Korean J Pain 2022;35(2):140-151
https://doi.org/10.3344/kjp.2022.35.2.140
pISSN 2005-9159 eISSN 2093-0569

KJP

Experimental Research Article

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Antinociceptive, anti-inflammatory, and cytotoxic properties of *Origanum vulgare* essential oil, rich with β-caryophyllene and β-caryophyllene oxide

Armenuhi Moghrovyan[1], Lilya Parseghyan[2], Gohar Sevoyan[3], Anna Darbinyan[2], Naira Sahakyan[4], Monica Gaboyan[5], Zaruhi Karabekian[3], and Armen Voskanyan[2]

[1]*Department of Pharmacognosy, Yerevan State Medical University after M. Heratsi, Yerevan, Armenia*
[2]*Orbeli Institute of Physiology, Laboratory of Physiologically Active Substances Investigations, Yerevan, Armenia*
[3]*Orbeli Institute of Physiology, Laboratory of Tissue Engineering, Yerevan, Armenia*
[4]*Yerevan State University, Research Institute of Biology, Faculty of Biology, Yerevan, Armenia*
[5]*Yerevan State Medical University after M. Heratsi, Faculty of Pharmacy, Yerevan, Armenia*

Received November 23, 2021
Revised February 1, 2022
Accepted February 2, 2022

Handling Editor: Jong Yeon Park

Correspondence
Armen Voskanyan
Orbeli Institute of Physiology, National Academy of Sciences, 22 Orbeli Bros. str. 0028 Yerevan, Armenia
Tel: +374 94818197
Fax: +37410 2802050
E-mail: arminvosking@gmail.com

Background: Essential oils are of great interest for their analgesic and anti-inflammatory properties. We aimed to study the content of the essential oil of the *Origanum vulgare* of the Armenian highlands (OVA) in different periods of vegetation and to investigate its antinociceptive and anti-inflammatory effects in mice (*in vivo*) and cytotoxic action in cultured cells (*in vitro*). OVA essential oil was extracted from fresh plant material by hydro-distillation.
Methods: For OVA essential oil contents determination the gas chromatography-mass spectrometry method was used. Formalin and hot plate tests and analysis of cell viability using the methyl-thiazolyl-tetrazolium (MTT) assay were used.
Results: The maximal content of β-caryophyllene and β-caryophyllene oxide in OVA essential oil was revealed in the period of blossoming (8.18% and 13.36%, correspondently). In the formalin test, 4% OVA essential oil solution (3.5 mg/mouse) exerts significant antinociceptive and anti-inflammatory effects (P = 0.003). MTT assay shows approximately 60% cytotoxicity in HeLa and Vero cells for 2.0 μL/mL OVA essential oil in media.
Conclusions: The wild oregano herb of Armenian highlands, harvested in the blossoming period, may be considered as a valuable source for developing pain-relieving preparations.

Key Words: Analgesia; Anti-Inflammatory Agents; Beta-Caryophyllene; Caryophyllene Oxide; Cell Survival; Gas Chromatography-Mass Spectrometry; Nociception; Oils, Volatile; Origanum; Pain; Pain Measurement.

INTRODUCTION

Interest in the raw materials of natural herbs, already great, is increasing day by day [1]. The flora of Armenia is rich in its variety of herbal raw materials [2]. Plants of the *Lamiaceae* family are valuable medicinal, mostly aromatic plants, many of which produce essential oils, used in traditional and modern medicine, as well as in the food, cosmetics, and pharmaceutical industries [3].

Origanum vulgare, belonging to the Lamiaceae fam-

Author contributions: Armenuhi Moghrovyan: Writing/manuscript preparation; Lilya Parseghyan: Investigation; Gohar Sevoyan: Investigation; Anna Darbinyan: Investigation; Naira Sahakyan: Writing/manuscript preparation; Monica Gaboyan: Investigation; Zaruhi Karabekian: Project administration; Armen Voskanyan: Supervision.

ily, is widely spread in Armenia, where its raw material resources have been investigated [4]. Nowadays, the research on oregano is of a great scientific interest. The essential oil extracted from this plant has positive effects as antioxidant, and as an anti-inflammatory, anti-diabetic, anti-proliferative and antibacterial agent [5,6]. According to European and Russian Pharmacopeias, the essential oil of *O. vulgare* contains thymol and carvacrol, which serve as a basis for the classification of herbal raw material [7]. However, modern scientific perceptions about oregano are based on other criteria, taking into a consideration the chemotype of the plant. The literature data suggest four chemotypes of oregano: the first chemotype is distinguished by the high content of thymol, the second possess high content of carvacrol, the third chemotype has moderate thymol content, and the fourth is characterized by a low or complete absence of phenol in the essential oil and a high content of sesquiterpenes [8].

The composition of biologically active substances in plants are defined by growth, as well as climatic and other environment factors [9] which have great influence on the essential oil production dynamics [10]. The content of essential oil from the *Origanum vulgare* of the Armenian highlands (OVA) differs from the essential oils of the same plants represented in the territories of other countries of the region (Turkey, Iran, and Georgia) [11]. It is known that essential oils have painkiller and anti-inflammatory properties [12]. The rather high percentage of β-caryophyllene, β-caryophyllene epoxide, and some other physiologically active substances suggest that OVA essential oil may have a pronounced antinociceptive and anti-inflammatory effect. β-caryophyllene is a substance belonging to the class of cannabinoids and have expressed analgesic effects. Therefore, the antinociceptive effect of *O. vulgare* can be of particular interest.

The exact mechanism of the essential oil's influence on the endocannabinoid system activity is still poorly understood [13]. There are two types of cannabinoid receptors. Type 1 (CB1) cannabinoid receptors are predominantly present in the central nervous system, making them a potential target for neuropsychological disorders and neurodegenerative diseases. Therefore, CB1 activation can cause psychosis and panic in some cases. On the other hand, inhibition of CB1 can lead to depressive or anxious behavior [14]. Cannabinoid receptors of the type 2 (CB2) are widely represented in the immune system and the periphery (in leukocytes and lymphocytes, mast cells, and microglia), which determines their participation in immune modulation [15]. CB2 is a promising target for treating inflammatory diseases, neuropathic pain, and immune modulation [16]. CB2 activation can affect different signaling pathways. Cannabinoids are able to modulate the function of immune cells and thus influence the secretion of cytokines. Inflammation plays a decisive role in the defense of the immune system against damaging factors such as pathogen attack and mechanical injury. Exogenous as well as endogenous cannabinoids can modulate pain and inflammation both through a direct influence on appropriate receptors as well as by inhibiting inflammatory neuropeptide release [17].

