ORIGINAL ARTICLE

In vitro investigation of anti-inflammatory and COX-2 inhibitory potential of flower extracts of *Matricaria recutita*

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Abstract

Introduction: *Matricaria recutita* has been traditionally used as a long-term anti-inflammatory herb, thus giving scope for anti-inflammatory studies. The study was conducted to investigate *in vitro* anti-inflammatory and cyclooxygenase-2 (COX-2) inhibitory activities of dried flower extracts of *Matricaria recutita.* **Materials and Methods:** For the present investigation, various extracts were obtained by successive Soxhlet extraction using solvents with increasing polarity, namely, petroleum ether, chloroform, ethyl acetate, methanol, and aqueous (AE). 300 and 500 µg/ml concentrations of the extracts were used for the study. *In vitro* anti-inflammatory activity was studied by membrane stabilization of human red blood cells (HRBCs). The percentage of membrane stabilization was compared with standard drug diclofenac sodium at a concentration of 300 and 500 µg/ml. *In vitro* COX-2 inhibitory activity was evaluated by enzyme immunoassay (EIA). **Results:** AE at a concentration of 500 µg/ml showed maximum membrane stabilization of 82.43% and 68.62% of promising COX-2 inhibition in comparative with remaining extracts. **Discussion:** The inhibition of membrane stabilization is the measure of anti-inflammatory activity due to analogous nature of lysosomal membrane which releases lysosomal enzymes responsible for inflammation with HRBC membrane. Thus, significant inhibition of HRBC membrane directly correlates the significant anti-inflammatory capacity of the extract. Remarkable inhibition of COX-2 inhibition by EIA states that *Matricaria recutita* possesses specific COX-2 inhibitory activity. **Conclusion:** The results observed thus suggest that the AE of dried flowers of *Matricaria recutita* possesses promising *in vitro* anti-inflammatory and significant COX-2 inhibitory potential.

Key words: Cyclooxygenase-2, enzyme immunoassay, *in vitro* anti-inflammatory, *Matricaria recutita*, membrane stabilization

INTRODUCTION

A protective response against physical trauma, harmful chemicals, and microorganisms resulting in the injury of tissue may be defined as inflammation. Involvement of a large number of chemotactic, vasoactive, and proliferative factors at different stages has been observed during anti-inflammatory action.[1] Initiation of inflammatory response may be due to release of chemicals such as prostaglandins (PGs), leukotrienes, histamine, bradykinin, platelet-activating factor, and interleukin-1 from the injured tissues and migrating cells.[2] The two distinct isoforms of cyclooxygenase (COX) are COX-1 which is constitutive and involved in normal cellular homeostasis and COX-2 which is inducible and responsible for biosynthesis of PGs in acute inflammatory conditions and is believed to be target for anti-inflammatory drugs.[3] Most of the currently used anti-inflammatory drugs are either steroidal or non-steroidal and have undesirable side effects ranging from gastrointestinal irritation to cardiovascular effects. Hence, natural compounds derived from plants or other sources are especially important to be developed

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into anti-inflammatory drugs. Moreover, considering the resulting side effects of COX-1 inhibitors, herbs selectively inhibiting COX-2 enzyme need to be studied. More diverse chemical structures are found in plants which would be absolutely specific in inhibiting a particular enzyme. Such compounds may contain chemical groups susceptible to chemical or biological transformation. Their derivatives may exhibit novel properties which may be advantageous in the pharmacological view. *Matricaria recutita* commonly called as chamomile has a long history as a traditional medicine with anti-inflammatory and analgesic activity. It has been used for centuries for its analgesic and anti-inflammatory properties.[4,5] It is most widely used all over the world, well documented, and included in pharmacopeia of 26 countries.[6] Conventionally, it has been used to treat inflammatory diseases for long term.[7] Externally, chamomile is used for wounds, eczema, skin irritations, ulcers, neuralgia, gout, sciatica, leg ulcers, hemorrhoids, rheumatic pain, and mastitis.[8] German Commission E approved the use of chamomile in external use to treat inflammation of skin, mucous membrane, anogenital area, respiratory tract, bacterial skin diseases, gastrointestinal spasms, and inflammatory diseases related to gastrointestinal tract. Internally, it has been used to relieve insomnia, hysteria, anxiety, nightmares, convulsions, and delirium. It has been used as a digestive aid as well as, to treat various gastrointestinal disturbances such as flatulence, diarrhoea, motion sickness, indigestion, anorexia, nausea, and vomiting. Based on the scientific literature reviewed, no study reported the inflammatory and specific COX-2 inhibitory activity of *Matricaria recutita* by *in vitro* methods. Hence, an attempt was made to study the potential of chamomile for inflammatory and specific COX-2 inhibitory response by *in vitro* methods so that the significance of the herbal extracts toward anti-inflammatory activity can be justified.

