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Chemical Characterization and *In vitro* Cytotoxicity to Mexican Oregano Essential Oil (*Lippia graveolens* Kunth) against Mouse 3T3-L1 Preadipocytes Cells Line

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ABSTRACT

Background: The Mexican oregano essential oil (MOEO) (*Lippia graveolens* Kunth) has documented antioxidant, anti-inflammatory and cytotoxic activity. The objective of this study was to determine the chemical composition of MOEO from plants grown in greenhouses and of natural origin (wild) in northern Mexico, by gas chromatography coupled to mass spectrometry and to evaluate its effect on the viability of 3T3-L1 cells by the bromide assay of 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium.

Methods: The aerial part of *L. graveolens* plants developed in greenhouse and wild conditions was collected. The MOEO was extracted and analyzed by gas chromatography coupled to mass spectrometry. Cytotoxicity was determined in the 3T3-L1 preadipocytes cell line exposed to concentrations between 0.0 and 15.00 µg mL^{-1} of MOEO, after 24 and 48 hours of exposure. The statistical analysis included an analysis of variance and Tukey's HSD post hoc test ($P \leq 0.05$) in the SPSS v. 20 software.

Result: The extraction of the MOEO presented a yield of 45.63±5.21 mL kg^{-1} dry weight and a density of 995±2.98 µg mL^{-1}. The chromatographic analysis identified 34 chemical components, of which the highest relative proportions corresponded to thymol (59.45% in greenhouse and 47.53% in wild) and carvacrol (9.27% in greenhouse and 12.14% in wild). At 24 hours of exposure, concentrations below 1.00 µg mL^{-1} registered average cell viabilities of 99.02% (greenhouse) and 90.98% (wild), while concentrations above 1.00 µg mL^{-1} registered 69.80% (greenhouse) and 79.62% (wild). Regarding the 48 hours, concentrations below 1.00 µg mL^{-1} showed viabilities of 25.81% (greenhouse) and 21.87% (wild), while concentrations above 1.00 µg mL^{-1} registered 61.09% (greenhouse) and 64.64% (wild).

Key words: Carvacrol, Greenhouse, Inhibition of cell viability, Thymol, Wild plants.

INTRODUCTION

Since the beginning of civilization, natural products have played a fundamental role in traditional medicine. However, since 2004, when the World Health Organization endorsed the use of complementary therapies, interest in products of natural origin has grown considerably (WHO, 2023). In this sense, the development of research related to plant-derived bioactive compounds has gained recognition for their potential for the development of treatments against different diseases in an accessible, affordable way and with a safe therapeutic profile (Ho *et al.*, 2021). Within these plants, the essential oil (EO) of *Lippia graveolens* Kunth, commonly known as Mexican oregano, is used as a raw material to produce cosmetics, pharmaceuticals and liquors. Additionally, it has been documented that the essential oils of this species have antioxidant potential and anti-inflammatory and cytotoxic activity (Marin-Tinoco *et al.*, 2023; Rojo-Ruvalcaba *et al.*, 2025).

Lippia graveolens EOs have been evaluated in cell cultures to quantify their effect on cell viability (cytotoxicity) (Zapata *et al.*, 2009). Quantification has been performed using colorimetric techniques such as the 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide reduction assay or the sulforrodamine B test, reporting a reduction in the percentage of viable cells (Frías-Zepeda *et al.*, 2022). In *in vitro* cytotoxicity assays, the cellular response varied depending on the type of cell line (CL), the period of exposure and the concentration of the

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administered extract (Elshafie *et al.*, 2017; Rojo-Ruvalcaba *et al.*, 2025). Experiments carried out on CLs treated with monoterpene constituents isolated from oregano EO (OEO), such as thymol and carvacrol, showed an inhibition of cell proliferation and alteration of signaling pathways at micromolar concentrations (Elshafie *et al.*, 2017). This contributes to characterizing the cytotoxic profile of isolated terpene compounds and plant-based extracts (Rojo-Ruvalcaba *et al.*, 2025).