Pain relief is a major challenge in the current health care system. Pain is probably the most common symptomatic reason to seek medical consultation. Despite improved knowledge of the underlying mechanisms and better treatments, many people who have any type of pain receive inadequate care and non-effective drugs. There is a proven causal link between pain and inflammation. In inflammation, key players are macrophages, T-lymphocytes, cytokines, and chemokines. In the spinal cord and brain, microglia and astrocytes are involved in these processes too. Many physiologically active substances reduce inflammation, resulting in pain relief [18].

The aim of this research was to reveal the chemical composition of OVA essential oil, and to study its possible analgesic, cytotoxic, and anti-inflammatory properties.

MATERIALS AND METHODS

1. Experimental animals

Male outbred albino mice (4–5 weeks old, 20 ± 2 g) were used throughout these experiments. Mice were obtained from L. A. Orbeli Institute of Physiology National Academy of Sciences of the Republic of Armenia (NAS RA). The animals were maintained in a room with a controlled temperature (22°C ± 2°C) and a 12-hour light/dark cycle, and all mice had unlimited access to food and water. Twelve hours before each experiment, the animals received only water to avoid possible interference of the food with the absorption of the drug. The study was conducted according to the "Principles of Laboratory Animal Care" and was carried out in accordance with the European Communities Council Directive of September 22, 2010 (2010/63/EU) and was approved by the Institutional Review Board of the Orbeli Institute of Physiology of NAS RA (protocol code N4, date of approval: 22.07.2021).

For the formalin test, eleven groups (six mice in each group) were investigated. During the implementation of the hot plate test, there were two groups of mice (twelve in each). Totally, ninety mice were used. No animal was euthanized during experiments.

2. Plant material

The wild *O. vulgare* plant served as the investigation material, and was harvested between May and August 2018 in different phenological periods (pre-blossoming, blossoming, and fruiting, Gegharkunik region, village Chkalovka, 1930 meter above sea level). The identification of the plant was carried out at the Department of Pharmacognosy, Yerevan State Medical University, Yerevan (Armenia), and the plant samples were deposited and are available at the Herbarium of the Institute of Botany, National Academy of Sciences of Armenia, Yerevan (Armenia): voucher specimen number is ERE 191395.

3. Raw materials preparation

The primary processing of the raw materials was carried out immediately after collection: discarding organic and mineral mixtures, washing, and drying [19].

4. Essential oil extraction

OVA essential oil was extracted from fresh plant material (aerial parts only) by hydro-distillation for 3 hours, using a Clevenger-type apparatus. The distilled essential oil had been dehydrated with anhydrous sodium sulfate and stored at 4°C in dark, airtight bottles until further analysis [7].

5. Determination of essential oil chemical composition

The essential oil composition was analyzed at the FDA Analytical laboratory (Tonus-Les LLC., Yerevan, Armenia). The gas chromatography (GC) analysis was carried out using a Bruker gas chromatograph (Bruker 450-GC; Bruker Corporation, Billerica, MA), fitted with 60 m × 0.25 mm × 0.25 μm OPTIMA-FFAP column (Macherey-Nagel, Düren, Germany). The oven temperature varied from 40 to 220°C with a scanning rate of 3°C/min, evaporator temperature was 220°C. Helium (purity 5.6) was used as a carrier gas at a flow rate of 1 mL/min. The GC was equipped with a Hewlett–Packard 5972 Series mass spectrometry (MS) detector. The MS operating parameters were an ionization voltage of 70 eV and an ion source temperature of 250°C. The diluted samples of essential oils of 2 μL had been injected manually. To avoid overloading the GC column, the essential oils were diluted 1:50 (v/v) in methanol. The identification of peaks was carried out based on a library search using the NIST-2013 [20]. It was also determined retention time and retention index would be basic parameters for the descriptive components, which are very important for the chemometrics of essential oils [21]. The substances are presented in ascending order of retention indices (**Table 1**).

6. Chemicals, compounds, and drugs

As a standard analgesic sodium metamizole (Analgin®; Yerevan Chemical-Pharmaceutical Firm, Yerevan, Armenia) and as a standard anti-inflammatory agent diclofenac sodium (Diclofenac®; Hemofarm A.D., Vršac, Serbia) (positive controls) were used. The formalin (Sigma-Aldrich, St. Louis, MO) and other chemicals were analytical or sequencing grade.

7. The formalin test

The formalin test was used to evaluate the antinociceptive properties of the OVA essential oil after intraperitoneal injection in mice [22]. Counting the bites/licks of the hind paw was used for estimating nociceptive behavior. An intraplantar injection was performed on the hind paw of the mice, using 20 μL of 5% formalin. The nociceptive behavior (the biting/licking number) was recorded for 45 minutes. Sodium metamizole (Analgin®, 8.3 mg/kg) and sodium diclofenac (Diclofenac®, 10 mg/kg) were used as standard drugs. Dosage selection was based on protocols widely used in the literature [23]. The initial mixture of OVA essential oil for the experimental groups were made up of 50 μL dimethyl sulfoxide (DMSO), 100 μL Tween 80, and 50, 100, 150, 200, and 250 μL of OVA essential oil in 1 mL saline (respectively for each group). Then, the volume of the solution was brought to 5 mL using saline. Tested solutions were injected intraperitoneally (15 minutes and 30 minutes before formalin injection), with the concentrations of OVA essential oil being 2%, 3%, 4%, and 5% per mouse, respectively. The actual amount of essential oil administered to one mouse corresponded to 2.0, 3.0, 4.0, and 5.0 μL (essential oil density is equal to 0.87, the OVA essential oil mass was 1.74, 2.60, 3.48, and 4.35 mg/mouse) according to one or another group of experimental animals. The whole experiment lasted 60 or 75 minutes from the moment the essential oil was injected. The quantity of biting/licking of the hind paw was recorded for 45 minutes after intraplantar injection of formalin.

8. The hot plate test

This test was used to assess acute thermal pain [24]. The mice (20 ± 2 g) were placed into a Plexiglas cylinder, one by one, on a surface maintained at a stable 55°C. An intraperitoneal injection of 0.1 mL 4% OVA essential oil was made in the experimental group after 15 minutes. The time la-

Table 1. The concentrations, RT, and RI of chemical composition of the OVA essential oil in different vegetation periods (compounds with concentration more than 1.0%)

