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RESEARCH ARTICLE

Origanum vulgare L.: *In vitro* Assessment of Cytotoxicity, Molecular Docking Studies, Antioxidant and Anti-inflammatory Activity in LPS Stimulated RAW 264.7 Cells

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ARTICLE HISTORY

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Abstract: ***Background*:** Inflammation involves a dynamic network that is highly regulated by signals that initiate the inflammation process as well as signals that downregulate it. However, an imbalance between the two leads to tissue damage. Throughout the world, inflammatory disease becomes common in the aging society. The drugs which are used clinically have serious side effects. Natural products or compounds derived from natural products show diversity in structure and play an important role in drug discovery and development.

***Objective*:** *Oreganum Vulgare* is used in traditional medicine for various ailments including respiratory and rheumatic disorders, severe cold, suppression of tumors. The current study aims to evaluate the anti-inflammatory potential by evaluating various *in vitro* parameters.

***Methods*:** Inflammation-induced in macrophages *via* LPS is the most accepted model for evaluating the anti-inflammatory activity of various plant extracts and lead compounds.

***Results*:** The extracts (OVEE, OVEAF) as well as the isolated compound(OVRA)of *Oreganum Vulgare* inhibit the pro-inflammatory cytokines (IL-6 and TNF-α) and NO without affecting cell viability.

***Conclusion*:** Our study established that the leaf extracts of *Oreganum vulgare* L. exhibit anti-inflammatory activity and thus confirm its importance in traditional medicine.

Keywords: Phytoconstituents, IL-6, LPS, traditional medicine, inflammation, anti-inflammatory.

1. INTRODUCTION

Inflammation is a combination of highly regulated sequences of events provoked by a variety of stimuli, which include microbial, allergic, metabolic, autoimmune, constitutive, and physical factors. The sequence of events is distinguished by five classical inflammatory signs, including redness, pain, swelling, and heat, as described by Aulis Cornelius Celsius. The fifth sign, loss of function, was later on added by Virchow during the 19th century. The response to inflammation occurs in vascular connective tissue, including circulating cell, plasma, and blood vessels, which corresponds to the recruitment of leukocytes, increased vascular permeability, and enhanced microvascular caliber followed by the secretion of various inflammatory mediators [1-7]. As inflammation is the body's primary response to various harmful stimuli involving the innate and adaptive immune system, healing of damaged organs takes place. However, this physiological side of inflammation relies on the presence of endogenous suppressors of pro-inflammatory signaling pathways [8]. When physiological suppressors fail, uncontrolled inflammation leads to necrosis, fibrosis, apoptosis, and finally, organ destruction [9]. So the inflammation, after accomplishing its function, must be terminated to prevent damage to tissues as the persistence of this process may lead to chronic inflammation, which is associated with various chronic diseases including asthma, atherosclerosis, arthritis, ulcerative colitis, Crohn's disease and even cancer [10-14]. Chronic inflammation is a type of innate immunity that involves macrophages, which played an important role in the initiation, progression, and remission of inflammation. Inflammatory cytokines like TNF-α, interferon-gamma, and bacterial lipopolysaccharides are mainly involved in the activation of macrophages apart from various inflammatory me-

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diators, including nitric oxide (NO), reactive oxygen species (ROS), Prostaglandin E_2, TNF-α, and IL-6 [15]. Lipopolysaccharide (LPS), an endotoxin present in gram-negative bacterial cell wall, stimulates macrophages to release large amounts of pro-inflammatory cytokines such as TNF-α, IL-6 as well as inflammatory mediators NO and PGE_2. So for the treatment of various inflammatory disorders, the suppression of macrophage activation may have a valuable therapeutic potential. Therefore, inflammation-induced in macrophages via LPS is the most accepted model for evaluation of the anti-inflammatory activity of various plant extracts and lead compounds along with their mechanism. For the treatment of various inflammatory diseases, many types of drugs such as nonsteroidal anti-inflammatory drugs(NSAIDs), corticosteroids, immunosuppressants and biologicals are used. However, all these drugs possess serious adverse effects in which gastrointestinal, renal, and cardiovascular toxicity are the most common [16, 17]. Due to these side effects associated with the currently available anti-inflammatory drugs, there is a need for the development of novel anti-inflammatory drugs. Plants offer a valuable source of novel bioactive secondary metabolites, Besides natural products play a predominant role in the development of drugs for treating human disease [18, 19]. So currently, researches are mainly confined to identify plant-based molecules to treat various disorders. In this direction, we have investigated the mechanism behind the anti-inflammatory of *Origanum vulgare*, which has been reported to possess various therapeutic activities including body pain, blood pressure, microbial infection, toothache, Rheumatism, Pyrexia, antiseptic, germicidal, and cardiac stimulant [20-26].

Origanum vulgare, also known as flavoring herb, belongs to the family labiate. The herb is native to the Mediterranean and European regions. In India, the herb mainly grows in the Himalayan region from Kashmir to Sikkim at an altitude of 1600-3800m [27-30]. *Origanum vulgare* is a perennial herb that grows 70-100 cm in height. The leaves of the herb are ovate and the flowers are pale white to pink in color [31-33]. The herb is widely used in traditional medicine due to its various health effects, including digestive and bronchial disorders, for relieving cold, respiratory and rheumatic disorders, for the suppression of various tumors [34-38]. The current study aims to evaluate the anti-inflammatory potential by evaluating various *in vitro* parameters. Preliminary phytochemical investigation of the ethanolic leaf extract, as well as the respective fractions, was performed. No attempts have been made yet to study in detail the *in vitro* anti-inflammatory activity of *Origanum vulgare* to the best of the author's knowledge. Keeping this into consideration along with its medicinal importance, the current study aims to study the anti-inflammatory activity of ethanolic (OVEE), ethyl acetate extract(OVEAF), as well as the isolated compound (OVRA) of *Origanum vulgare* for its anti-inflammatory property.

