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The possible anti-inflammatory properties of hydro-ethanolic extract of Oregano

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ABSTRACT

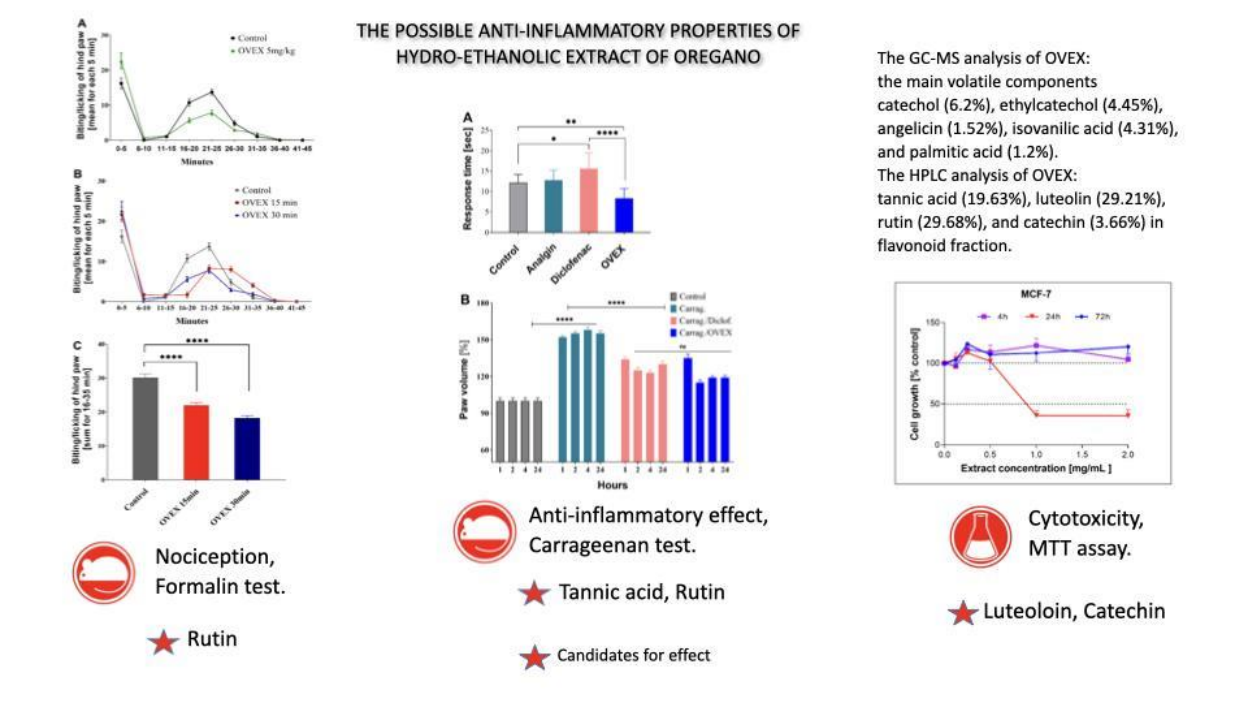
Backgrounds: Plant compounds are of great value due to their analgesic, anti-inflammatory, cytotoxic, and other characteristics. This study aimed to examine the chemical composition and impact of the *Origanum vulgare* hydro-ethanolic extract on nociceptive behavior and anti-inflammatory properties in *in vivo* mice models.

Methods: Gas chromatography-mass spectrometry and high-performance liquid chromatography techniques were employed to determine the major active phytochemicals of the extract. The effects of the extract on nociceptive reactions were evaluated using formalin and hot plate tests, while the anti-inflammatory effect was determined using the Carrageenan test. The methyl-thiazolyl-tetrazolium assay was applied to analyze growth-inhibiting properties of the extract on MCF-7 breast cancer cells.