Compound	Pre-blossoming		Blossoming		Fruiting	
	Concentration (%)	RT/RI	Concentration (%)	RT/RI	Concentration (%)	RT/RI
(+)-Sabinene	2.42	13.43/971	3.1	3.36/897	3.29	13.36/897
β-Pinene	2.06	15/983	-	-	-	-
trans-β-Ocimene	6.13	18.37/1,041	3.81	19.35/978	2.61	18.4/1,042
3-Octanol	-	-	2.37	26.69/979	-	-
2-Hexenal diethyl acetal, trans	-	-	3.27	21.22/993	-	-
β-Ocimene	4.04	19.19/1,043	-	-	5.97	19.27/1,050
o-Cymene	2.22	20.32/1,045	5.22	20.43/1,045	9.41	20.33/1,045
Eucalyptol	-	-	1.95	17.7/1,059	4.18	17.68/1,037
α-Terpinolene	-	-	-	-	1.0	20.88/1,089
β-Linalool	-	-	2.90	34.26/1,083	1.18	34.21/1,100
trans-β-Terpineol	1.01	21.79/1,125	-	-	-	-
Neo-allo-ocimene	1.65	25.35/1,131	-	-	-	-
p-Menthan-3-one	1.18	30.43/1,275	-	-	-	-
L-4-terpineol	2.19	37.14/1,335	2.34	37.19/1,137	2.18	37.12/1,137
cis-β-Terpineol	-	-	2.57	30.42/1,158	-	-
Carvacrol/Isothymol	-	-	2.38	62.23/1,262	-	-
α-Terpineol	-	-	-	-	1.74	41.53/1,181
(-)-β-Bourbonene	-	-	2.21	32.77/1,339	1.13	32.68/1,380
Dihydroedulan II	-	-	1.93	31.68/1,342	-	-
Elixene	1.27	30.68/1,375	-	-	1.75	42.89/1,375
β-Caryophyllene	7.0	36.7/1,417	8.18	36.9/1,494	6.41	36.62/1,410
γ-Elemene	1.18	42.89/1,433	-	-	-	-
α-Humulene	1.68	39.97/1,456	2.68	40.06/1,456	1.53	39.93/1,455
l-β-Bisabolene	-	-	3.24	42.47/1,501	2.18	42.31/1,561
Germacrene D	3.80	41.83/1,482	3.80	41.92/1,515	9.22	41.8/1,515
β-Caryophyllene epoxide	5.6	53.91/1,517	13.36	54.18/1,517	11.2	53.91/1,517
cis-Z-α-Bisabolene epoxide	1.97	81.02/1,536	-	-	-	-
Isoaromadendrene epoxide	3.72	82.25/1,579	-	-	-	-
Ent-Spathulenol	6.75	59.2/1,590	3.77	59.26/1,536	2.51	59.15/1,590
tau-Cadinol	1.48	60.76/1,628	-	-	1.53	60.75/1,628
α-Cadinol	6.9	61.41/1,652	-	-	6.93	63.09/1,653
α-Humulene epoxide II	-	-	2.38	56.17/1,579	-	-
Palmitic acid	-	-	2.52	84.11/1,968	-	-

RT: retention times, RI: retention indexes, OVA: *Origanum vulgare* of the Armenian highlands.

tency of hind paw licking and/or shaking, or jumping, was recorded, and at this point the animal was immediately removed from the hot plate to avoid further damage and the next animal was placed on it.

9. The investigation of cytotoxicity and growth inhibition

The HeLa (human cervical cancer cells) and Vero cells (African green monkey's kidney epithelial cells; American Type Culture Collection, Manassas, VA) were used. Both cell lines were cultivated in Dulbecco's modified Eagle medium with 5% v/v fetal bovine serum (Gibco/Termo Fisher Scientefic, Rochester, MN), 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C in a humidified atmosphere containing 5% CO_2 (Memmert ICO105). The metabolic effects of OVA essential oil were assessed by methylthiazolyl-tetrazolium (MTT) colorimetric assay [25]. In all experiments both HeLa and Vero cells were seeded at 1 × 10^5 cells/mL (1 × 10^4/well of 96-well plate) a day before the MTT assay. Initial studies to test the influence of different concentrations of DMSO as a solvent of essential oil in the culture media of the HeLa cells were carried out. The 0.1% concentration of DMSO proved to be comfortable for the proliferation of both HeLa and Vero. In the first series of experiments, twenty-four hours after culture seeding, the media was replaced, and 100 μL of fresh media containing essential oil were added to each well at the following v/v ratios: 0.1, 0.25, 0.5, 1.0, and 2.0 μL/mL. The data of cytotoxicity was evaluated after twenty-four hours of essential oil addition (totally after 48 hours). In the second series of experiments, culture seeding in the media containing the essential oil was carried out at the same ratios: 0.1, 0.25, 0.5, 1.0, and 2.0 μL/mL and data of growth inhibition was evaluated after twenty-four hours.