2. MATERIALS AND METHODS

2.1. Chemicals

In the current study, all the chemicals, as well as reagents, are of high purity. Dulbecco's eagle medium (DMEM) and phosphate buffer saline (PBS) were purchased from Sigma, UK. ELISA kits, IL-6, Human TNF-α were purchased from Invitrogen (USA). From GIBCO USA, fetal bovine serum was obtained and lipopolysaccharide (LPS) *E. Coli* was taken from callbiochem (USA). Griess Reagent was purchased from Promega (USA) and MTT from Calbiochem (San Diego CA). Besides, other chemicals that were used in the study were of research-grade. From the central drug house(CDH), ferric chloride, glacial acetic acid, and chloroform were purchased. Ascorbic acid, ethanol, methanol, picric acid, calcium chloride, sodium chloride, 2,2 diphenyl picryl hyradyzl (DPPH), sucrose, and trichloroacetic acid were procured from Merk. From Qualigens, potassium dihydrogen phosphate, hydrogen peroxide, and hydrochloric acid were purchased. Ferric nitrate, ethylene diamine tetraacetate, potassium chloride, sodium hydroxide, dimethyl sulfoxide, and sodium dihydrogen monophosphate were purchased from Himedia. From Sisco research laboratories (SRL), ascorbic acid was purchased and from Rankem along with ethyl acetate and hexane.

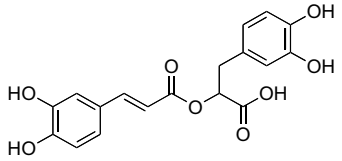


Fig. (1). Chemical structure of Rosmarinic acid.

2.2. Plant Material and Preparation of Extract

The leaf part of *Origanum vulgare* was procured from Srinagar (Kashmir), India in September 2018 and was identified and authenticated by Akthar Ahmad (department of taxonomy University of Kashmir) with voucher specimen number 2696-(KASH). The leaves were immediately shade dried and powdered with the help of a pulverizer. The powdered material (4kg) was extracted with ethanol (3 times, 4L each) at 25º C for 72 hours. The extract was filtered via Whatman filter paper and the filtrate obtained was concentrated in a rotary vacuum evaporator to get the dried extract (yield 9.47%). Through liquid-liquid fractionation of the ethanolic extract(OVEE), ethyl acetate (OVEAF) was prepared and both were dried on a rotary vapor. For further use, these dried extracts were stored at -20ºC.

3. EXPERIMENTAL

3.1. Isolation And Characterization of Compounds from Ethyl Acetate Fraction

The ethyl acetate extract fraction (OVEAF, 25g) was subjected to sub-fractionation using vacuum liquid chromatography(VLC) on silica gel (60-120) which was packed in chloroform and run in chloroform with increased amounts of methanol (from 0 to 100%) and finally washed with 100% methanol (each 500ml). A total of 8 fractions were collected (each 250ml) and were evaluated on TLC using solvent system chloroform: methanol: formic acid in different ratios (7:2.5:0.5, 5.5:3.5:1). The chromatograms were derivatized with NP reagent. Fractions which showed similar TLC profile were combined to obtain four (F1, F2, F3, F4) pooled fractions. The combined fractions F3 and F4 having similar TLC profile (7 gr) were chromatographed onto a Sephadex

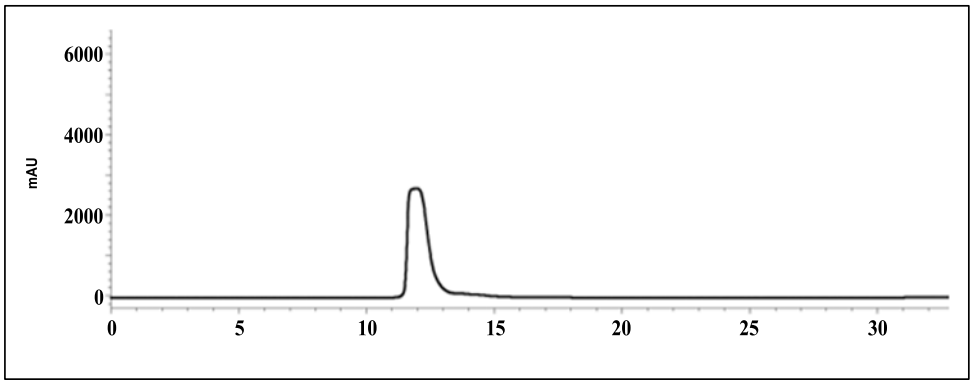


Fig. (2). HPLC Purity profile of Rosmarinic acid (OVRA) DAD: Signal B, 254 nm/Bw:16 nm).