Results: Various flavonoids and organic acids were identified in *O. vulgare* hydro-ethanolic extract. The extract possessed low cytotoxicity on MCF-7 cells as only 1 mg/mL or higher concentrations of the extract induced considerable growth inhibition. The formalin and hot plate tests demonstrated some sensitizing effects of the investigated extract (5 mg/kg) on mice. Moreover, the Carrageenan test revealed pronounced anti-inflammatory properties of the *O. vulgare* extract.

Conclusion: The potent biological activity of the wild oregano herb extract makes it a promising source for the development of medicinal preparations.

Keywords: Nociceptive behavior, anti-inflammatory effect, Oregano, flavonoids, chemical composition, cytotoxicity, *in vivo* mice models.



INTRODUCTION

The development of new biologically active preparations that have fewer harmful side effects remains a popular pursuit. Despite the challenge posed by the low bioavailability of plant-based compounds, they remain beneficial for treating and preventing a variety of illnesses [1,2].

Origanum vulgare L. (oregano) is a perennial herb that is indigenous to the Mediterranean region and western Eurasia [3,4]. It has been utilized in traditional medicine across several countries, including Armenia, for its preventive and therapeutic effects in bronchial and digestive disorders, colds, and stomach upsets [3,4]. Recent studies have also recommended its use in treating "civilization diseases" such as cancer, diabetes, and neurodegenerative disorders [3–6]. The aerial parts of the plant contain numerous medicinally beneficial volatile and non-volatile components, including essential oils (EO), phenolic substances, flavonoids, tannins, sterols, and high quantities of terpenoids [3,7,8]. The amount of bioactive substances present can vary depending on factors such as the location of harvest, collection time, the presence of different chemotypes within the same species, and other variables [7–9]. A study conducted by Moghrovyan *et al*. [7] identified more than 180 types of substances, primarily terpenoid in nature, in the EO of oregano grown in the high-altitude Armenian landscape. The EO of Armenian oregano is

primarily composed of different sesqui- and monoterpenes, including β-caryophyllene epoxide (13.3%), β-caryophyllene (8.2%), and o-cymene (5.2%) [7]. However, other sources have suggested that the primary constituents of oregano EO are carvacrol and thymol, which could be explained by cultivation conditions and other external factors [10,11]. On the other hand, oregano hydro-alcoholic extracts are predominantly composed of phenolic acid derivatives and flavonoids such as catechins and rosmarinic acid (HPLC analysis) [12].

In a previous study, we demonstrated the significant antinociceptive and anti-inflammatory effects of essential oil extracted from O. vulgare, sourced from the Armenian highlands, which was abundant in β-caryophyllene and β-caryophyllene [8]. High anti-inflammatory and pain-relieving activity of *O. vulgare* EO was shown in several other research works as well [13–16]. According to the available data within the literature, analgesic properties of Oregano essential oil is mainly due to carvacrol monoterpene contained in EO of Oregano [16]. Although high bioactive properties are within *O. vulgare* EO, it also exhibited high cytotoxicity and, therefore, its consideration for potential medical applications as pain-relieving and/or anti-inflammatory preparation is quite limited [8]. However, there was lack of data about analgesic and anti-inflammatory properties of hydro-alcoholic extract of the herb. We hypothesized that the hydro-alcoholic extract of *O. vulgare*, which may have lower cytotoxicity, could still exhibit antinociceptive and anti-inflammatory effects. Therefore, the present study aimed to explore the antinociceptive, anti-inflammatory, and cytotoxic properties of hydro-alcoholic extracts from O. *vulgare* harvested at the Armenian flora.

MATERIALS AND METHODS

Chemicals, reagents, and drugs*:* Sodium metamizole (Analgin, "Yerevan chemical-pharmaceutical firm" OJSC, Yerevan, Armenia) and sodium diclofenac (Diclofenac, Hemofarm A.D., Vršac, Serbia) were used as standard analgesic and anti-inflammatory drugs, respectively. The λ-carrageenan and all other chemicals and standards used in the study were purchased from Sigma-Aldrich Co. ltd.