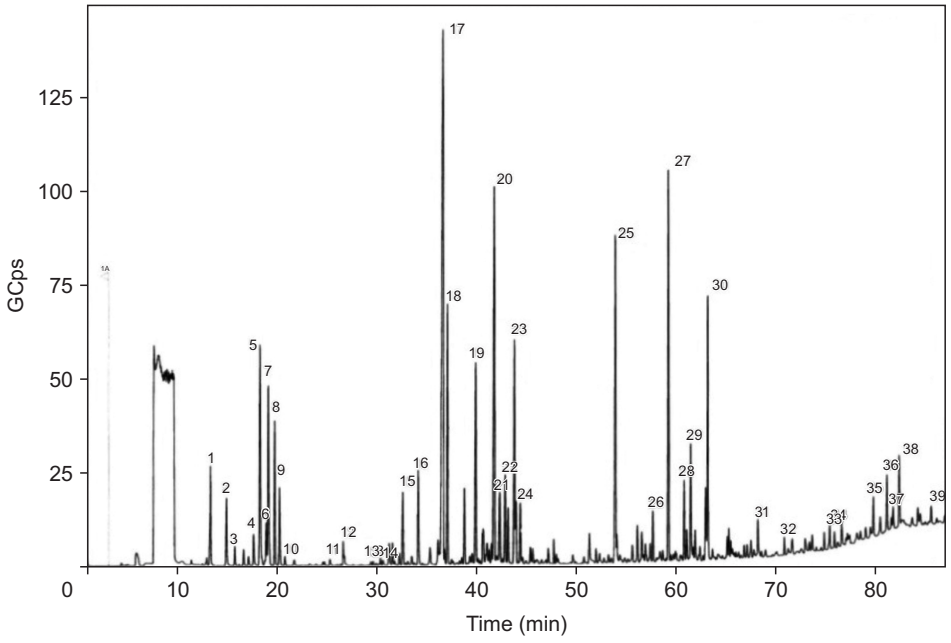

Fig. 1. GC-MS chromotograph of OVA essential oil in the period of pre-blossoming. 1: (+)-Sabinene, 2: β-Pinene, 3: α-Terpinolene, 4: Eucalyptol, 5: trans-β-Ocimene, 6: 1-Isopropyl-4-methyl-1,4-cyclohexadiene, 7: β-Ocimene, 8: Ethyl amyl ketone, 9: o-Cymene, 10: trans-β-Terpineol, 11: Neo-allo-ocimene, 12: 3-Octanol, 13: p-Menthan-3-one, 14: Elixene, 15: (-)-β-Bourbonene, 16: β-Linalool, 17: β-Caryophyllene, 18: L-4-terpineol, 19: α-Humulen, 20: D-Germacrene, 21: α-Muurolene, 22: γ-Elemene, 23: 1-Isopropyl-4,7-dimethyl-1,2,3,5,6,8a-hexahydronaphtalene, 24: (R)-(+)-β-Citronellol, 25: β-Caryophyllene epoxide, 26: d-Viridiflorol, 27: Ent-Spathulenol, 28: 1-Methyl-4-(methylethyl)-(E)-2-cyclohexenol, 29: tau-Cadinol, 30: α-Cadinol, 31: 6-Isopropyl-4,8a-dimethyl-1,2,3,5,6,7,8,8a-octahydro-2-naphthalenol, 32: Methyl 1,5,5-trimethyl-6-[(1E)-3-methyl-1,3-butadienyl]-7-oxabicyclo[4.1.0.]hept-2-yl ether, 33: trans-Phytol, 34: Diepicadrene-1-oxide, 35: (3E)-4-(1,5-Dihydroxy-2,6,6-trimethyl-2-cyclohexen-1-yl)-3-buten-2-one, 36: cis-Z-α-Bisabolene epoxide, 37: 3-[(2E)-2-Dodecenyl]dihydro-2,5-furandione, 38: Isoaromadendrene epoxide, 39: 2-Methyl-4-(2,6,6-trimethylcyclohex-1-enyl)but-2-en-1-ol. GC-MS: gas chromatography–mass spectrometry, OVA: *Origanum vulgare* of the Armenian highlands.

10. Statistical analysis

Data analysis was performed by Graph Pad Prism 8 software (Graph Pad Software Inc., San Diego, CA). The results of the formalin test observations at each minute were averaged both for the entire experimental period (45 minutes) and in 5 minutes intervals (for the analysis of dynamic changes in nociceptive behavior). One-way analysis of variance followed by the Bonferroni multiple comparison test was used for statistical analysis. Values of $P < 0.05$ were considered as significant. Results are given as mean ± standard error of mean (SEM).

RESULTS

1. Chemical composition of the OVA essential oil in different periods of vegetation: GC-MS analysis

Since most plants at different stages of vegetation differ in the quality and quantity of the chemical composition of essential oils, their determination in experimental mixtures is necessary. Depending on when the plant is collected, the essential oil in one case can demonstrate pronounced antibacterial properties, in another case analgesic properties, and the third case anti-cancer effects, *etc*.

2. Pre-blossoming

The results of the quantitative and qualitative analysis of essential oil obtained in the pre-blossoming period have shown that OVA essential oil contains 20 components with concentrations of more than 1.0% (total amount 74.99%). Another 125 components were found with concentrations lower than 1.0% (total amount 25.0%) (**Fig. 1**).

3. Blossoming

The results of the quantitative and qualitative analysis of essential oil obtained in the blossoming period have shown that OVA essential oil contains 20 components with concentrations of more than 1.0% (total amount 80.89%). Another 120 components were found with concentrations lower than 1.0% (total amount 19.0%) (**Fig. 2**).

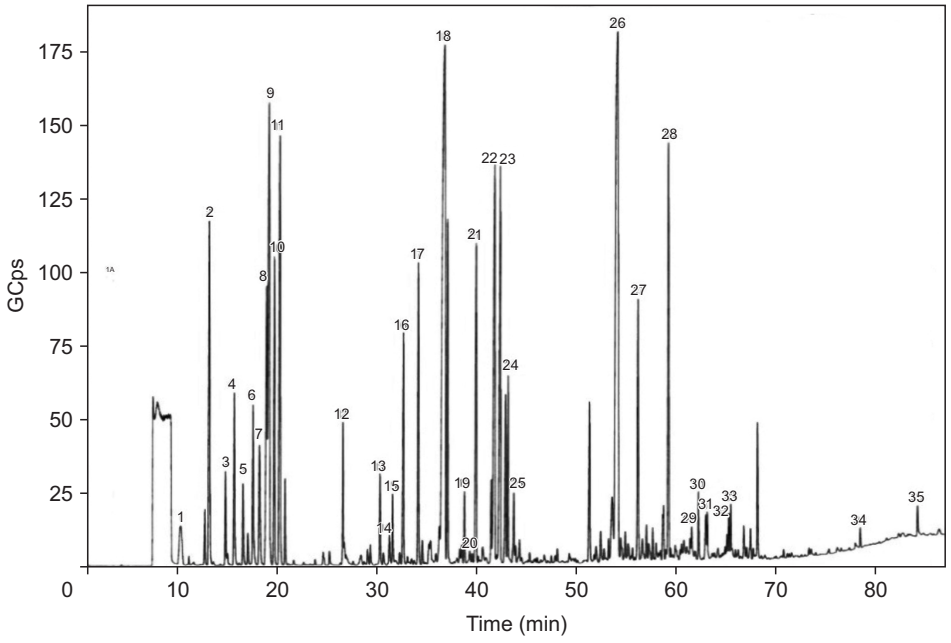

Fig. 2. GC-MS chromotograph of OVA essential oil in the period of blossoming. 1: β-Thujene, 2: (+)-Sabinene, 3: β-Pinene, 4: α-Terpinolene, 5: D-Limonene, 6: Eucalyptol, 7: γ-Terpinene, 8: Ethyl amyl ketone, 9: o-Cymene, 10: 4-methyl-3-(1-methylethylidene)-1-cyclohexene, 11: 2-Hexenal diethyl acetal, trans, 12: 3-Octanol, 13: cis-β-Terpineol, 14: alfa-Copaene, 15: Dihydroedulan II, 16: (-)-β-Bourbonene, 17: β-Linalool, 18: β-Caryophyllene, 19: L-4-terpineol, 20: Alloaromadendrene, 21: Humulen, 22: Germacrene D, 23: l-β-Bisabolene, 24: Elixene, 25: α-trans-Farnesene, 26: β-Caryophyllene epoxide, 27: α-Humulene epoxide II, 28: Ent-Spathulenol, 29: Isoaromadendrene epoxide, 30: Carvacrol/Isothymol, 31: α-Cadinol, 32: Ledene oxide-(II), 33: Tetracyclo[6.3.2.0(2,5).0(1,8)]tridecan-9-ol,4,4-dimethyl-, 34: Dibutyl phthalate, 35: Palmitic acid. GC-MS: gas chromatography–mass spectrometry, OVA: *Origanum vulgare* of the Armenian highlands.

4. Fruiting

The results of the quantitative and qualitative analysis of essential oil obtained in the fruiting period have shown that OVA essential oil contains 20 components with concentrations of more than 1.0% (total amount 84.38%). Another 101 components were found with concentrations lower than 1.0% (total amount 15.6%) (**Fig. 3**). The GC-MS analysis of OVA essential oil additionally showed that it contains acyclic sesquiterpenes, acyclic sesquiterpene alcohols, bicyclic sesquiterpenoids, tricyclic sesquiterpenoids, tricyclic sesquiterpene alcohols, monocyclic terpenoids, bicyclic monoterpenoids, acyclic monoterpene alcohols, monocyclic monoterpene ketones, monoterpene acids, and aromatic monoterpenoids. The dominant components of OVA essential oil during different vegetation periods are shown in **Table 1**.