In this sense, the 3T3-L1 CL, represents an *in vitro* model for the study of the cytotoxic capacity of natural compounds (Yamaguchi *et al.*, 2017). It has been shown that the exposure of 3T3-L1 cells to terpenoid compounds influences the proliferation rate and the deposition of intracellular lipids (Morissette *et al.*, 2024). Additionally, it has allowed the determination of non-lethal concentration ranges, before evaluating metabolic responses. In this way, the use of 3T3-L1 cells has facilitated the evaluation of cytotoxicity and interaction of secondary molecules of plant origin on cells in the preadipocyte stage (Murillo *et al.*, 2026).

Although there are reports on the cytotoxic effect of essential oils and bioactive compounds of *L. graveolens*, information about the action of this species' EO on mouse 3T3-L1 preadipocyte cells is limited (Frías-Zepeda *et al.*, 2022). Therefore, the objectives of this study were to chemically characterize the constituents of Mexican OEO (MOEO) (*L. graveolens*) of plants grown in greenhouses and plants naturally developed in a semi-arid zone of northern Mexico, by gas chromatography coupled to mass spectrometry and to evaluate the cytotoxic effect of this EO on the viability of the mouse 3T3-L1 preadipocyte CL using the MTT photocolorimetric technique. Investigating these characteristics is of vital importance to develop new natural treatment opportunities for human health (Hussien and Tantawy, 2026; Kala and Juyal, 2026). This should be based on a holistic and sustainable vision aligned with the objectives of sustainable development (Armijo-Nájera *et al.*, 2026).

MATERIALS AND METHODS

Location of collection site and identification of plant material

The greenhouse where the oregano samples were grown and collected was located at the facilities of the Technological University of Rodeo (25.1650° LN, 104.5558° LW, 1342 masl). The collection site of the samples of naturally produced oregano corresponded to a natural community of wild oregano (25.0233° LN, 104.4758° LW, 1471 masl). Both sites are located in the municipality of Rodeo, Durango, Mexico. The climate of the study area is characterized by being semi-arid with an average annual temperature of 19.1°C, a maximum of 25.5°C and a minimum of 12.1°C; the average annual rainfall is 417.3 mm. The precipitation regime occurs mainly from June to September (SMN, 2010).

The collection was carried out on the same day for both sites (November 21, 2025). The entire aerial plant (stems, leaves, flowers and fruits) was collected, then dehydrated at room temperature and in the shade for four days, to continue with the separation of the stems from the rest of the collected parts. The stems were discarded and 350 g of the rest were weighed on an analytical balance with an accuracy of 0.0001 g (Denver Instrument Company). Finally, they were labeled and stored in clean and closed paper bags until use. All samples were made in quadruplicate. With the help of a botanical press, samples of mature oregano plants from both sites were sent to the Laboratory of Botany and Systematics of the Faculty of Biological Sciences of the Juarez University of the State of Durango, located in Gómez Palacio, Durango, Mexico, where they were identified. The results of the identification indicated that the plants of both sampling sites corresponded to Mexican oregano (*Lippia graveolens* Kunth).

Mexican oregano essential oil extraction

OEO´s were extracted according to the methodology reported by Marin-Tinoco and others in 2023.

Mexican oregano essential oil chromatographic analysis

OEO´s were chromatographically analyzed according to the methodology reported by Marin-Tinoco *et al.* (2023).