Collection and extraction of plant material*:* *O. vulgare* L. aerial parts were harvested from Gegharkunik province (Armenia) (v. Lichk, 1940 m height above mean sea level, 40016'36" latitude N end 45022'77" longitude E) during the blossoming period (2021, July). The plant was identified at the Department of Pharmacognosy, Yerevan State Medical University in Armenia, the plant samples were stored (the voucher specimen number is ERE 191395) and can be requested from the Herbarium of the Institute of Botany, National Academy of Sciences of Armenia in Yerevan. Plant raw material was processed immediately by discarding organic and mineral mixtures, washing, and drying [17].

The treatment of the plant material was carried out according to the Quality Control WHO, 2011. The extraction was implemented using 50% ethanol as solvent as described [17]. The obtained dried extracts were stored at 4ºC until further use.

The qualitative and quantitative phytochemical characterization of the volatile compounds in O. vulgare extract by GC-MS method: The quantitative and qualitative investigation of the volatile extractive substances from oregano was carried out by the GC-MS method as described previously [8].

The quantitative chemical characterization of O. vulgare extract by HPLC technique: The quantitative study of the main constituents of plant extract was implemented by the High-performance liquid chromatography (HPLC) method as described by Tsaturyan et al. [18] in the

Laboratory of bioactive substance purification and certification SPC "Armbiotechnology" NAS RA, SNPO, using the Waters 2695 Separation Module (USA) HPLC device. As standards luteolin, rutin, quercetin, catechin, tannic, and gallic acids (purity 99%) were applied.

The analytical procedure for organic acids (oxalic, tartaric, citric, malic, succinic, and fumaric acids) was performed by applying the chromatographic conditions as previously described [18].

Cell cultures: Human breast cancer MCF-7 (ATCC HTB-22, Manassas, VA, USA) cells were maintained in EMEM (Minimal Essential Medium Eagle) medium supplemented with L-glutamine (2 mmol/L), sodium pyruvate (200 mg/L), fetal bovine serum (10%), 0.01 mg/mL human recombinant insulin, and antibiotics (100 U/mL penicillin and 100 µg/ml streptomycin). Cells were maintained at 37 °C under a humidified atmosphere with 5% CO_2 in S-Bt Smart Biotherm Incubator, (Biosan, Latvia) as described before [19]. MCF-7 cells were chosen considering the fact that they are well-established and widely used cell line models. Additionally, in our further studies, we plan to use *in vivo* rat models of breast cancer to asses both anti-inflammatory and anticancer properties of the oregano extract.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay: The MTT test was performed to assess the inhibition of growth of MCF-7 cells exposed for 4, 24, or 72 h to different concentrations (2, 1, 0.5, 0.25, and 0.125 mg/mL) of *O. vulgare* extract. Treatments were performed as four technical replicates. Three independent replicates of each treatment were performed. Cytotoxicity was expressed as percent growth inhibition of cells exposed to tested plant extract compared to control cells treated with the appropriate volume of solvent only (1% ethanol in the final mixture), whose growth was regarded as 100%.

Experimental animals: For *in vivo* experiments, male outbred albino mice weighing 20±2 g were sourced from L. A. Orbeli Institute of Physiology NAS RA. Animal maintenance was performed as indicated earlier [8]. 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). The number of experimental groups was 13 (6 animals in each).

The in vivo formalin test: The antinociceptive properties of *O. vulgare* hydro-ethanolic extract (OVEX) were assessed in mice through the formalin test following intraperitoneal (IP) injection [20,21]. Nociceptive behavior was assessed as described previously [8]. The selection of doses for standard medications was guided by widely-used protocols [22]. For initial assessment and selections of effective doses for further studies, different concentrations of *O. vulgare* dried extract for the experimental groups were tested ranging from 2.5 to 200 mg/kg. The saline solution of OVEX contained 1% DMSO. Each mouse was injected with the above-indicated aliquot of 0.1 mL [8]. Tested solutions were injected IP 15 or 30 minutes before formalin injection.