The results of the GC-MS analysis of OVA essential oil showed that it contains acyclic sesquiterpenes, acyclic sesquiterpene alcohols, bicyclic sesquiterpenoids, tricyclic sesquiterpenoids, tricyclic sesquiterpene alcohols, monocyclic terpenoids, bicyclic monoterpenoids, acyclic monoterpene alcohols, monocyclic monoterpene ketones, monoterpene acids, and aromatic monoterpenoids. As can be seen, the content of essential oil components is quite variable during different stages of growth. For the major components (with contents more than 5% in the essential oil at different periods), the data is summarized in **Fig. 4**. The maximal amounts of β-caryophyllene and β-caryophyllene epoxide are present in the OVA essential oil in the blossoming period. Therefore, the essential oil of *O. vulgare* distilled in this period was chosen for further investigations.

5. Analgesic and anti-inflammatory properties of OVA essential oil: the formalin test

First, the formalin test for the intact mice was carried out. The analgesics were injected intraperitoneally (15 minutes or 30 minutes before the formalin intraplantar injection). The analgesic effects of Analgin and Diclofenac pretreatment 15 minutes before the formalin intraplantar injection are shown in **Fig. 5**.

It is known that only local anesthetics affect the acute pain phase [26]. Both Diclofenac and Analgin showed a significant pain relief effect in the second phase (16–25 minutes, $P = 0.028$). Then the analgesic effects of different doses of OVA essential oil were tested (**Fig. 6**). As can be seen from the data, the 4% OVA essential oil has the most effective analgesic effect, but there is a delay in the second phase, so it was decided to conduct a study of OVA essential oil antinociceptive action 30 minutes before the

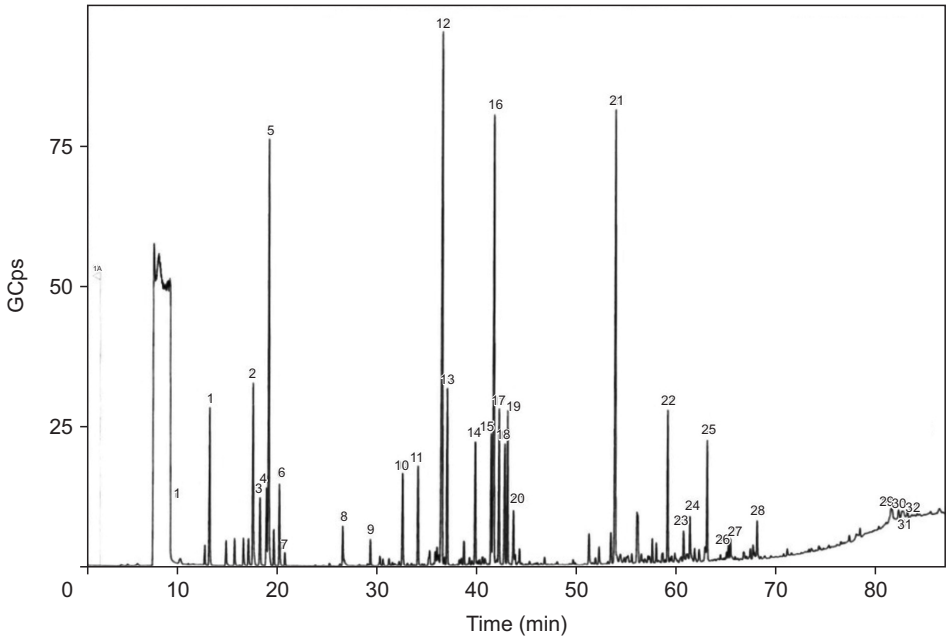

Fig. 3. GC-MS chromotograph of OVA essential oil in the period of fruiting. 1: Sabinene, 2: Eucalyptol, 3: trans-β-Ocimene, 4: γ-Terpinene, 5: β-Ocimene, 6: o-Cymene, 7: α-Terpinolene, 8: Ethylamylcarbinol, 9: Matsutake, 10: (-)-β-Bourbonene, 11: β-Linalool, 12: β-Caryophyllene, 13: L-4-terpineol, 14: α-Humulene, 15: α-Terpineol, 16: Germacrene D, 17: l-β-Bisabolene, 18: Elixene, 19: trans-α-Farnesene, 20: (+)-δ-Cadinene, 21: β-Caryophyllene epoxide, 22: Ent-Spathulenol, 23: tau-Cadinol, 24: 6-Isopropenyl-4,8a-dimethyl-3,5,6,7,8,8a-hexahydro-2-(1H)-naphthalenone, 25: α-Cadinol, 26: Tricyclo[5.2.2.0(1,6)]undecan-3-ol, 2-methylene-6,8,8-trimethyl, 27: Tetracyclo[6.3.2.0(2,5).0(1,8)]tridecan-9-ol,4,4-dimethyl, 28: trans-Z-α-Bisabolene epoxide, 29: 15,15`-Bi-1,4,7,10,13-pentaoxacyclohexadecane, 30: 3,6,9,12,15,18,21-Heptaoxatritriacontan-1-ol, 31: 3-Ethyl-5-(2-ethylbutyl)octadecane, 32: Isodecyl octyl phthalate. GC-MS: gas chromatography–mass spectrometry, OVA: *Origanum vulgare* of the Armenian highlands.

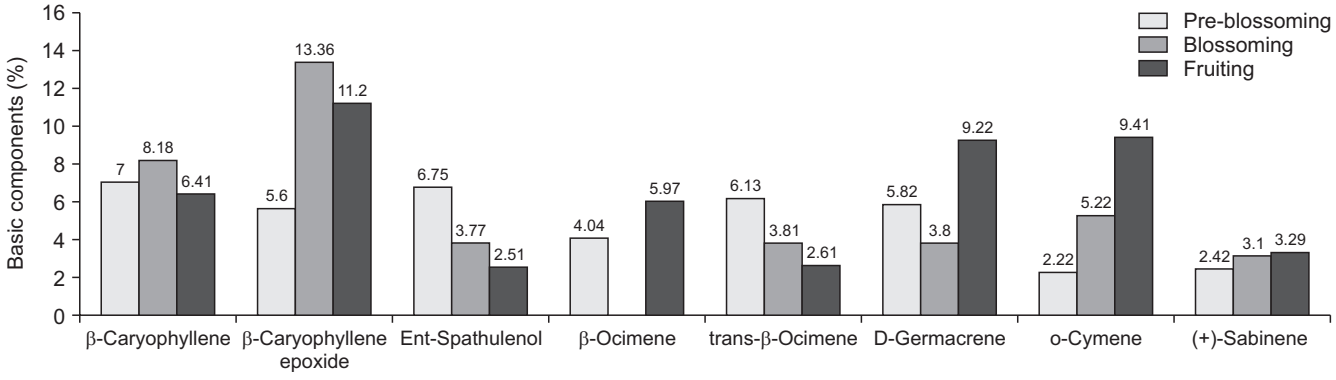

Fig. 4. The percentage of OVA essential oil dominant components in different vegetation periods. OVA: *Origanum vulgare* of the Armenian highlands.

