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Evaluation of *in vitro* anti-inflammatory and COX-2 inhibitory activity of leaves of *Origanum vulgare*

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Abstract
The study was conducted to investigate *in vitro* anti-inflammatory and COX-2 inhibitory activities of dried leaves of *Origanum vulgare.* For the present investigation, 300 µg / ml and 500 µg / ml of petroleum ether, chloroform, ethyl acetate, methanol and water extracts were used. The extracts were obtained by successive soxhlet extraction. *In vitro* anti-inflammatory activity was studied by membrane stabilization of HRBCs. The percentage of membrane stabilization was compared with standard drug Diclofenac sodium at a concentration of 300 µg / ml and 500 µg / ml. *In vitro* COX-2 inhibitory activity was evaluated by enzyme immunoassay. Methanolic extract at a concentration of 500 µg / ml showed significant membrane stabilization of 84.34% and 74.52% of remarkable COX-2 inhibition. The results observed thus, suggest that the methanolic extract of dried leaves of *Origanum vulgare,* possesses promising *in vitro* anti-inflammatory and COX-2 inhibitory activity.

Keywords: Membrane stabilization, enzyme immunoassay, *Origanum vulgare, in vitro* cox-2 inhibition

1. Introduction
Inflammation may be defined as the attempt of the body to self protect against harmful stimuli, which include damaged cells, pathogens, irritants. In this process body's white blood cells release chemicals into the blood or affected tissues to get rid of foreign substances. A cascade of reactions like, enzyme activation, mediator release, fluid extravasations, cell migration, tissue breakdown and repair occur during this response as a process of host defence [1]. These various reactions involved form a basis for therapeutic targeting, interference of which may suppress the inflammatory response. Non steroidal anti-inflammatory drugs (NSAIDs) widely used as anti-inflammatory agents act by inhibiting the prostaglandin (PG) synthesis secondary to their inhibition of the enzyme cyclooxygenase (COX). Two distinct isoforms of cyclooxygenase, COX-1 and COX-2 have been identified of which, COX-1 expressed constitutively in most tissues, regulates normal functioning of the GIT, kidneys and platelets. In contrast, inducible COX-2 expressed in response to inflammation, and to some extent in other tissues including kidneys and brain. Traditional NSAIDs are non-selective and inhibit both COX-1 and COX-2. However, most common complications associated with usage of NSAID involve gastrointestinal bleeding, ulceration and perforation. To avoid NSAID induced side effects specific COX-2 inhibitors were introduced. However, modern specific COX-2 inhibitors reported to have detrimental effects on cardiovascular and renal function [2]. Due to potential problems associated with modern anti-inflammatory agents search for novel agents especially from natural sources has been intensified as more potent drugs with less side effects can be elucidated. Most of the plants have a folklore value against various ailments and have not been explored and *Origanum vulgare* commonly known as oregano or wild marjoram, belonging to family *Lamiaceae* is one such herb which has been traditionally used as an anti-inflammatory agent. For thousands of years oregano has been used for it's effective culinary, cosmetic and folklore medicine [3]. *Oregano* which is rich in essential oils, have been reported to neutralise the COX-2 enzyme which is associated with tissue inflammation [4]. It has been reported to possess Chemical constituents like rosmarinic acid, oleanolic acid, ursolic acid, essential oil rich in monoterpenic phenol called carvacrol, flavonoid luteolin, caffeic acid, eugenol [5, 6]. *In vitro* anti-inflammatory effect was demonstrated which was due to inhibition of aldose reductase and lipoxygenase [7]. In a study, oregano reduced harmful reactive oxygen species including free radicals [8]. Trans-sabinene hydrate, Thymol, Carvacrol isolated from *Origanum vulgare* Decreased proinflammatory agents, TNF-α, IL-1β & IL-6 Cytokine synthesis as well as increased production of anti-inflammatory cytokine IL-10 [9]. In context to the literature reviewed and scientific investigations observed, much light has not been focused on the anti-inflammatory and

COX-2 inhibitory activity of *Origanum vulgare.* Hence an attempt was made to study the the anti-inflammatory and COX-2 inhibitory activity by *in vitro* methods in order to validate the folkloric uses of this herb as an anti-inflammatory agent and establish the possible mechanisms of pharmacological action.

2. Material and methods: For the present investigation, Oregano leaves were obtained from Munnalal dawasaz, Hyderabad. The collected leaves of oregano were thoroughly checked for any foreign matter and shade dried. After complete drying the drug was powdered coarsely by using a laboratory grinder and sieved. 50 g of leaf powder was extracted by successive soxhlation with solvents petroleum ether (60 - 80 ^{o}C), chloroform, ethyl acetate, methanol and water for 8 hours. The extracts obtained were further concentrated in vacuo by using rotary vacuum evaporator. The extracts were then dried in a desiccator and stored.