Culture of 3T3-L1 preadipocyte cells

The 3T3-L1 preadipocyte CL (ATCC® CL-173™) was obtained directly from the American Type Culture Collection (ATCC, Manassas, VA, USA). Upon receipt, the cells were handled and cultured in strict accordance with the manufacturer's instructions. All experiments were conducted within four months of receiving the preadipocyte cells and without undergoing prolonged serial passaging; therefore, additional short tandem repeat profiling was not required. Furthermore, all experiments were performed using mycoplasma-free cells. The cells were maintained in Basal Medium I (BMI), consisting of Dulbecco's Modified Eagle Medium (Gibco, Rockville, MD, USA) supplemented with 10% calf serum (Gibco, Rockville, MD, USA), 100 U mL^{-1} penicillin, 100 µg mL^{-1} streptomycin, 50 µg mL^{-1} gentamicin and 125 µg mL^{-1} amphotericin (Sigma-Aldrich, St. Louis, MO, USA). Cultures were incubated at 37°C in a humidified atmosphere containing 5% CO_2. The culture medium was replaced every 48 hours. Subculturing was performed using a solution of 0.25% trypsin and 0.53 mM EDTA. Cell counting was carried out using a Neubauer hemocytometer under a light microscope.

Mexican oregano essential oil cytotoxic activity

Cytotoxicity was evaluated by a cell viability assay of 3T3-L1 preadipocytic cells in response to different concentrations of MOEO (0.0, 0.015, 0.025, 0.05, 0.125, 0.50, 1.50, 5.00, 10.00 and 15.00 µg mL^{-1}). The technique used for this evaluation was the photocolorimetric technique of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium

bromide) (Sigma, New Jersey, USA) described by Arunachalam and Sreeja in 2025. For this purpose, 3T3-L1 cells were cultured at a density of 2,000 mL^{-1} cells in 96-well plates; and incubated for 24 hours at 37°C in a humid atmosphere with 5% CO_2. Subsequently, 100 µg of the concentrations included in the study were added. The plates were incubated for a period of 24 or 48 hours at 37°C; and then stirred for one minute in an analog microplate shaker CVP-9001 (CS Scientific, Vela Quin, Mexico City, Mexico). For the determination of 50% inhibition of cell viability (IC_{50}). The IC_{50} values for each origin of oregano oil were obtained by simple linear regression analysis of dose-response curves generated with absorbance data. All procedures were performed quadruplicate.

Statistical analysis

The normal distribution of each variable was checked using the Shapiro-Wilk test. In addition, the Levene test was used to verify the equality of variances. Subsequently, a unidirectional and bidirectional analysis of variance was performed. When a significant effect was detected, pairwise comparisons were made between the means using Tukey's post hoc test of honestly significant difference (HSD). The differences were considered statistically significant at a level of P≤00.05. For the presentation of the information, the mean and standard error of each concentration of each origin of oregano oil were determined. All statistical analyses were performed using SPSS v. 20 (SPSS Inc., IL, USA).

RESULTS AND DISCUSSION

Main chemical composition of the mexican oregano essential oil

The average yield obtained during the MOEO extraction process was 45.63±5.21 mL kg^{-1} dry weight. The calculated density was 995±2.98 µg mL^{-1}. The chromatographic analysis of the MOEO identified 34 components (data not shown). Table 1 presents the mean values of the main 20 chemical components, which are ordered according to the percentage in the sample from highest to lowest. Where, the combined analysis of gas chromatography and mass spectrometry showed that six compounds (*i.e.* thymol, carvacrol, o-cymene, gamma-terpinene, beta-myrcene and eucalyptol) represented 86.97 and 92.08%, for the MOEO grown in greenhouses and for the one of natural origin (wild), respectively. Thymol stands out, which evidenced a global mean concentration of 53.49% (Table 1).

In general, the yield and chemical composition of EOs of the same species vary considerably, mainly due to different environmental factors (Fikry *et al.*, 2019). Therefore, it is necessary to examine these fluctuations in species of economic and social relevance such as Mexican oregano. The chromatographic analysis of the MOEO revealed that the majority constituents, for the two sampled sites, were thymol and carvacrol. This is relevant, considering that a high content of carvacrol has been associated with greater biological activity in the oil (Walczak *et al.*, 2021). Additionally, interspecies chemical differences

Table 1: Main chemical components in Mexican oregano essential oil (*Lippia graveolens* Kunth) from plants grown in greenhouse (GH) and occurring naturally (Wild) in semiarid areas in northern Mexico, analyzed with gas chromatography-mass spectrophotometry.