formalin intra plantar injection. The results are shown in **Fig. 7**. We also performed a study of the analgesic effect of 5% OVA essential oil to determine the optimal dose, but after intra peritoneal injection of 5% OVA essential oil, the animals' condition had deteriorated markedly, which suggested that this dose probably had a toxic effect and was not suitable for provided study. Thus, according to our results, the 4% OVA essential oil (30 minutes before the formalin intraplantar injection) has the highest analgesic therapeutic potential, which may be compared with standard analgesics (**Fig. 8**). The significance of this data is given in **Fig. 9**.

6. Analgesic and anti-inflammatory properties of OVA essential oil: hot plate test

According to obtained data, there was no significant difference between the intact and experimental groups. So, the antinociceptive effect of OVA essential oil is not related to pain and heat sensitive TRPV1 receptors. The results are shown in **Fig. 10**.

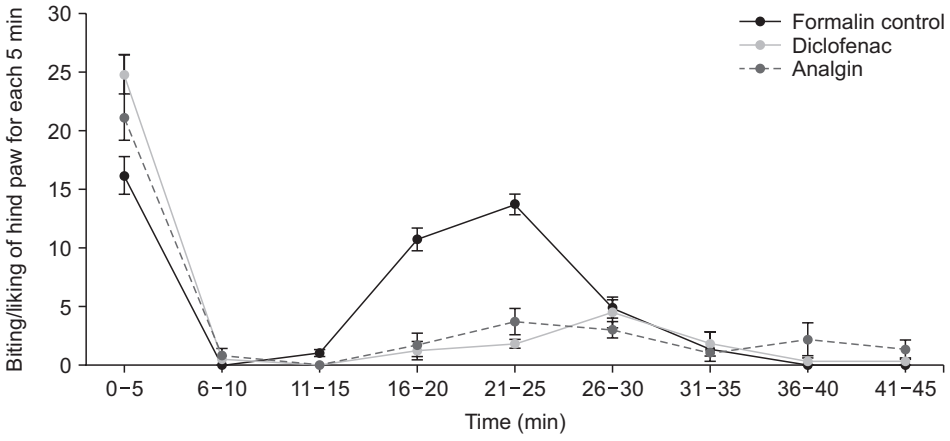

Fig. 5. The analgesic effects of Analgin and Diclofenac pretreatment (15 minutes) *vs.* formalin. The data represent mean ± standard error of mean.

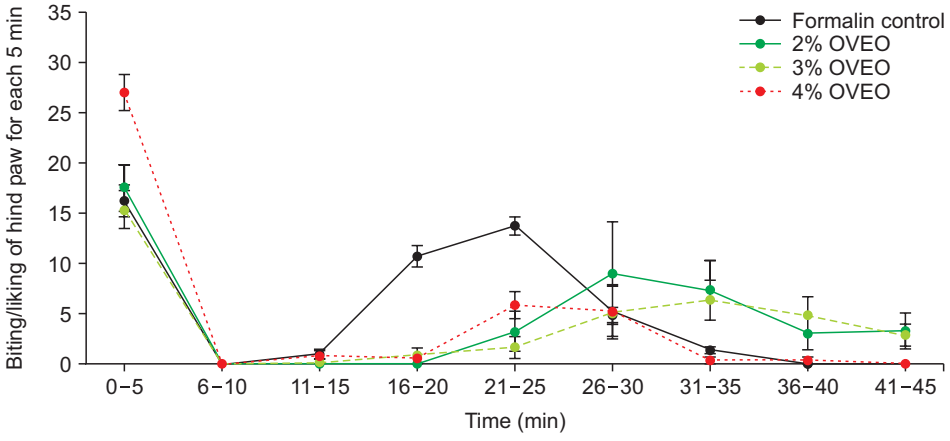

Fig. 6. The analgesic properties of various doses of OVA essential oil (OVEO), 15 minutes before formalin injection. The data represent mean ± standard error of mean. OVA: *Origanum vulgare* of the Armenian highlands.

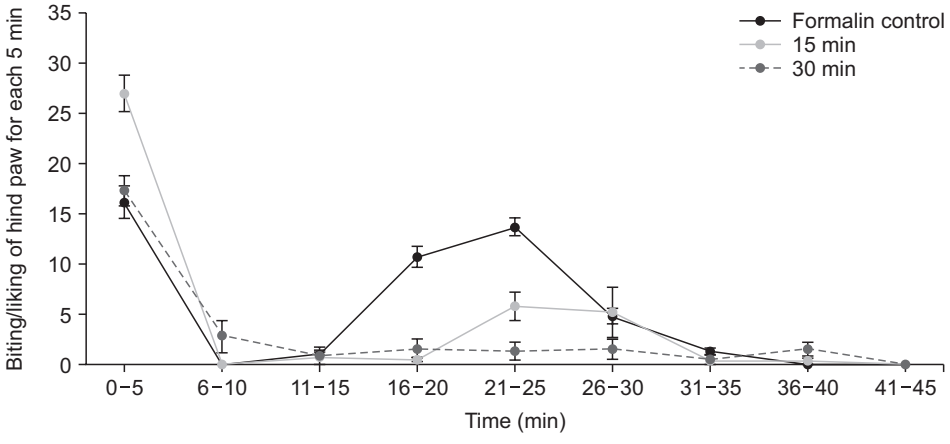

Fig. 7. The analgesic effect of 4% OVA essential oil 15 and 30 minutes before formalin injection. The data represent mean ± standard error of mean. OVA: *Origanum vulgare* of the Armenian highlands.

