflammatory activity of the minor components of EOZ when tested alone in various animal models. For example, β-caryophyllene showed marked oral antinociceptive properties in the hot-plate and formalin tests; and also exhibited activity in the constriction injury of the sciatic nerve model in mice throughout the opioid and endocannabinoid pathways (32). D-limonene inhibited the acetic acid-induced nociception and the second phase of formalin test in mice (33); and also induced an anti-hyperalgesic effect in a model of neuropathic pain in rats (34). In mice, eucalyptol inhibited chemical nociception induced by intraplantar formalin and intraperitoneal acetic acid administration; moreover, its anti-inflammatory activity has been demonstrated in the carrageenan-induced paw edema and cotton pellet-induced granuloma models (35).

Whether or not DME acts synergistically with the minor compounds remains an open question. However, in this case, the pharmacological action of DME was higher than that of the complex mixture suggesting its important role in the overall activity of the oil.

Previous reports showed that DME possesses antifungal, antifeedant, and antihyperglycemic activities (14,36,37); however, this work describe for the first time its antinociceptive and anti-inflammatory effects. Further research on the potential of DME to treat different types of pain, including neuropathic pain.

**UPLC analysis and method validation**

Although, DME and ZGE are important active principles of the traditional preparation of *Z. grandiflora* (Figure 7), ZGE was chosen as the analytical marker, considering its higher yields in the aqueous extract. The most suitable parameters for separating ZGE were achieved using an ACQUITY UPLC BEH Phenyl column and a binary mobile phase consisting of acetonitrile and water with 0.1% formic acid. Under these conditions, effective baseline resolution was achieved for ZGE (Figure 8). Comprehensive validation of the method for quantitative analysis was carried out according to the ICH guidelines (2005).

The system’s linearity was tested in the concentration range between 5 and 100 μg/ml and was found linear in the concentration range used. The calibration curves showed a good linear relationship between the peak areas and the tested concentration range. The linear regression equation was \( y = 12.962x - 8079 \) \( (R^2 = 0.9994) \) for PDA detection (254 nm). LOD value for ZGE was 1.33 μg/ml, whereas the LOQ value was 4.01 μg/ml.

Recovery assays tested the linearity of the analytical method. The linear regression equation was \( y = 1.007x - 0.0018 \) \( (R^2 = 0.9999) \) for PDA detection (254 nm). Recovery experiments evaluated the accuracy of the analytical method by spiking three samples at different concentration levels (10, 50, and 75 μg/ml) of the standard to the dichloromethane soluble fraction by triplicate. The results showed that the method was reliable and reproducible, with recoveries from 100.1% to 101.0% (CV ≤ 1.8%).

The reproducibility and repeatability of the analytical method were evaluated in terms of the intermediate precision by analyzing 12 replicates of the stock solution (50 μg/ml) on two different days. The overall intra- and inter-day variations of the standard (CV) were 0.57% and 0.95%, respectively.
Altogether, the described information revealed that the method developed was precise, accurate, and linear for the quantitative analysis of ZGE in the crude drug of *Z. grandiflora*.

**Acknowledgments**

This work was supported by grants from CONACyT CY011226 and DGAPA IN217320 awarded to R.M. VIRP acknowledges a fellowship from CONACyT to pursue graduate studies (no. 769727). The authors also recognize the valuable academic support of Araceli Pérez-Vásquez, Mariesa Gutierrez, Rosa Isela del Villar, Nayeli López Balbiaux, Isabel Rivero-Cruz, and Georgina Duarte from Facultad de Química. We recognize the valuable help of Adalberto Felix-Leyva from Cinvestav, South campus, for helping with the animal surgical procedures. EL and RB thank their collaborators, Alicia Melgoza Castillo, for the assistance in fieldwork and Virginia Evangelista for elaborating the plant distribution map.

**Disclosure statement**

No potential conflict of interest was reported by the author(s).

**Funding**

The work was supported by the Consejo Nacional de Ciencia y Tecnología [CY011226] Dirección General de Asuntos del Personal Académico, Universidad Nacional Autónoma de México [IN217320].

**ORCID**

Valeria Reyes-Pérez [http://orcid.org/0000-0002-5535-7627](http://orcid.org/0000-0002-5535-7627)

Vinicio Granados-Soto [http://orcid.org/0000-0003-1038-8400](http://orcid.org/0000-0003-1038-8400)

Rachel Mata [http://orcid.org/0000-0002-2861-2768](http://orcid.org/0000-0002-2861-2768)

Myrna Deciga-Campos [http://orcid.org/0000-0002-1467-9486](http://orcid.org/0000-0002-1467-9486)