LH-20 column (70 × 2.5 cm) eluting with methanol (100%), and after repeated chromatography on a Sephadex LH-20 (50 × 2 cm) column with methanol (100%) as an eluent, Rosmarinic acid(OVRA) (Fig. **1**) ($C_{18}H_{16}O_8$) white amorphous powder was obtained with percentage purity more than 95% as shown in (Fig. **2**). ESI-MS m/z 361 $[M+H]^+$:^{1}H-NMR (DMSO-d_6) 6.44 (1H, d, J = 15.8 Hz), 7.05 (lH, *b*s), 7.00 (1H, bs), 6.77 (1H, d, J = 8.1 Hz), 6.68 (lH, *b*s), 6.63 (1H, d, J = 7.88 Hz), 6.52 (lH, d, J = 7.72 Hz), 6.23 (lH, d. J = 15.9 Hz), 5.02 (1H, dd, J = 8.52, 3.48 Hz), 2.99 (1H, dd, J = 3.04, 14.08 Hz), 2.88 (1H, dd, J = 14.1, 9.04 Hz); ^{13}C-NMR (DMSO-d_6) 171.4, 165.8, 148.4, 145.4, 145.4, 144.7, 143.7, 127.7, 125.2, 121.3, 119.8, 116.5, 115.6, 115.2, 114.6, 113.5, 73.4, 36.2.

3.2. Antioxidant Activity

3.2.1. ABTS Radical Cation Scavenging Activity

ABTS [2,2-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)] scavenging activity of the (OVEE), (OVEAF) and compound (OVRA) was evaluated according to radical cation decolorization assay [39]. To generate radical cations for the assay, aqueous solutions of potassium sulfate (2.45 mmol/l) and ABTS (7 mmol/l) in the ratio of 1: 1 were mixed to prepare the final solution and then kept at room temperature for at least 6 h under dark conditions. The stock solution of ABTS was further diluted with ethanol followed by equilibration at 30^0 C. With the help of Ascorbic acid as a reference standard, aliquots of (OVEE, OVEAF) extract, and (OVRA) compound (0.1ml) of different concentrations (20,40,60,80,100 µg/ml) were mixed with diluted radical cation solution(2.9ml) of ABTS and kept under incubation at 30^0 C for 20 minutes and absorbance was measured at 734nm. The following formula was used to calculate the ability of OVEE, OVEAF, and OVRA to reduce ABTS cation radical.

$$\text{Scavenging } \% = \frac{(Ac - Aa)}{Ac} \text{ X } 100$$

A_c = Absorbance of control, A_a = Absorbance of the sample.

3.2.2. DPPH Free Radical Scavenging Activity

The free radical scavenging effects of OVEE, OVEAF, and OVRA were determined by measuring their free radical effects using DPPH (1,1-diphenyl-2-dipicrylhydrazyl free radical [40]. In this experiment, the working concentration of DPPH (1 mg/mL), Ascorbic acid (1 mg/mL), and test sample (10 mg/mL) was prepared. The given protocol was followed for making aliquots of 1 ml of the desired concentration (5-100 µg/mL) of the test sample with methanol and DPPH and incubated the mixture for 15 min at room temperature in the dark. The discoloration of DPPH was taken as the indicator of antioxidant activity. The optical density was recorded at 517 nm using an ELISA reader (Multiscan Spectrum; Thermo Electron Corporation, USA). All the samples were investigated in triplicate. The percentage of DPPH radical scavenging activity was determined by using the following formula.

$$\% \text{ Scavenging activity} = \frac{(\text{Ac} - \text{As})}{\text{Ac}} \text{ X } 100$$

At = Absorbance of sample; Ao = Absorbance of control

3.3. Cells and Cell Culture

RAW 264.7 mouse murine macrophage cell line was procured from ATCC, Manassas, VA, USA, and cultured in Dulbeccos eagle's medium (DMEM, Sigma, UK) and is supplemented with fetal bovine serum 10% and 1% of streptomycin and penicillin. At 37^0 C, the cells were incubated in a humidified incubator along with 5% CO_2.

3.4. Measurement of Cell Viability by MTT Assay

The viability of cells was determined by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) reduction assay [41]. In brief, RAW 264.7 cells at a density of 16000 cells /well were seeded into 96-well plates and were allowed to adhere in a CO_2 incubator at 37 ^{0} C for a time period of 24 h. After 24 h of incubation, the cells were treated with different concentrations of extracts (0-100µM/ml) and isolated compound with or without LPS for another 24 h. Afterward, 20µL of MTT (0.5mg/ml in PBS, PH 7.4) was added and kept for incubation for another 4h at 37 ^{0} C. Finally, the supernatant was removed and 100 µL of dimethyl sulfoxide (DMSO) was added to each well to dissolve the formazan crystals which form after the addition of MTT. The absorbance was measured by using the Synergy Mx plate reader at 570 nm. The results were expressed as a percentage of cell viability by using LPS induced group as a

control group. Three replicates were carried for each treatment.

3.5. NO Assay

For the evaluation of NO production in RAW 264.7 cells, the concentration of nitrite was measured in the supernatant as an indicator using the Griess reaction. In brief, RAW 264.7 macrophage cells (2 x 10^5 cells/well) were treated with different concentrations of extracts as well as the isolated compound in the presence or absence of LPS (1ug/ml) 1 h before LPS treatment and then kept in incubation for 24 h. Dexamethasone in different concentrations was used as a positive control. After the incubation period, the supernatant (100µL) was collected by centrifugation at 1000 rpm and mixed with Griess reagent (0.1% N-1- naphthyl ethylenediamine dihydrochloride and 1% sulphanilamide in 5% phosphoric acid) and kept for incubation for 10 minutes at room temperature in the dark. By using the Synergy Mx Plate reader, the absorbance was measured at 540 nm. With respect to the standard concentration curve of sodium nitrite ($NaNo_2$), the concentration of nitrite was calculated [42].