7. The cytotoxic properties of OVA essential oil: MTT assay

The essential oil of *O. vulgare* from the Armenian highlands may be a candidate for being a pain-killer drug, so its action on the survival and proliferation of cells is of great interest. The growth-inhibitory and cytotoxic properties of OVA essential oil diluted in cultural media by the MTT assay were investigated. Data are shown in **Fig. 11** and **Fig. 12**. According to our results, the 1.0–2.0 μL content of essential oil in each well (100 μL) showed a certain influence on both cancer and non-cancer cells. When cells were already attached, all doses showed about 30% of the cytotoxic effect of OVA essential oil both for cancer (HeLa) and non-cancer (Vero) cells. When cells were seeded in media with preliminarily added OVA essential oil, cancer

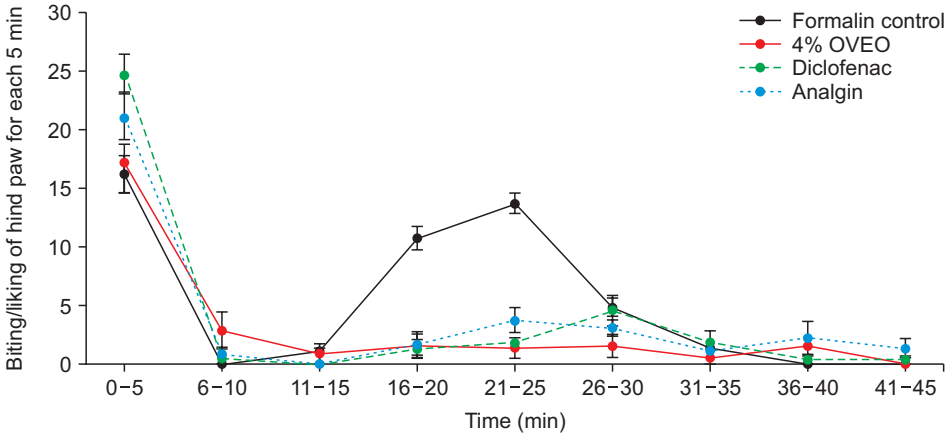

Fig. 8. Comparing the analgesic effect of 4% OVA essential oil (OVEO) with standard analgesics. The data represent mean ± standard error of mean. OVA: *Origanum vulgare* of the Armenian highlands.

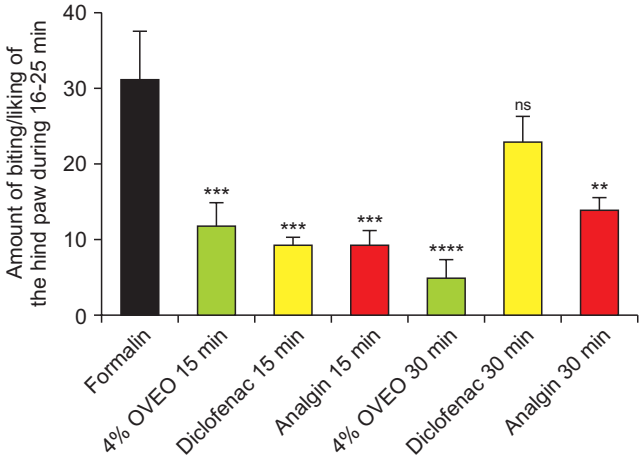

Fig. 9. Significance of obtained data in the second phase of the formalin test. The data represent mean ± standard error of mean ($P < 0.05$). OVA: *Origanum vulgare* of the Armenian highlands, OVEO: OVA essential oil, ns: not significant. **$P < 0.005$, ***$P < 0.0005$, ****$P < 0.0001$.

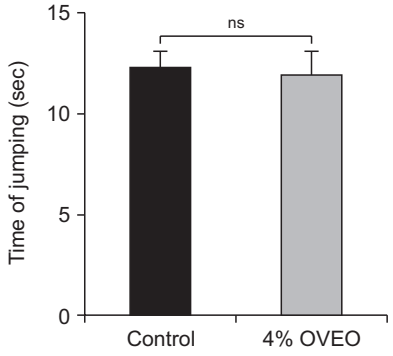

Fig. 10. Effect of the OVA essential oil in hot plate test in mice. Control (saline, 0.1 mL, intra peritoneal [IP]) and experimental (0.1 mL, 4% OVEO, IP) groups were injected 15 minutes before the behavioral test. The data represent mean ± standard error of mean. OVA: *Origanum vulgare* of the Armenian highlands, OVEO: OVA essential oil, ns: not significant.

cells (HeLa) showed significantly higher viability (about 60%, $P = 0.041$) than non-cancer (Vero) cells (about 35%), when growing in media with OVA essential oil (**Fig. 13**). So, OVA essential oil shows growth-inhibitory effect on both HeLa and Vero cells.

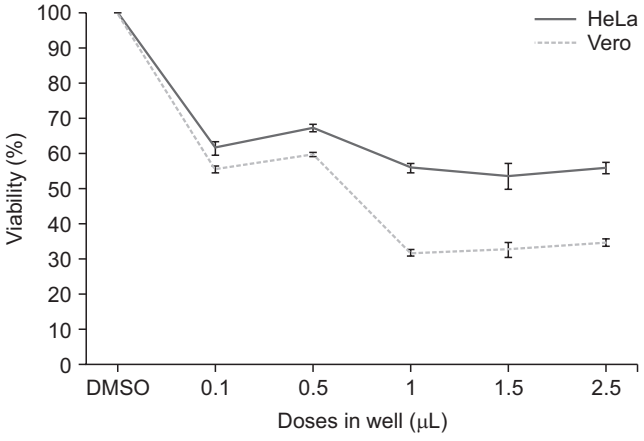

Fig. 11. The growth-inhibitory effect of OVA essential oil on HeLa and Vero cells viability (media with OVA essential oil preliminary addition). OVA: *Origanum vulgare* of the Armenian highlands. The data represent mean ± standard error of mean.

DISCUSSION

The results of the present investigation directly confirm that the essential oil is exposed to quantitative and qualitative changes depending on vegetation period of plant (**Fig. 4**). The amount of the essential oil of investigated aerial parts of the oregano of the Armenian highlands changes depending on differing hours of daylight, air temperature, amount of light received, moisture, and the intensity of solar radiation. The variability of scientific data is often defined by climatic features [27].

The results of our investigations showed that the wild *O.*

vulgare of Armenia belongs to the fourth chemotype (with the prevailing content of sesquiterpenoids and the very low quantity of phenolic compounds). Carvacrol, which has been discovered only in the blossoming period, was quantitatively equal to 2.38% and thymol was absent. The latter has been discovered in the fruiting period, but only in small amounts (0.01%), and in this case carvacrol was absent. It has been confirmed once more that the qualitative and quantitative composition of the essential oil of *O. vulgare* depends most on the geographical location of the plant's growing area. The yield and the quality of the essential oil of *O. vulgare* is defined by ontogenetic factors, external environmental conditions, individual development periods, the geographical position of the growing location, as is also described in the data in the literature.

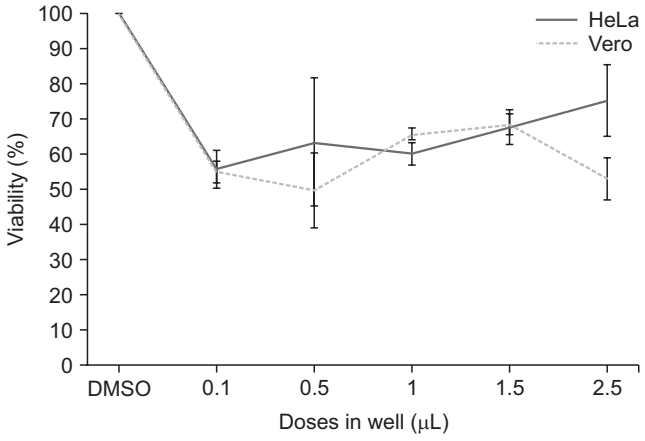

Fig. 12. The cytotoxic effect of OVA essential oil on HeLa and Vero cells viability (media without OVA essential oil addition). OVA: *Origanum vulgare* of the Armenian highlands. The data represent mean ± standard error of mean.