Retention time (min)	Compound name	Percentage in the sample (Relative areas)	
		GH	Wild
9.79	Thymol	59.45	47.53
9.90	Carvacrol	9.27	12.14
5.04	o-Cymene	7.23	24.73
5.65	Gamma-Terpinene	6.41	2.53
4.53	Beta-Myrcene	2.42	1.67
5.13	Eucalyptol	2.19	3.48
5.77	Bicyclo[3.1.0]hexan-2-ol, 2-methyl-5-(1-methylethyl)-, (1.alpha.,2.alpha.,5.alpha.)-	2.84	0.08
7.68	Terpinen-4-ol	1.62	1.33
4.92	Cyclohexene, 1-methyl-4-(1- methylethylidene)-	1.45	0.52
11.67	Caryophyllene	1.16	1.69
10.67	Phenol, 5-methyl-2-(1-methylethyl)-, acetate	0.71	0.07
3.48	Bicyclo[3.1.0]hex-2-ene, 2-methyl-5-(1- methylethyl)-	0.70	0.08
12.19	Humulene	0.67	1.08
8.71	Benzene, 2-methoxy-4-methyl-1-(1- methylethyl)-	0.66	0.19
6.40	Linalool	0.43	0.89
6.16	(+)-4-Carene	0.27	0.18
7.46	endo-Borneol	0.25	0.15
2.89	3-Methyl-3-buten-1-ol, acetate	0.24	0.15
3.56	Alpha-Pinene	0.23	0.40
4.80	3-Carene	0.18	0.14

are usually attributed to the geographical origin and phenological state of the plant (Tellez-Monzon and Nolazco-Cama, 2017). The above is observed in the information contained in Table 2, which shows the chemical polymorphism of the EO of this species; by comparing the concentrations of thymol and carvacrol, determined in this study and what has been reported, for these two bioactive compounds, by another research.

Cytotoxic effect of mexican oregano essential oil on 3T3-L1 cells

As shown in Fig 1a, at 24 hours of exposure, concentrations below 1.00 µg mL^{-1} showed an average viability percentage of 99.02 and 90.98% for MOEO from greenhouse and wild, respectively. This percentage showed a significant decrease with concentrations above 1.00 µg mL^{-1}, where MOEO from greenhouses registered an average cell viability of 69.80% and wild MOEO of 79.62% (Fig 1a). Regarding the results obtained for 48 hours of exposure, the trend showed differences with respect to 24 hours (Fig 1b). Considering that the percentage of viability for concentrations less than 1.00 µg mL^{-1} were 25.81 and 21.87% for the MOEO from greenhouse and wild, respectively. This percentage evidenced a significant increase with concentrations greater than 1.00 µg mL^{-1}, where the MOEO from the greenhouse registered an average cell viability of 61.09% and the wild MOEO of 64.64% (Fig 1b). In addition, Fig 1c includes the calculated values of the probability by the bidirectional analysis of variance performed. Fig 2 presents images of the cytotoxic activity of greenhouse MOEO at a concentration of 0.50 µg mL^{-1}, on the mouse 3T3-L1 preadipocyte cell line after 24 and 48 hours of incubation.

In this same context, wild MOEO was more cytotoxic than greenhouse MOEO in both evaluated periods (*i.e.* 24 and 48 hours), considering that values of 1.08 (r^2: 0.80) and 0.11 (r^2: 0.80) were calculated for the IC_{50} at 24 hours of exposure for greenhouse and wild MOEO, respectively. When calculating the IC_{50} for the 48 hours of exposure, the reported values were 0.57 (r^2: 0.90) for the greenhouse MOEO and 0.10 (r^2: 0.79) for the wild MOEO. These low IC_{50} values suggest that the cytotoxic activity of MOEO is not only due to its major components, but also to a synergism between major and minority components (Zapata *et al.*, 2009).