The percentage inhibition of NO was calculated with the following formula:

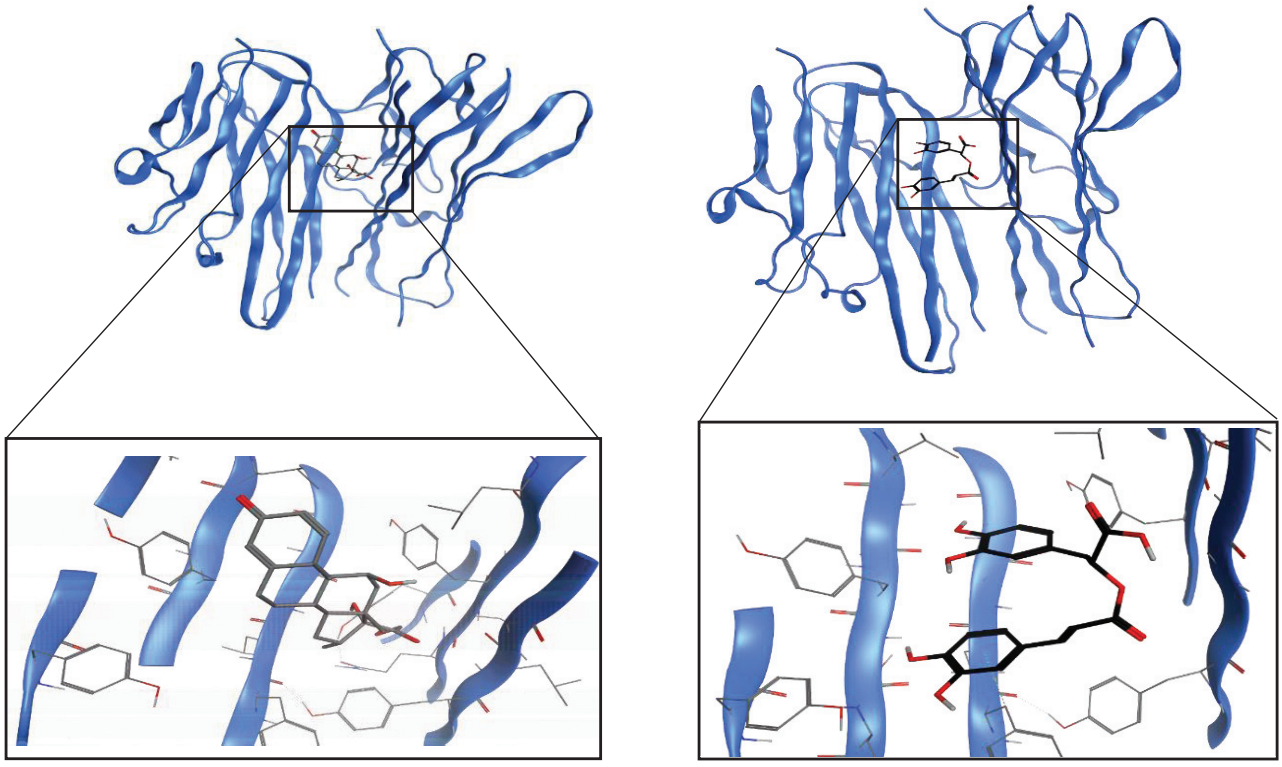
$$\text{NO inhibition (\%)} = \frac{(NO_2)\ \text{control} - (NO_2)\ \text{sample}}{(NO_2)\ \text{control}} \text{ X } 100$$

3.6. Determination of Production of Cytokines in RAW 264.7 Cells

The inhibitory effect of OVEE, OVEAF, and OVRA on the production of cytokines (TNF-α, IL-6) was determined by enzyme-linked immunosorbent assay(ELISA) kit. In a 96-well plate, RAW 264.7 cells were seeded at a density of 2 x 10^5 cells/well and left for incubation overnight. The cells were then pretreated with OVEE, OVEAF, and OVRA for 1hr before stimulation with LPS for 24 h to induce inflammation. To collect the supernatant, the culture plate was then centrifuged at 1500rpm and assayed according to the protocol of the manufacturer (Invitrogen) to measure the amount of TNF-α and IL-6 produced in each sample. The whole experiment was carried out in triplicate.