The in vivo hot plate test: The acute thermal pain was evaluated by conducting the hot plate test [21,23]. The dose of 5 mg/kg OVEX was injected (0.1 mL, IP) 15 minutes before to being transferred to the hot plate. The experimental procedure was described previously [8].

The in vivo carrageenan test: The carrageenan test was used for the determination of anti-inflammatory effect of the OVEX [24,25]. In the negative control group, 1% λ-carrageenan solution was injected into the central pad of the mouse hind paw (50 µL) and 0.1 ml saline solution

was injected IP, which resulted in excess swelling that reached maximum volume in 3–5 hours and decreased in 24 hours. In the positive control group, the mice were injected with 0.1 mL diclofenac (10 mg/kg, IP) immediately after the carrageenan IPL injection. The experimental animals were injected with 5 mg/kg of OVEX solution. The volume of the edema was measured using a digital plethysmometer (Ptm-Xn, Milton Enterprises, India) in 1, 2, 4, and 24 hours after the carrageenan injection.

Statistical analysis: The results represent as mean ± standard error or standard deviations of at least three independent experiments. All statistical analyses were carried out using GraphPad Prism 8 software (GraphPad Software, Inc., USA). A p-value of less than 0.05 was considered significant.

RESULTS

Chemical composition of O. vulgare extract: The GC-MS analysis showed that the main volatile components of *O. vulgare* extract were catechol (6.2%), ethyl catechol (4.45%), angelicin (1.52%), isovaleric acid (4.31%), and palmitic acid (1.2%). The HPLC analysis of *O. vulgare* extract revealed that the concentration of some well-known flavonoids with a plethora of biological activities was present in high levels. These compounds were: tannic acid (19.63%), luteolin (29.21%), rutin (29.68%), and Catechin (3.66%) in flavonoid fraction.

Cytotoxicity of OVEX by MTT assay: According to obtained data the 50/50 hydro-ethanolic extract of *O. vulgare* aerial parts has not exhibited noticeable growth inhibitory effect on MCF-7 cell line (Fig.1) at almost all tested concentrations and exposure times.

Considerable cytotoxic activity (about 60%) was found only at 24 h exposure time on the cells treated by quite high concentrations of plant extracts (started from 1 mg/mL). After the 4 and 72 h exposure time, the cell growth inhibitory activity of the extract was not detected even at the tested highest concentration (2 mg/mL). The IC50 values for MCF-7 cells were detectible only for 24 h exposure time and amounted to 1.629 mg/mL.

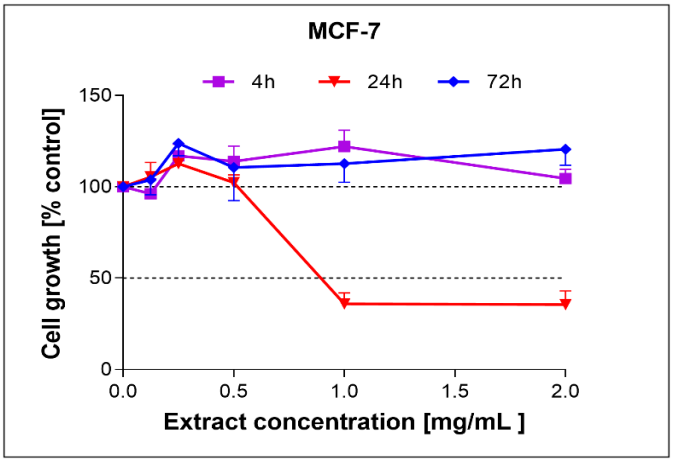


Figure 1. Inhibition of growth of MCF-7 cells treated by *O. vulgare* aerial part extract after the 4, 24, and 72 h exposure time. Results represent means ± SD from three independent experiments. SD values did not exceed 15%.

Analgesic action: The study had shown that comparatively high doses of OVEX (10 to 200 mg/kg) were toxic and caused disturbances in mice behavior. Therefore, 5 mg/kg, dose was chosen for further experiments. In the first phase of the formalin test, OVEX was injected 30 min before formalin injection. According

to obtained data OVEX at concentration of 5 mg/kg exhibited analgesic action (Fig. 2).