This certain correlation of the main components of the essential oil of Armenian *O. vulgare* with the prevailing content of sesquiterpenoids, such as β-caryophyllene and β-caryophyllene epoxide, gives accented possibilities for investigating it as an analgesic and anti-inflammatory agent in mice.

It was shown that essential oil expresses significant antinociceptive effects for all concentrations of OVA essential oil (2%, 3%, and 4%). As was mentioned above, a 5% solution of OVA essential oil had manifested slight signs of behavioral discomfort in tested mice and we decided do not to exceed the concentration of 4%, despite the fact that it is known that, for example, β-caryophyllene is not toxic even in much higher doses [28]. In our case, the injected effective dose of whole OVA essential oil was equal to 3.5 mg/mouse. This means approximately 286 μg/mouse in terms of β-caryophyllene (8.18%) and 467.6 μg/mouse in terms of β-caryophyllene oxide (13.36%). Based on scientific data it is known that β-caryophyllene's effect on pain-like behavior and its anti-inflammatory action is mediated by interaction with CB2 receptors and then β-endorphin release, which lead to activation of the opioid receptors. In contrast, β-caryophyllene oxide expresses its antinociceptive and anti-inflammatory effect through interaction with central pain receptors. In addition, it is known, that both β-caryophyllene and β-caryophyllene oxide decrease the release of inflammatory mediators of pain [15].

The formalin test (consists of Phase 1 [0–5 minutes] and Phase 2 [15–45 minutes]) produces painful behavior in animals. These phases are separated by a quiet phase called the interphase, in which nociceptive reactions decrease or disappear completely [29]. The formalin test allows investigation of nociceptive behavior in the time period (second phase of formalin action) when inflammatory processes

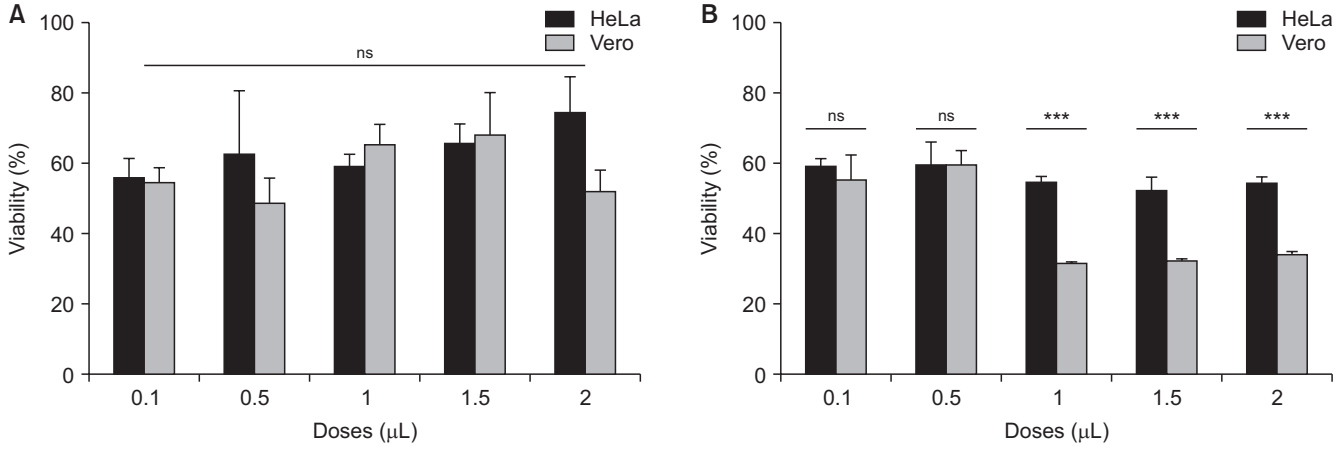

Fig. 13. Significance of cytotoxic effect of OVA essential oil. (A) The essential oil added after cell attachment. (B) The essential oil added before cell attachment ($P < 0.05$). The data represent mean ± standard error of mean. OVA: *Origanum vulgare* of the Armenian highlands, ns: not significant. ***$P < 0.0005$.

and inflammation-induced pain are developing. On the basis of these data, it was chosen as the preferable test for our investigations. The results obtained in the second phase of the formalin test suggested that the antinociceptive effect of OVA essential oil is related to the inhibition of the biosynthesis of pain mediators, such as prostaglandins and prostacyclins. In the hot plate test, it seems that OVA essential oil components did not interact with heat-sensitive TRPV1 receptors, and in this case we received a null result. Presumably, OVA essential oil exerts its analgesic effect by another route, probably the CB2 receptor-activating mechanism.

There are certain articles and reviews describing the anticancer properties of β-caryophyllene and β-caryophyllene oxide [15,30]. This study has shown the cytotoxic effects of OVA essential oil on a cancer cell line *in vitro*. The maximal tested dose was equal to 2.0 μL of OVA essential oil per well (100 μL), which was a 2% solution in cell media.

When OVA essential oil solution was added to the well after 24 hours of cultivation (with already attached cells), all investigated doses show the cytotoxic effect for cancer and non-cancer cells. When cells were seeded in media with preliminarily added OVA essential oil in the same concentration (2%), cancer cells showed a significantly higher viability (about 60%, $P = 0.030$) than non-cancer cells (about 35%). By contrast, OVA essential oil exerts a highly expressed and significant antinociceptive effect *in vivo*. In conclusion, OVA essential oil exerts a highly expressed and significant antinociceptive effect *in vivo*. So, the wild oregano herb of the Armenian highlands, harvested in the blossoming period, may be considered as a valuable source for developing pain-relieving preparations.

CONFLICT OF INTEREST

No potential conflict of interest relevant to this article was reported.

FUNDING

No funding to declare.

ORCID

Armenuhi Moghrovyan, https://orcid.org/0000-0002-1117-3690
Lilya Parseghyan, https://orcid.org/0000-0003-2838-6788
Gohar Sevoyan, https://orcid.org/0000-0002-3446-6314
Anna Darbinyan, https://orcid.org/0000-0002-6047-8714
Naira Sahakyan, https://orcid.org/0000-0002-7370-1380
Monica Gaboyan, https://orcid.org/0000-0002-3846-3866
Zaruhi Karabekian, https://orcid.org/0000-0002-8693-7288
Armen Voskanyan, https://orcid.org/0000-0002-4395-538X

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