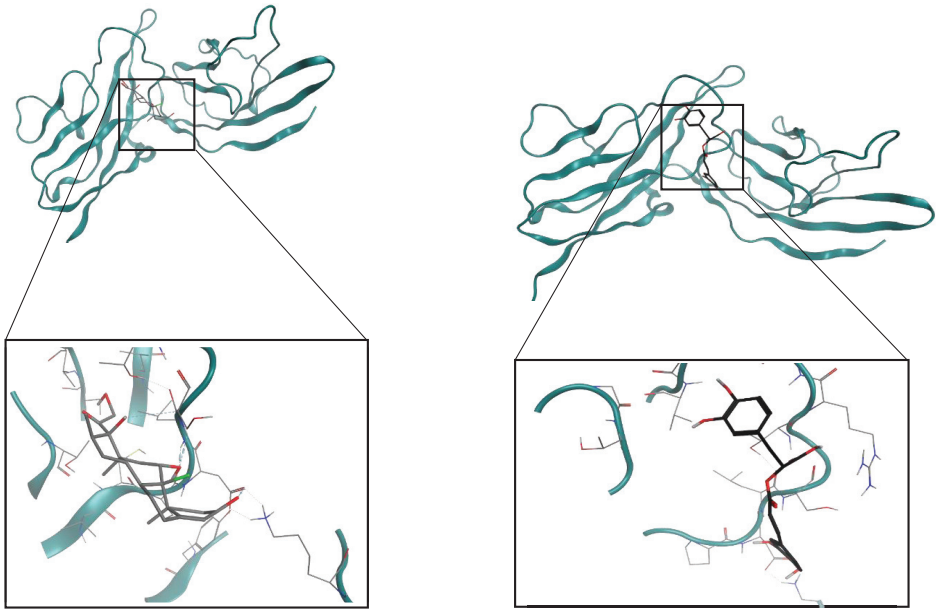
3.7. *In Silico* Studies

Rosmarinic acid and dexamethasone were docked on TNF-α (PDB ID: 2AZ5) (Fig. **3a**) and IL-1β (PDB ID: 3O4O) (Fig. **3b**) by employing Molecular Operating Environment(MOE) 2019.01 docking program. The structures of TNF-α and IL-1β retrieved from the RCBS Protein database were fixed by repairing all the breaks in these structures. After removing water molecules and adding hydrogen atoms, the partial charges were added to the proteins. The protein structures were minimized using Optimized potential for liquid simulations (OPLS) force field in MOE2019.01. The 3D dimensional structure of rosmarinic acid and dexamethasone was downloaded from the NCBI's PubChem



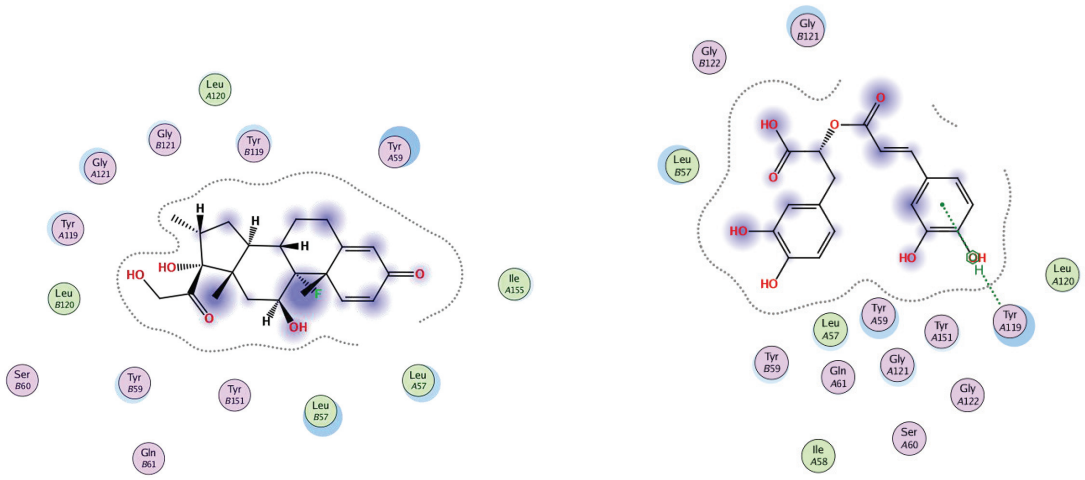
TNF-α Dexamethasone complex TNF-α Rosmarinic acid

Fig. (3a). Dexamethasone and rosmarinic acid docked on a co-crystallized ligand site in TNF-α. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).



IL-1β Dexamethasone complex IL-1β Rosmarinic acid

Fig. (3b). Dexamethasone and rosmarinic acid docked on a co-crystallized ligand site in IL-1β. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).



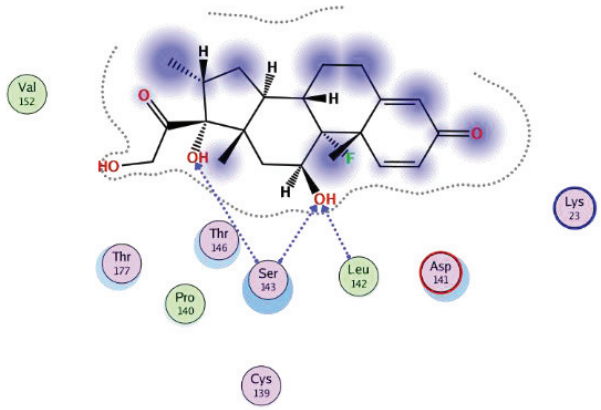
Dexamethasone Rosmarinic acid

Fig. (4a). Ligand interaction with TNF-α. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