The sum of biting/licking amount during the second phase of the formalin test (inflammatory pain) showed a decrease in painful action of formalin after injection of 5 mg/kg OVEX (Fig. 2).

During previous studies on *O. vulgare* essential oil (OVEO) [8] we compared two versions of OVEO injection – 15 min and 30 min before formalin injection. Therefore, in the current study comparative tests with OVEX were done in the same manner. The selected dose of OVEX was tested for both 15 min and 30 min before formalin injection (Fig. 2). In the experimental group the slight antinociceptive effect was gained, which was expressed more in the case of OVEX IP injection in 30 min compared to 15 min before formalin IPL injection (OVEX dose was 5 mg/kg).

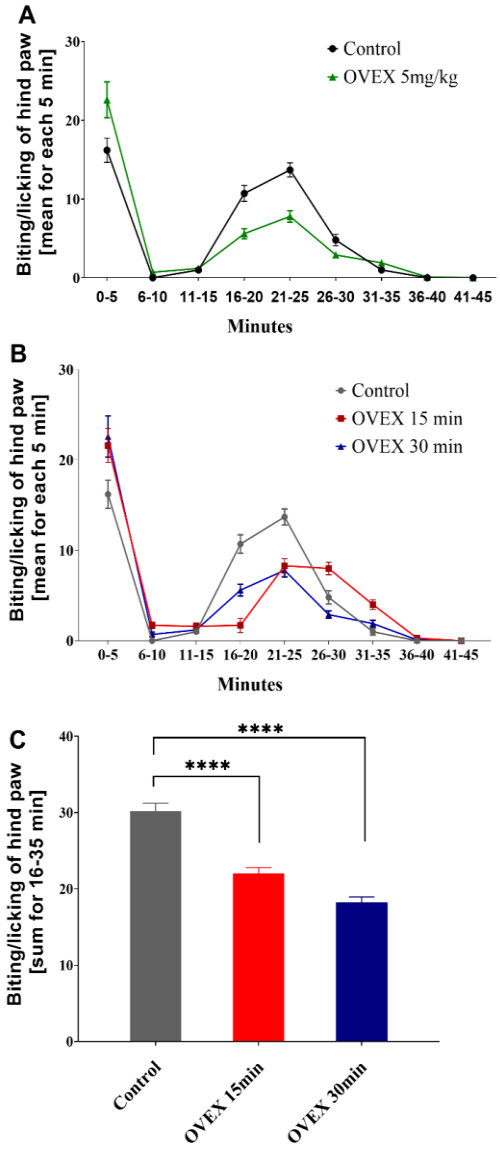


Figure 2. Formalin test results for biting/licking behavior after 30 min of OVEX injection (A) and for the different timing of injection (B), and the evaluation of various timing protocols for the administration of formalin in the second phase (C). In the C the administration of OVEX was carried out intraperitoneally at a dose of 5 mg/kg, with the onset of administration occurring either 15 minutes or 30 minutes before the intraperitoneal injection of formalin. n=6. Data represent mean ± standard error. Dunnett's multiple comparison tests were used for C. A level of $p<0.05$ was considered statistically significant. ****$p < 0.0001$.

The sum of biting/licking amount during the second phase of the formalin test (inflammatory pain), showed a more effective anti-pain behavior of 5 mg/kg dose OVEX IP injection 30 min compared to 15 min before formalin IPL injection (Fig. 2).

In the hot plate test experiments, the OVEX exhibited slight but significant sensitizing action in mice to thermal stimuli. The response (latent) time of experimental mice of escaping behavior was shorter (8.3 sec), than the same one for control animals (12.5 sec, Fig. 2).

The anti-inflammatory action of OVEX was compared with diclofenac. Both OVEX and diclofenac were injected IP immediately after the carrageenan IPL injection and volumetric data were obtained in 1, 2, 4, and 24 h after the injection (Fig. 3).