2.2 Chemical and reagents

Diclofenac sodium was obtained as a gift sample from Mangalam Drugs and Pharmaceuticals Ltd, Wapi, Gujarat. All the solvents were procured from E. Merck, Mumbai. The colorimetric human COX-2 inhibitor screening kit (Item No. 560131) used for *in vitro* COX-2 inhibitory activity was manufactured by Cayman Chemical, USA. The contents of the kit includes, PG screening EIA antiserum, PG screening AChE tracer, PG screening EIA standard, EIA buffer concentrate, wash buffer concentrate, polysorbate 20, mouse anti rabbit IgG coated plate, 96- well cover sheet, Ellman's reagent, reaction buffer, COX-1 (ovine), COX-2 (human recombinant), heme, arachidonic acid (substrate), potassium hydroxide, hydrochloric acid, stannous chloride.

2.2.1 Investigation of *In Vitro* Anti-inflammatory activity

Evaluation of *In vitro* anti-inflammatory activity of the extracts was studied by membrane stabilisation method [10]. The method involves stabilisation of human red blood cells which is the measure of anti-inflammatory response, as HRBC membrane and lysosomal membrane are analogous to each other [11]. Thus, lysosomal membrane stabilisation prevents the lysosomal enzymes release, responsible for inflammation thus, resulting in anti-inflammatory response.

2.2.2 Preparation of HRBCs (human red blood cells)

Blood (5 ml) was collected from healthy human donors and centrifuged. With the help of sterile pipettes the supernatant was carefully pipetted and centrifugation was repeated by resuspending the packed cells in an equal volume of isosaline. The process was repeated 4 times until clear supernatant were observed. A 10% HRBC suspension was then prepared with normal saline and kept at 4 ^{0}C until use.

2.2.3 Effect of plant extracts on HRBC system

4.5 ml of reaction mixture was prepared which includes, 2ml hyposaline (0.25% w/v NaCl), 1 ml of isosaline buffer solution, pH 7.4 (6.0 g TRIS, 5.8g NaCl, HCl to regulate the pH and water to make 1000 ml) and varying volumes of the extract solution in isotonic buffer (concentration = 10mg/ ml) to make the volume to 4.0 ml. Then 0.5 ml of 10% HRBC in normal saline was added. Two controls were performed. One with 1.0 ml of isosaline buffer instead of extract (control 1) and the other one with 1 ml of extract solution and without red blood cells (control 2). The mixture was incubated at 56 ^{0}C for 30 min. The tubes were cooled under running water for 20 min. The mixture was centrifuged, and the absorbance of the supernatant was read at 560 nm. The percentage of membrane stabilization was determined using the formula

$$100 - \frac{\text{(Extract absorbance value – control 1 absorbance value)}}{\text{Control 2 absorbance value}} \times 100$$

The control 1 represents 100% HRBC lysis. Standard drug, diclofenac sodium was used to compare the HRBC membrane stabilizing of the test drug.

2.2.4 Evaluation of *in vitro* COX – 2 inhibitory activity

In vitro COX-2 inhibition was evaluated by enzyme immunoassay [12]. The capacity of the test compound to inhibit COX-2 (human recombinant) was investigated by using enzymes immunoassay (EIA) kit (Catalogue No.560131, Cayman Chemical, Ann Arbor, MI, USA) following the Manufacturer's instructions. The test compound was dissolved in DMSO, and the solution was made at the final concentration of 10 µM. Reaction buffer solution (960µl, 0.1M Tris-HCL, pH-8 containing 5mM EDTA and 2 mM phenol) containing COX-2 enzymes (10 µl) in the presence of heme (10 µl) was added with 10 µl of 10 µM test drug solution. These solutions were incubated for a period of 10 min at 37 ^{o}C after then 10 µl of AA solution was added followed by stopping the COX reaction by addition of 50 µl of 1 M HCL. Conversion of arachidonic acid (AA) to PGH_2 is catalysed by Cyclooxygenase followed by reduction of PGH_2 to $PGF_{2\alpha}$ by stannous chloride. The $PGF_{2\alpha}$, produced from the PGH_2 by reduction with stannous chloride (100 µl), was measured by enzyme immunoassay. This was based on the competition between PGs and PG-acetyl cholinesterase conjugation (PG tracer) for the limited amount of PG antiserum. The amount of PG tracer that is able to bind to the PG antiserum is inversely proportional to the concentration of PGs in the well since the concentration of PGs tracer is held constant while the concentration of PGs varies. This antibodies-PG complex bind to mouse anti-rabbit monoclonal antibodies that has been previously attached to the well. The plate is washed to remove any unbound reagents and then Ellman's reagents, which contain the substrate to acetylcholine esterase, are added to the well. The product of this enzymatic reaction produced a distinct yellow colour, determined by spectrophotometrically (Micro titre Plate reader) at 412 nm, is proportional to the amount of PG tracer bound to the well, which is inversely proportional to the amount of free PGs present in the well during the incubation:

$$\text{Absorbance } \alpha \text{ [Bound PG tracer] } \alpha \text{ 1/PGs.}$$

Percentage inhibition was calculated by the comparison of compound treated by control incubations.

2.3 Statistical analysis: Data were expressed as mean ± SEM, where, n = 6, statistical analysis was performed by one way analysis of variance (ANOVA) and significance in the results was observed by Tukey's multiple comparison test. All the statistical analysis was performed by using Graph Pad Prism version 5.0 software.

3. Results: By performing *in vitro* anti-inflammatory studies by HRBC membrane stabilization method the percentage of membrane stabilization for methanolic extract of oregano, was significant. The membrane stabilization capability increased

with the concentration of the extract and the percentage protection of the methanolic extract at a concentration of 500 µg /ml was comparable to 500 µg of standard drug diclofenac. The percentage of membrane stabilization of various extracts represented in table 1. Further, significant COX-2 inhibitory response was observed for methanolic extract at a concentration of 500 µg /ml. Percentage of COX-2 inhibition of the various oregano extracts represented in table 2.

Table 1: Percentage of membrane stabilisation of various extracts.

Extract	% protection Concentration (µg/ml)	
	300	500
PEE	20.80±0.01[a]	23.90 ±0.03[a]
CE	30.75±0.04 [a]	34.95±0.02 [a]
EE	45.44±0.02 [a]	46.05±0.03 [a]
ME	79.44±0.01 [a]	84.34±0.01 [a]
AE	49.26±0.02 [a]	52.43±0.01 [a]
Diclofenac sodium (Standard)	87.07±0.01 [a]	92.29±0.01 [a]

PEE – petroleum ether extract. CE – chloroform extract, EE – ethyl acetate extract, ME – methanolic extract, AE – aqueous extract.

Values expressed in mean ± SEM where, n=6, statistical significance done by ANOVA followed by Tukey's multiple comparison test. ([a]$P \leq 0.01$, [b]$P \leq 0.05$ compared with standard)

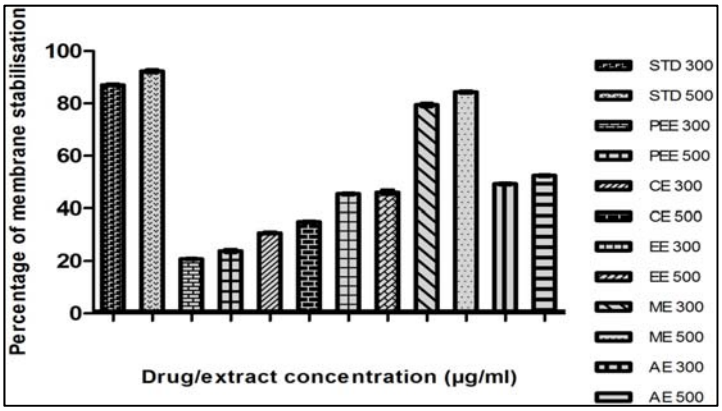

Fig 1: *In vitro* anti-inflammatory response of various extracts of *Origanum vulgare*"

Table 2: Percentage COX-2 inhibition of various extracts

Extract	% COX-2 inhibition Concentration (µg/ml)	
	300	500
PEE	45.62±0.01[a]	49.93 ±0.02 [a]
CE	35.68±0.03 [a]	39.62±0.04 [a]
EE	48.65±0.01 [a]	54.79±0.02 [a]
ME	68.86±0.01 [a]	74.52±0.03 [a]
AE	50.36±0.05[b]	52.72±0.05 [b]

Values expressed in mean ± SEM where, n=6, statistical significance done by ANOVA followed by Tukey's multiple comparison test. ([a]$P \leq 0.01$, [b]$P \leq 0.05$ compared with control)