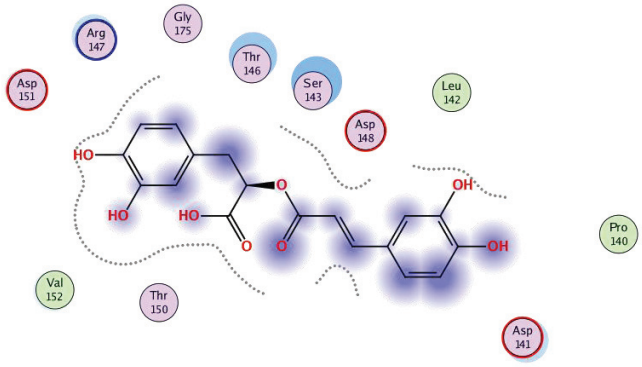
database [43, 44] and was converted into mol2 format by using Open Babel 2.4.1 [45] Linux version and minimized in MOE2019.01 by using MMFF94x force field. After protein and ligand preparation, the ligands rosmarinic acid and dexamethasone were docked on a co-crystallized ligand site in TNF-α. While as in the case of IL-1β, the site finder of MOE 2019.01 and Meta Pocket 2.0 [46], a meta approach to improve protein-ligand binding site prediction, Omics, 13(4), 325-330) server was used for the binding site. After verifying the binding site based on score function and literature, the rosmarinic acid and dexamethasone were docked at the defined site. In both docking cases, i.e. TNF- and IL-1β, a total of 100 solutions were generated and then clustered based on rmsd and scoring. The most populated cluster was selected and the representative member of which is shown in (Figs. **4a**, **4b**). The docking pose shows the interaction of rosmarinic acid and dexamethasone with TNF- and IL-β. The binding affinity of rosmarinic acid and dexamethasone with TNF- and IL-1β was calculated using Cyscore 2.0 [47].

3.8. Statistical Analysis

All the results were expressed as (mean ± of SD). Graphpad prism 8 and Microsoft Excel were used to calculate SD. A P-value of less than 0.5 was considered significant. All the experiments were carried out in triplicates.



Dexamethasone



Rosmarinic acid

Fig. (4b). Ligand interaction with IL-1β receptor. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

4. RESULTS

4.1. Anti-oxidant Activity

The anti-oxidant capacity of natural products is mainly determined by the ABTS assay. The ABTS radical scavenging activity of OVEE, OVEAF, OVRA is shown (Fig. **5a**) along with Ascorbic acid as a standard. Comparable with ascorbic acid (93.02± 3.21), the extracts OVEE (59.83±3), OVEAF (79.76±3.555) and compound OVRA (88.22±44) exhibit significant ABTS radical scavenging activity at 100µg/ml concentration. The extracts OVEE (57.31±3.44), OVEAF (64.45±3.12) and compound OVRA (73.88±3.44) also exhibit significant DPPH radical scavenging activity at 100µg/ml concentration as shown in Fig. (**5b**) compared to ascorbic acid (93.22±4.55). The antioxidant activity of compound OVRA increases with an increase in concentration. DPPH changes its color from violet to yellow on the acceptance of hydrogen or electron. The ability of a compound to carry out such a reaction makes it an antioxidant. Compounds including ascorbic acid, pyrogallol, glutathione, and tocopherol act as hydrogen donors, and commonly used standards to carry out DPPH assay.

4.2. Effect on Cell Viability

To find the safety level of OVEE, OVEAF, and OVRA, MTT assay was performed. Raw 264.7 cells were subjected to different concentrations (1-10 mg/ml) of OVEE, OVEAF, and OVRA(1-10µM) for a time period of 48h. At low concentrations (1-10µM), we observed that cell viability did not get affected and the cell viability was more than 80% at 10µM. Therefore, to investigate the anti-inflammatory

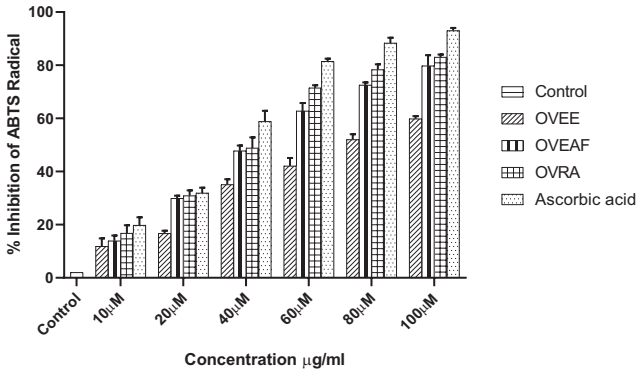


Fig. (5a). Percent inhibition of ABTS radical scavenging activity by OVEE, OVEAF, OVRA, and known antioxidant Ascorbic acid measured at 517 nm. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

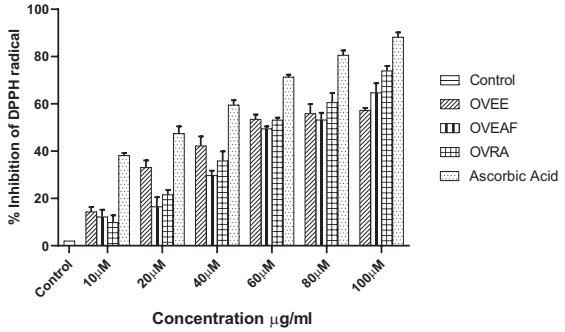


Fig. (5b). Percent inhibition of DPPH free radical by OVEE, OVEAF, OVRA, and known antioxidant Ascorbic acid measured at 517 nm. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

activity of OVEE, OVEAF, and OVRA, on RAW 264.7 cells, our data showed no toxic effect at a concentration of (1-10μM) as shown in (Fig. **6**).