According to obtained data during the 24 h registration period, OVEX showed a significant anti-inflammatory effect.

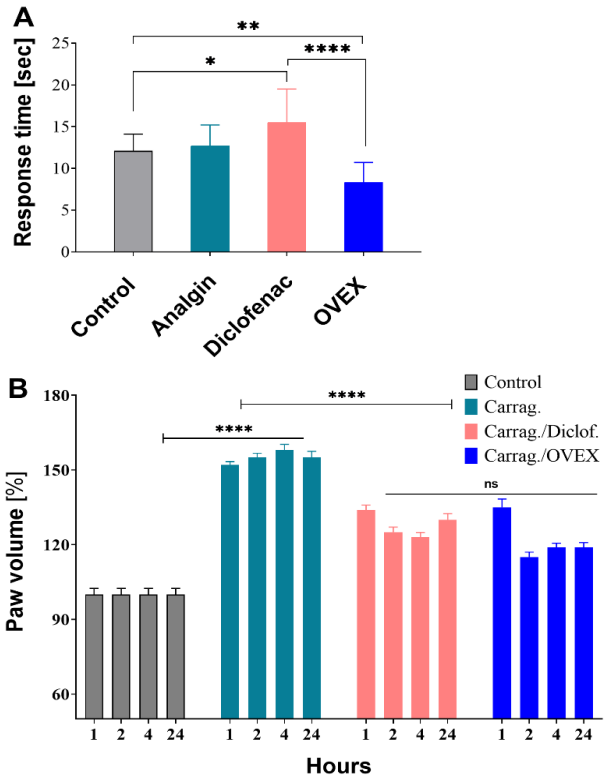


Figure 3. The comparative data for analgin, diclofenac, and OVEX solution effects in 5mg/kg dose in hot plate test (A, n=6) and Diclofenac and OVEX inhibition of inflammatory edema, evoked by carrageenan injection (B, n=9). Data represents mean ± standard error. Tukey's multiple comparison tests were used for A (One-way Anova) and B (Two-way Anova). A level of $p<0.05$ was considered statistically significant. *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$, NS: not significant.

DISCUSSION

In our previous research work strong antinociceptive and anti-inflammatory effects of *O. vulgare* essential oil was reported [8]. However, promising biological activities of OVEO was accompanied with strong cytotoxicity, making it difficult to consider for practical applications. The high cytotoxicity of OVEO was also confirmed by literature data. For instance, Elansary et al. [26] showed strong cytotoxic activity of *O. vulgare* essential oil against MCF-7 cell line (IC50 value was 8.11 µg/mL). Therefore, the cell growth inhibiting, antinociceptive and anti-inflammatory effects of 50/50 hydro-alcoholic crude extract of *O.*

vulgare were explored in the current study in order to assess its potential as an antinociceptive and anti-inflammatory preparation.

Our initial hypothesis was accurate as the OVEX possessed significantly lower cytotoxicity compared to OVEO. Hydro-ethanolic extract of aerial part of *O. vulgare* herb collected from Armenia exhibited low cytotoxicity in *in vitro* MTT assay. The IC50 value of OVEX was 1.629 mg/mL compared to the 8.11 µg/mL IC50 value of OVEO. Despite this, there are several reports showing considerable cytotoxic activity of *O. vulgare* alcoholic extracts against different cell lines [3,8,26–29]. For example, considerable cytotoxic effect of methanol extract of *O. vulgare* on HCT-116 cells was found, whereas it exhibited significantly lower cytotoxicity on MDA-MB-231 cells (IC50 value was about 500 µg/mL after 24 h exposure) [27]. Marrelli *et al*. [28] also showed considerable growth inhibitory activity of *O. vulgare* hydro-alcoholic extract (at 100 µg/mL concentration for 24 h exposure time) on MCF-7, HepG2 (hepatic cancer) and LoVo (colorectal cancer) cell lines. The differences in cytotoxicity of various *O. vulgare* plant samples can be due to the chemical composition of samples collected from different regions as well as extraction methods. In previous research, we have shown significant differences in the chemical composition of essential oils between Armenian and Turkish *O. vulgare* plants [7].