Regarding the results obtained at 24 hours, Frías-Zepeda *et al.* (2022) used non-tumor cell models and determined IC_{50} values at 24 hours, for *L. graveolens* extracts, of 59.86±1.22 µg mL^{-1} for the leaf extract and 50.77±1.37 µg mL^{-1} for the stem extract in normal HFF-1 fibroblasts. Accordingly, López Villarreal *et al.* (2022) recorded cellular viabilities in human gingival fibroblasts (HGF-1) of 84.60±3.21% at 125 µg mL^{-1} and 79.53±3.50% at 250 µg mL^{-1} after 24 hours of exposure to ethanolic extract of *L. graveolens*, obtaining an IC50 value of 1384.70 µg mL^{-1}. A study carried out with isolated monoterpene components present in OEO, reported IC_{50} values for carvacrol of 48.30±6.70 mg L^{-1} in HepG2 tumor cells and 90.50±9.90 mg L^{-1}

Table 2: Average thymol and carvacrol content in Mexican oregano essential oil (*Lippia graveolens* Kunth) from plants grown in greenhouse and occurring naturally in semiarid areas in northern Mexico, compared with other studies made in Mexico.

Source	Thymol (%)	Carvacrol (%)	Region/Country
This study	53.49	21.41	Durango, Mexico
Marin-Tinoco *et al.*, 2023	7.70	17.90	Durango, Mexico
Rodriguez-Canales *et al.*, 2025	6.89	18.75	Puebla, Mexico
Rojo-Ruvalcaba *et al.*, 2025	5.15	0.006	Jalisco, Mexico

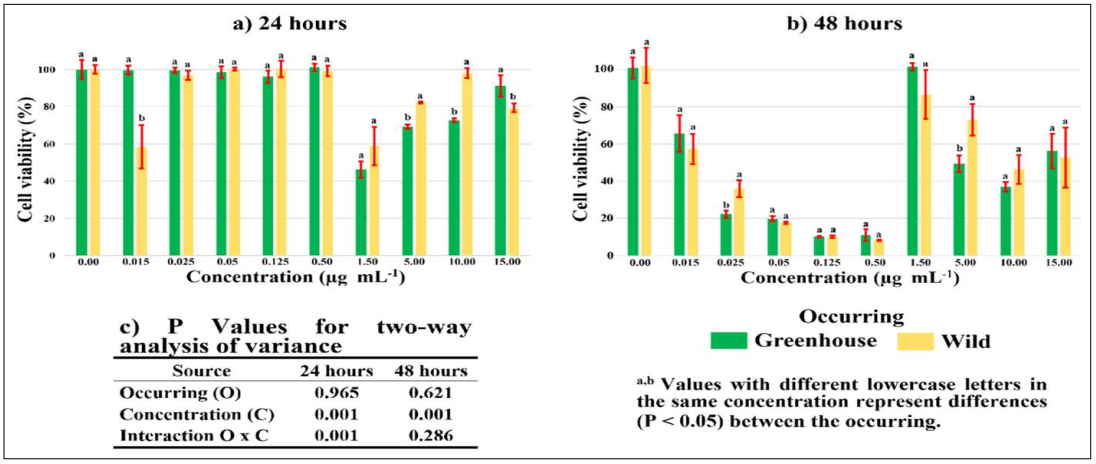


Fig 1: Cytotoxic effect of different concentrations of Mexican oregano essential oil (*Lippia graveolens* Kunth) from plants grown in greenhouse and occurring naturally (Wild) in semiarid areas in northern Mexico, in mouse 3T3-L1 preadipocyte cell line *in vitro*.



Where: (A) 3T3-L1 cell control (cells without treatment); (B) 3T3-L1 cells treated with 0.50 µg mL^{-1} after 24 hours of incubation; (C) 3T3-L1 cells treated with 0.50 µg mL^{-1} after 48 hours of incubation. Images obtained under the microscope (40×).