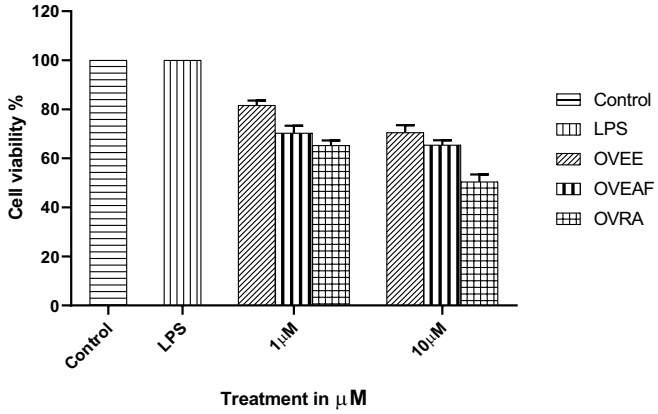


Fig. (6). Viability of RAW 264.7 cells after 24 h of treatment with OVEE, OVEAF, OVRA. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

4.3. Effect on NO Production in LPS- Stimulated RAW 264.7 Cells

Compared to LPS treatment alone, the production of NO was inhibited by OVEE and OVEAF as well as by the isolated compound (OVRA) more significantly. Moreover, the inhibition followed a dose-dependent pattern (Fig. **7**). For NO release, maximum inhibition of 54% was observed in an isolated compound followed by OVEE (47%), and OVEAF (43%), at 10μM, which is comparable to dexamethasone (65.7%) and L-NAME (60.5).

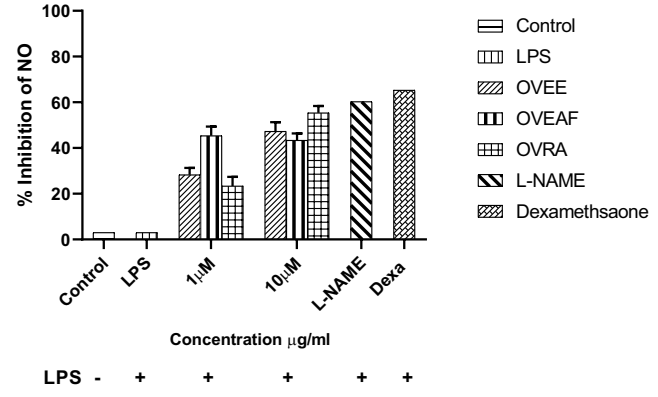


Fig. (7). Effects of extracts (OVEE, OVEAF) and isolated compound (OVRA) on NO production in LPS-stimulated RAW 264.7 macrophages. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

4.4. Effect on the Production of Pro-inflammatory Cytokines in LPS-stimulated RAW 264.7 Macrophages

The extracts (OVEE, OVEAF) and the isolated compound (OVRA) were observed for TNF-α and IL-6 inhibition in LPS stimulated RAW 264.7 cells. From the results, as shown in Fig. (**8a**, **8b**), it is evident that OVRA displayed maximum TNF-α inhibition of 68.15 % at 10 μM concentration. Besides, OVRA showed maximum IL-6 inhibitory up to 58.8% at 10 μM concentration. For (OVEAF), Maximum TNF-α and IL-6 inhibition was 73.2% and 50.3% at 10μM concentration, respectively. For (OVEE), TNF-α 65.8% and IL-6 52.3 were observed at 10 μM concentration. In LPS untreated cells, the levels of TNF-α and IL-6 were undetectable and served as a control. At 10μM concentration, there were no significant changes in cell viability on the treatment of RAW 264.7 cells with the extract as well as isolated compounds. The extract, which exhibits more than 40% inhibition of TNF-α and IL- 6 is considered more potent [48, 49].

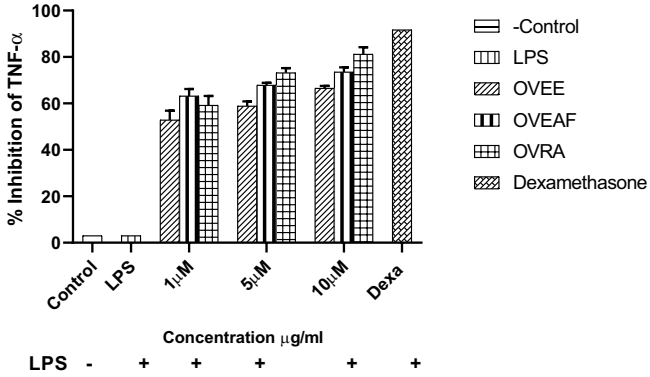


Fig. (8a). Inhibition of the LPS-Induced TNF-α Production in RAW 264.7 cells. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

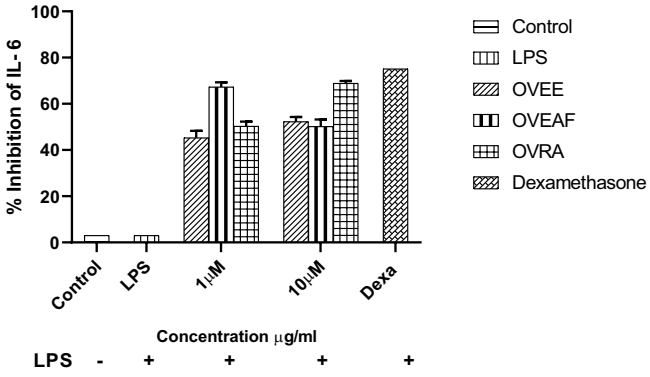


Fig. (8b). Inhibition of the LPS-Induced IL-6 Production in RAW 264.7 cells. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

4.5. Molecular Docking Studies

The docking pose shows the interaction of rosmarinic acid and dexamethasone with TNF- and IL-β. The binding affinity of rosmarinic acid and dexamethasone with TNF- and IL-1β was calculated using Cyscore 2.0 [47, 50]. From the docking score and cyscore analysis, it was found that rosmarinic acid had greater affinity towards TNF-α and IL-1β than dexamethasone (Table **1**), which was also verified from *in vitro* results.