Some literature data indicates the antinociceptive effect of oregano extract in dose-dependent manner in formalin and other tests [29]. However, in our investigations we got opposite results: unlike the essential oil, the water-alcohol extract of oregano did not show significant analgesic effect. Moreover, it caused some weak sensitization of peripheral afferent receptors, in particular TRPV1 channels, as can be seen from the studies of the Hot Plate and the formalin test. Possibly, this kind of effect can be associated with the chemical composition of OVEX. On the other hand, OVEX extracted from plants harvested from Armenian highlands contains various valuable metabolites which can be useful for the application for other purposes. For instance, angelicin is a furanocoumarin, and it can be used as an anticancer agent and local analgesic [30]. The presence of isovanilic and palmitic acids can be responsible for anti-inflammatory and antioxidant effects of *O. vulgare* extract [31]. HPLC analyses allowed for the determination of other major active components, all of which are responsible for the high antioxidant and antibacterial capacity of OVEX reported previously [1,7].

Although OVEX did not express considerable antinociceptive properties, thd carrageenan test revealed anti-inflammatory activity of the extract. This property raises its potential application probability in the development of different preparations with anti-inflammatory, anti-microbial, and antioxidant properties in the auxiliary therapy of different diseases.

Phytochemical characterization of bioactive phytochemicals containing in OVEX confirming its potential possible medical applications. Previously, we showed that the percent yield of the extractive substances of *O. vulgare* aerial parts during the blossoming period was 31% [7]. Moreover, based on our previous report [7] predetermined flavonoid content in the extract, was 3.9 % by catechin equivalents, the total flavonoid content by luteolin equivalents was 3.8%, and the content of total tannins was 19%. In the current study we showed high content of tannic acid, luteolin, rutin, and catechin in the extract. These compounds expressed strong bioactive properties based on literature data. For example, tannic acid was described to possessing antiviral, antibacterial, antioxidant, and anti-inflammatory properties [32–34]. Luteolin is well known as an antioxidant, anti-inflammatory, antimicrobial, anticancer, and a cancer chemo-preventive agent [35,36]. Based on the literature, rutin exhibits anti-nociceptive, antioxidant, anti-inflammatory, neuroprotective,

antidiabetic, and anticancer activities [37,38]. Many authors [39–41] report about high and antioxidant and anticancer properties of catechin, one of the other major constituents of OVEX.

CONCLUSION

Thus, although the aerial part hydro-alcoholic extract *O. vulgare* herb growing in Armenia did not possess considerable antinociceptive properties, however, it has low cytotoxicity compared to the essential oil of the herb. Moreover, *O. vulgare* extract exhibited significant anti-inflammatory properties as evidenced by the carrageenan test. It also exhibited high antioxidant and antimicrobial properties shown earlier. Therefore, the remarkable biological activities displayed by the extract derived from wild oregano herb hold great promise for its utilization in the development of medicinal formulations, which could serve as an auxiliary therapy for different diseases.

Abbreviations: OVEX - *O. vulgare* hydro-ethanolic extract; OVEO - *O. vulgare* essential oil; IP – intraperitoneal; IC50 - Half maximal inhibitory concentration; MTT - 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MCF-7 - human breast cancer; EO - essential oil; GC–MS - Gas chromatography–mass spectrometry; HPLC - High Performance Liquid Chromatography; FC - Folin–Ciocalteu; GA - gallic acid; DMSO - Dimethyl sulfoxide.

Authors Contribution: All authors contributed to the study's conception and design. LP, AV, AM, MG, NS and AD, carried out the investigations and analyzed the outcomes. LP, AV, MG and NS wrote the manuscript. AV, NA and NS directed the experiments, corrected, and edited the manuscript. All authors revised and accepted the final version of the manuscript.

Competing Interests: The authors declare no conflict of interest.

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All authors contributed to the article formation equally. The final version of the manuscript was revised and approved by all authors.

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