Fig 2: Cytotoxic effect of Mexican oregano essential oil (MOEO) (*Lippia graveolens* Kunth) from plants grown in green house in semiarid areas in northern Mexico, in mouse 3T3-L1 preadipocyte cell line *in vitro*.

in non-tumor HEK293 cells, while for thymol they reported IC_{50} values of 289.50±6.60 and 939.80±29.60 mg L^{-1} for HepG2 and HEK293, respectively (Elshafie *et al.*, 2017). However, another study conducted on 3T3-L1 preadipocytes, the viability evaluation performed with terpene compounds showed no reduction in cell viability at concentrations from 0 to 100 µM (Murillo *et al.*, 2026).

Considering the evidence obtained at 48 hours of exposure, the temporal pattern obtained is consistent with what was reported by Frías-Zepeda *et al.* (2022), in the SK-LU-1 lung carcinoma line, where IC_{50} values of *L. graveolens* stem extract ranged from 7.80±1.32 µg mL^{-1} at 24 hours to 0.81±0.28 µg mL^{-1} at 48 hours, while in normal HFF-1 cells the IC_{50} remained at values above 300 µg mL^{-1} at 48 hours. Similarly, in normal fibroblasts NIH3T3, Criollo-Mendoza *et al.* (2022), recorded a cell viability of 75% at concentrations of 175 and 200 µg mL^{-1} of polyphenol-rich extract of *L. graveolens*. In another study carried out, using extracts of the oregano species *Lippia origanoides*, the research team determined that the essential oil of the trans-β-caryophyllene/p-cymene chemotype presented at 48 hours, an IC_{50} value of 9.10±1.00 µg mL^{-1} in HeLa cells and 64.40±7.80 µg mL^{-1} in non-tumor Vero cells, registering a selectivity index of 7.10 (Zapata *et al.*, 2009).

Likewise, a study conducted on THP-1 macrophages reported that concentrations of oregano extract (*Origanum vulgare*) greater than 30 µg mL^{-1} reduced cell viability at 24 and 48 hours in a dose-dependent manner, while concentrations of up to 30 µg mL^{-1} did not affect viability (Ocaña-Fuentes *et al.*, 2010). On the other hand, Rojo-Ruvalcaba *et al.* (2025) reported that the infusion of *L. graveolens* presented an IC_{50} value of 0.18 mg mL^{-1} in the noncancerous HaCaT CL at 48 hours, registering a cytotoxicity percentage of 12.07% measured by the release of lactate dehydrogenase, unlike pure carvacrol that showed an IC_{50} of 110 µM and a cytotoxicity of 57.66%. In general, the IC_{50}, determined in this study, were lower than those reported in the consulted studies. Although it was not specifically determined, it could be assumed that the monoterpene components present in MOEO, such as thymol and carvacrol, have lipophilic properties that facilitate their interaction with the phospholipid bilayer of cell membranes (Elshafie *et al.*, 2017).

CONCLUSION

The chromatographic analysis of the MOEO extracted from plant material collected in Rodeo, Durango, northern Mexico, identified 34 chemical constituents, of which thymol (53.49%) and carvacrol (21.41%) corresponded to the components with the highest relative proportion. The cytotoxic effect showed quantitative variations depending on the time of exposure and the concentration. These results establish the chemical characterization of the MOEO and its cytotoxic profile, determining the concentration ranges and response times in 3T3-L1 preadipocytic cells for subsequent metabolic investigations. However, additional studies are required to identify the metabolites present in MOEOs with biological activity and their mechanisms of molecular action, in order to develop a formulation based on natural products as a therapeutic alternative.

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Disclaimers

The views and conclusions expressed in this article are solely those of the authors and do not necessarily represent the views of their affiliated institutions. The authors are responsible for the accuracy and completeness of the information provided, but do not accept any liability for any direct or indirect losses resulting from the use of this content.

Informed consent

No informed consent was required for this study. Whereas all experiments were conducted using the 3T3-L1 mouse cell line. This did not involve the participation of humans or live animals.

Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this article. No funding or sponsorship influenced the design of the study, data collection, analysis, decision to publish, or preparation of the manuscript.

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