**References**

1. D.F. Day, *Zinnia grandiflora* - general notes. Botanical Gazette, 9, 29 (1884).
2. J. Torrey, *Botany of the Boundary*. In: *Report on the United States and Mexican Boundary Survey: Made Under the Direction of the Secretary of the Interior*. Edt., W.H. Emory, pp. 27–270, House Exec. C. Wendell, Washington DC (1859).
3. G.O. Miller, *Landscaping with Native Plants of Texas*, Voyageur Press, Minneapolis, MN (2013).
4. A.R. Smith, *Zinnia*. In: *Flora of North America - Asteraceae* pp. 71–74, Vol. 21. Flora of North American Editorial Committee, NY (2006).
5. M.C. Stevenson, *Ethnobotany of the Zuni Indians*. In: *30th Annual Report of the Bureau of American Ethnology, 1908-1909*. Edit., W.H. Holmes, pp. 31–102, Government Printing Office, Washington DC, (1915).
6. G.R. Swank, *The Ethnobotany of the Acoma and Laguna Indians*. University of New Mexico Digital Repository, New Mexico, (1932).
7. L.C. Wyman and S.K. Harris, *Navajo Indian Medical Ethnobotany*, University of New Mexico Press, New Mexico (1941).
8. F.H. Elmore, *Ethnobotany of the Navajo*. University of New Mexico Press Bulletin 392, New Mexico (1994).
9. R.M. Zingg, *Mexican folk remedies of Chihuahua*, Journal of the Washington Academy of Sciences. 22, 174–181 (1932).
10. B.P. Bashyal, S.P. McLaughlin and A.A.L. Gunatilaka, *Zinga granadinosides A–C*, cytotoxic δ-elemanolide-type sesquiterpene lactones from *Zinnia grandiflora*, Journal of Natural Products. 69(12), 1820–1822 (2006). doi:10.1021/np0603626.
11. V. Reyes-Pérez, A. Pérez-Vásquez, M. Déciga-Campos, R. Bye, E. Linares and R. Mata, *Antinociceptive Potential of Zinnia grandiflora*. Journal of Natural Products Journal of Natural Products, 82, 456–461 (2019).
12. V.I. Reyes-Pérez, V. Granados-Soto, M. Rangel-Grimaldo, M. Déciga-Campos and R. Mata, *Pharmacological analysis of the anti-inflammatory...*
and antiallodynic effects of zinigrandinolide E from Zinnia grandiflora in mice. Journal of Natural Products, 84, 713–723 (2021). doi:10.1021/acs.jnatprod.0c00793.

13. H. Álvarez-Osina, I. Rivero Cruz, G. Duarte, R. Bye and R. Mata, HPLC determination of the major active flavonoids and GC-MS analysis of volatile components of dysphania graveolens (amaranthaceae). Phytochemical Analysis, 24, 248–254 (2013). doi:10.1002/pca.2405.

14. S. Escandón-Rivera, A. Pérez-Vásquez, A. Navarrete, M. Hernández, E. Linares, R. Bye and R. Mata, Anti-Hyperglycemic activity of major compounds from Calea ternifolia. Molecules, 22, 1–13 (2017). doi:10.3390/molecules22020289.

15. International Conference on Harmonisation Validation of Analytical Procedures: Text and Methodology Q2 (R1), ICH Harmonised Tripartite Guideline, pp. 1–13. Geneva (2005).

16. M. Zimmermann, Ethical guidelines for investigations of experimental pain in conscious animals. Pain, 16, 109–110 (1983).

17. C.A. Winter, E.A. Risley and G.W. Nuss, Carrageenan-induced edema in hind paw, Experimental Biology and Medicine. 3(3), 544–547 (1962). doi:10.3181/00379727-111-27849.

18. S.R. Chaplain, F.W. Bach, J.W. Pogrel, J.M. Chung and T.L. Yaksh, Quantitative assessment of tactile allodynia in the rat paw. Journal of Neuroscience Methods, 53, 55–63 (1994). doi:10.1016/0165-2470(94)90144-9.

19. W.J. Dixon, Efficient analysis of experimental observations. Annual Review of Pharmacology and Toxicology, 20, 441–462 (1980). doi:10.1146/annurev.pa.20.040180.002301.

20. G.L. Ye, K.V. Savelieva, P. Vogel, K.B. Baker, S. Mason, T.H. Lanthorn and I. Rajan, Ligation of mouse L4 and L5 spinal nerves produces robust allodynia without major motor function deficit. Behavioural Brain Research, 276, 99–110 (2015). doi:10.1016/j.bbr.2014.04.039.

21. A.F. Feyaerts, W. Luyten and P. Van Dijck, Striking essential oil: tapping into a largely unexplored source for drug discovery. Scientific Reports, 10, 1–11 (2020). doi:10.1038/s41598-020-59332-5.

22. E.A. Souza-Silva, R. Jiang, A. Rodriguez-Lafuente, E. Gionfriddo and J. Pawlisyn, A critical review of the state of the art of solid-phase microextraction of complex matrices I. Environmental Analysis TrAc - Trends in Analytical Chemistry TrAc - Trends in Analytical Chemistry, 71, 224–235 (2015). doi:10.1016/j.trac.2015.04.016.