Table 1. Docking and Cyscore score along with ADME properties.

Compounds Name	Docking Score		Cyscore (Binding Affinity)		ADME Studies				
	TNF-α	IL-1β	TNF-α	IL-1β	Log $P_{o/w}$	Log S	Log K_p	TPSA	B.A Score
Dexamethasone	5.17	135.14	1.97	1.31	2.15	3.56	7.32 cm/s	94.83 Å²	0.55
Rosmarinic acid	5.78	199.19	2.24	1.70	1.58	5.04	6.82 cm/s	144.52 Å²	0.56

5. DISCUSSION

The process of inflammation is perceived as a complex series of events involving plasma and cellular derived events which varies from species to species, from one tissue to another tissue and even in the same tissue depending upon the nature of trauma. Despite the assortment of harmful substances and tissues entangled in the inflammation, similar chemical mediators are released in the preliminary event. After an injury, different types of chemical mediators that are biologically active responsible for the progression of inflammation are released.

Various extracts of plants and isolated compounds showed anti-inflammatory activity by inhibiting various pathways of inflammation, including pro-inflammatory cytokines like TNF-α, IL-6. We found that the anti-inflammatory activity of the extracts understudy is very much comparable with those extracts, including licorice, pomegranate, and nutmeg that are reported to have potent anti-inflammatory activity [51, 52]. Moreover, the isolated compound, rosmarinic acid, which exhibits anti-inflammatory activity by inhibiting IL-6 and TNF-α is also comparable with capsaicin, carnosic acid [53, 54].

In the amelioration of various inflammatory diseases, the demand for improved drugs has not been overwhelmed by current anti-inflammatory drugs. In the current study, we investigated the effects of *Oreganum Vulgare* extracts on the production of pro-inflammatory mediators in RAW 264.7 mouse macrophages stimulated by LPS. The study was carried out due to the medicinal importance of the plant in curing various ailments. Traditionally, *Origanum Vulgare* is used as a bronchodilator, in toothache, as antiseptic, in rheumatism, and fever [21, 26, 55, 56]. Moreover, in modern medicine, it is reported to have anti-oxidant and anti-aging activities [57]. However, no attempts have been made to study its anti-inflammatory activity. Consequently, the current study bestows with the evaluation of the anti-inflammatory of *Oreganum Vulgare*.

In RAW 264.7 mouse macrophages, the extracts of the *Oreganum Vulgare* were evaluated for their potential to reduce lipopolysaccharide(LPS) induced TNF-α and IL-6 production. RAW 264.7 cells upon activation with LPS releases TNF-α and IL-6. LPS, which is a component of a gram-negative bacterial cell wall, induces macrophages and monocytes, which play a pivotal role in the innate immune response. RAW 264.7 cells on stimulation with LPS leads to a series of intracellular events which result in the secretion of cytokines as well as other mediators of inflammation that eventually constitute the pro-inflammatory response. RAW 264.7 cells on pretreatment with *Oreganum Vulgare* extracts at various concentrations followed by LPS treatment for 24 h cause the downregulation of pro-inflammatory cytokines (IL-6 and TNF-α). At 10µg/ml, the extracts, along with the isolated compound, showed potent inhibition of cytokines. In LPS untreated cells, the levels of IL-6 and TNF-α were not detectable and served as the control. The MTT assay also showed that up to 10µg/ml concentration, the extracts did not affect the viability of RAW 264.7 cells. Moreover, both the extracts (OVEE, OVEAF) as well as the isolated compound(OVRA) suppress the production of NO, which is also released during the process of inflammation to activate the various macrophages. With reference to the standard drug dexamethasone, the inhibitory potential of extracts, as well as the isolated compound, was studied. Both the extracts (OVEE, OVEAF) and (OVRA) showed significant cytokines and NO inhibition.

Various extracts of plants and isolated compounds showed anti-inflammatory activity by inhibiting various pathways of inflammation, including pro-inflammatory cytokines like TNF-α, IL-6. We found that the anti-inflammatory activity of the extracts understudy is very much comparable with the extracts, including licorice, pomegranate, nutmeg, that are reported having potent anti-inflammatory activity.

CONCLUSION

In conclusion, both the extracts as well as the isolated compound of *Oreganum Vulgare* inhibit the pro-inflammatory cytokines (IL-6 and TNF-α) and NO without affecting cell viability. Hence our study established that the leaf extracts of *Oreganum Vulgare* exhibit anti-inflammatory activity and thus confirm its importance in traditional medicine. This study encourages furtherstudy of the molecular mechanisms involved, which further confirms its anti-inflammatory activity at the molecular level. The results show an ideal natural plant to treat various inflammatory diseases.

LIST OF ABBREVIATIONS

OVEE = *Origanum vulgare* L. ethanolic extract

OVEAF = *Origanum vulgare* L. ethyl acetate fraction

OVRA = *Origanum vulgare* L. rosmarinic acid

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No animals/humans were used in the studies that is the basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

Not applicable.

FUNDING

None.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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