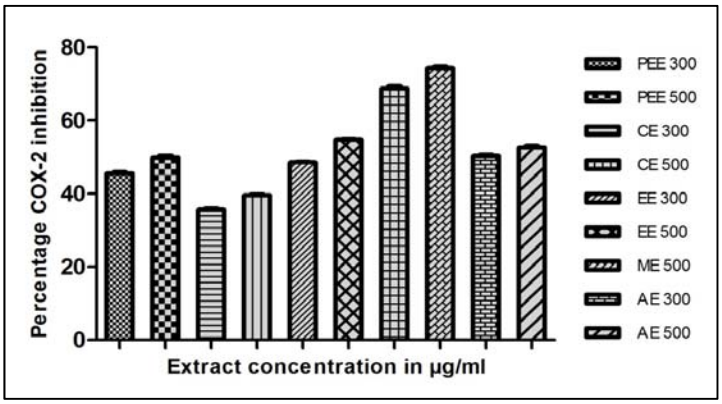

Fig 2: *In vitro* COX-2 inhibitory response of various extracts of *Origanum vulgare*

4. Discussion: The significant response in the membrane stabilization implies the anti-inflammatory potential of the drug. As stabilization of HRBCs is correlated with stabilization of lysosomal membrane, which is an important rate limiting step against inflammation it can be concluded that methanolic extracts of oregano showed promising anti-inflammatory effect. This potential of oregano towards anti-inflammatory response may be due to different active ingredients present in the methanolic extract. In order to analyze the possible mechanisms and the specificity of the herbal extract in inhibiting COX-2 enzyme, *in vitro* COX-2 anti-inflammatory activity was studied by COX catalyzed prostaglandin biosynthesis assay. The effective percentage inhibition of COX-2 enzyme contributes scope for elucidation of active ingredients involved in anti-inflammatory response. This study establishes an empirical evidence for the folkloric claims reporting the anti-inflammatory activity of oregano.

5. Conclusion: from the results of the present investigation it can be concluded that the methanolic extract of oregano possess anti-inflammatory activity mediated by COX-2 inhibition. Isolation of the active constituents from the crude extract may be helpful to develop novel COX-2 inhibitors with fewer side effects in comparative to modern anti-inflammatory drugs.

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7. References

1. Vane JR, Bolting RM. New insights into the mode of action of anti-inflammatory drugs. Inflamm. Res. 1995; 44(1):1-10.
2. Praveen Rao PN, Edward EK. Evolution of Nonsteroidal Anti Inflammatory Drugs (NSAIDs): Cyclooxygenase (COX) Inhibition and Beyond. J Pharm Pharmaceut Sci. 2008; 11(2):81s-110s.
3. Deighton N, Glidewell SM, Deans SG, Goodman BA. Identification by EPR spectroscopy of Carvacrol and Thymol as the major sources of free radicals in the oxidation of plant essential oils. J Sci Food Agric. 1993; 63:221-25.
4. Lagouri V, Dimitrios B. Nutrient antioxidants in Oregano. Int J Food Sci and Nutr. 1996; 47:493-97.
5. Shen D, Pan MH, Wu QL, Park CH, Juliani HR, Ho CT *et al*. LC-MS Method for the Simultaneous Quantitation of the Anti-inflammatory Constituents in Oregano (Origanum species). J Agric Food Chem. 2010; 58(12):7119-25.
6. Lopez-Lazarom. Distribution and Biological Activities of the Flavonoid Luteolin. Mini Rev Med Chem. 2009; 9(1):31-59.
7. Koukoulitsa C, Zika C, Hadjipavlou-Litina D, Demopoulos VJ, Skaltsa H. Inhibitory Effect of Polar Oregano Extracts on Aldose Reductase and Soyabean Lipoxygenase *in vitro*. Phytother Res. 2006; 20(7):605-6.
8. Osakabe N, Takano H, Sanbongi C, Yasuda A, Yanagisawa R, Inoue K *et al*. Anti-inflammatory and Anti-allergic Effect of Rosmarinic Acid (RA); Inhibition of Seasonal Allergic Rhino Conjunctivitis (SAR) and its Mechanism. Biofactors. 2004; 21(1-4):127-31.
9. Ocana FA. Supercritical Fluid Extraction of Oregano (*Origanum Vulgare*) Essential Oils: Anti-inflammatory Properties Based on Cytokine Response on THP-1 Macrophages. Food Chem Toxicol. 2010; 48(6):1568-75.
10. Mongeli E, Desmarchelier C, Coussio J, Ciccia G. Biological studies of *Bolax gummifera,* a plant of the Falkland islands used as a treatment of wounds. J Ethnopharmacol. 1997; 56:117-21.
11. Gandhisan R, Thamaraichelvan A, Baburaj C. Anti-inflammatory action of *Lannea coromandelica* HRBC membrane stabilization. Fitoterapia. 1991; 62:81-83.
12. Pradelles P, Grassi J, Maclouf JA. Enzyme immunoassay. Annal Chem. 1985; 57:1170-73.