23. E. Schmidt, Production of Essential Oils. In: Essential Oils: Science, Technology, and Applications. Edits, K. Hüsnü and G. Buchbauer, p. 127–162, CRC Press, New York (2015).

24. C.J. Morris, Carrageenan-Induced paw edema in the rat and mouse. Methods in Molecular Biology, 225, 115–121 (2003). doi:10.1385/1-59259-374-7:115.

25. I. Gilron and S.J.L. Flatters, Gabapentin and pregabalin for the treatment of neuropathic pain: a review of laboratory and clinical evidence, Pain Research and Management. 11suppl a, 16A–29A (2006). doi:10.1155/2006/651712.

26. T. Kiso, T. Watabiki, M. Tsukamoto, M. Okabe, M. Kagami, K. Nishimura, T. Aoki and N. Matsuoka, Pharmacological characterization and gene expression profiling of an L5/L6 spinal nerve ligation model for neuropathic pain in mice. Neuroscience, 153, 492–500 (2008). doi:10.1016/j.neuroscience.2008.02.031.

27. F. Bakkali, S. Averbeck, D. Averbeck and M. Idaomar, Biological effects of essential oils – a review, Food and Chemical Toxicology, 46(2), 446–475 (2008). doi:10.1016/j.fct.2007.09.016.

28. S.M. Njoroge, U. Ukeda and M. Sawamura, Changes of the volatile profile and artifact formation in daidai (citrus aurantium) cold-pressed peel oil on storage, Journal of Agricultural and Food Chemistry. 51(14), 4029–4035 (2003). doi:10.1021/jf021215q.

29. E.J. Lenardão, L. Savegnago, R.G. Jacob, F.N. Victoria and D.M. Martínez, Antinociceptive effect of essential oils and their constituents: an update review, Journal of the Brazilian Chemical Society. 27, 435–474 (2016).

30. D. Salvemini, Z.Q. Wang, P.S. Wyatt, D.M. Bourdon, M.H. Marino, P.T. Manning and M.G. Currie, Nitric oxide: a key mediator in the early and late phase of carrageenan-induced rat paw inflammation, British Journal of Pharmacology. 118(4), 829–838 (1996). doi:10.1111/j.1476-5381.1996.tb15475.x.

31. G. Moalem and D.J. Tracey, Immune and inflammatory mechanisms in neuropathic pain. Brain Research Reviews, 51, 240–264 (2006). doi:10.1016/j.brainresrev.2005.11.004.

32. L.I.G. Paula-Freire, M.L. Andersen, V.S. Gama, G. R. Molska and E.L.A. Carlini, The oral administration of trans-carvophyllene attenuates acute and chronic pain in mice. Phytomedicine, 21, 356–362 (2014). doi:10.1016/j.phymed.2013.08.006.

33. J.F. Do Amaral, M.I.G. Silva, M.R.D.A. Neto, P.F. Neto, B.A. Moura, C.T.V. Melo, F.L.O. De Araujo, D.P. De Sousa, P.F. De Vasconcelos, S.M.M. De Vasconcelos and F.C.F. De Sousa, Antinociceptive effect of the monoterpenic R-(+)-limonene in mice, Biological & Pharmaceutical Bulletin. 30(7), 1217–1220 (2007). doi:10.1248/bpb.30.1217.

34. A.C. Piccinelli, J.A. Santos, E.C. Konkiewitz, S. A. Oesterreich, A.S.N. Formaggio, J. Croda, E.B. Ziff and C.A.L. Kassuya, Antihyperalgesic and antidepressive actions of (R)-(+)-limonene, α-phellandrene, and essential oil from Schinus terebinthifolius fruits in a neuropathic pain model, Nutritional Neuroscience. 18(5), 217–224 (2015). doi:10.1177/14767383144000000119.

35. F.A. Santos and V.S.N. Rao, Antiinflammatory and antinociceptive effects of 1,8-cineole a terpenoid oxide present in many plant essential oils. Phytotherapy Research, 14, 240–244 (2000). doi:10.1002/1099-1573(200006)14:4<240:AID-PTR573>3.0.CO;2-X.

36. S.K. Agarwal, S. Verma, S.S. Singh, S. Kumar and G. Keshri, Antifertility chromene from Blepharispermum subessissile. Fitoterapia, 70, 435–437 (1999). doi:10.1016/S0367-326X(99)00064-7.

37. S.K. Agarwal, S. Verma, S.S. Singh, A.K. Tripathi, Z. K. Khan and S. Kumar, Antifeedant and antifungal activity of chromene compounds isolated from Blepharispermum subessissile. Journal of Ethnopharmacology, 71, 231–234 (2000). doi:10.1016/S0378-8741(00)00158-6.