MATERIALS AND METHODS

Collection and Extraction of Plant Material

For the present investigation, chamomile flowers were obtained from local market. The collected plant material was shade dried, after thoroughly checking for the presence of any foreign matter. After complete drying, a laboratory grinder was used to finely powder the drug. The fine powder was sieved, and 50 g of powdered drug was then extracted by successive soxhlation with petroleum ether (60-80°C), chloroform, ethyl acetate, methanol, and water for 8 h. The solvent extracts obtained were further concentrated in vacuo using rotary vacuum evaporator and then dried in a desiccator.

Chemical and Reagents

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

Evaluation of *In Vitro* Anti-inflammatory Activity

In vitro anti-inflammatory activity of the flower extracts of chamomile was studied by human red blood cell (HRBC) membrane stabilization method.[9] For this, HRBCs were prepared by collecting blood (5 ml) from healthy human donors. The blood collected was centrifuged, and the supernatant obtained was carefully pipetted out. The packed cells were then suspended in an equal volume of isosaline and centrifuged. The process was repeated 4 times until clear supernatant was observed. Then, with normal saline, a 10% HRBC suspension was prepared and kept at 4°C until use. The reaction mixture (4.5 ml) consisted of 2 ml hyposaline (0.25% w/v NaCl), 1 ml of isosaline buffer solution, pH 7.4 (6.0 g TRIS, 5.8 g NaCl, HCl to regulate the pH, and water to make 1000 ml), and varying volumes of the extract solution in isotonic buffer (concentration = 10 mg/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. Control 1 included 1.0 ml of isosaline buffer instead of extract, and Control 2 included 1 ml of extract solution and without red blood cells. The mixture was incubated for 30 min at 56°C. After cooling the tubes for 20 min under running water, 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:

$$100-\frac{\left(\begin{array}{l}\text{Extract absorbance value}-\\ \text{control 1 absorbance value}\end{array}\right)}{\text{Control 2 absorbance value}}\times 100$$

The control 1 represents 100% HRBC lysis. Diclofenac sodium was used as a standard drug.

Evaluation of *In Vitro* COX-2 Inhibitory Activity

In vitro COX-2 inhibitory activity was evaluated by EIA.[10] EIA kit (Catalogue No. 560131, Cayman Chemical, Ann Arbor, MI, USA) was used to study the ability of the test compound to inhibit COX-2 (human recombinant) according to the manufacturer's instructions. First step in the biosynthesis of the AA to PGH_2 is catalyzed by COX. The flower extracts of *Matricaria recutita* were dissolved in

DMSO, and the solution was made at the final concentration of 10 µM. Reaction buffer solution (960 µl, 0.1 M Tris-HCL, pH-8 containing 5 mM 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 10 min at 37°C. Then, 10 µl of AA solution was added, and then the COX reaction was stopped by adding 50 µl of 1 M HCL. The reduction of PGH_2 to $PGF_{2\alpha}$ by stannous chloride (100 µl) was measured by EIA. This was based on the competition between PGs and PG-acetyl cholinesterase conjugation (PG tracer) for the limited amount of PG antiserum. The ability of the amount of PG tracer 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 was washed to remove any unbound reagents; and then, Ellman's reagent, which contains the substrate to acetylcholine esterase, was added to the well. The distinct yellow color obtained as a result of the enzymatic reaction was determined spectrophotometrically (microtiter plate reader) at 412 nm, which is proportional to the amount of PG tracer bound to the well, and inversely proportional to the amount of free PGs present in the well during the incubation.

Absorbance α [Bound PG tracer]

Absorbance α 1/PGs.

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

Statistical Analysis

Data were analyzed by one-way analysis of variance and expressed as mean ± standard error mean, where, $n = 6$, $P \leq 0.05$ was considered statistically significant.

RESULTS

Aqueous extracts (AEs) of chamomile at a concentration of 500 µg/ml showed significant percentage of membrane stabilization in comparison to remaining extracts studied. The percentage protection of AEs at a concentration of 500 µg/ml was comparable to 500 µg of standard drug diclofenac. Results pertaining to the percentage of HRBCs membrane stabilization of various extracts studied are represented in Table 1. The COX-2 inhibitory capacity of the test extracts was studied by *in vitro* method using COX-catalyzed PG biosynthesis assay. Among all the herbal extracts studied for *in vitro* COX-2 inhibitory effect, the AE at a concentration of 500 µg/ml was found to show promising COX-2 inhibitory response in comparative with other extracts. COX-2 inhibition of the various extracts is represented in Table 2.

Table 1: Percentage of membrane stabilization of various extracts

% protection extract	Concentration (µg/ml)	
	300	500
PEE	48.51±0.01	53.72±0.03
CE	43.34±0.04	51.75±0.02
EA	65.24±0.02	69.68±0.03
ME	56.67±0.01	61.37±0.03
AE	72.25±0.02	82.43±0.01
Diclofenac sodium (standard)	84.73±0.01	88.45±0.01

PEE: Petroleum ether extract, CE: Chloroform extract, EA: Ethyl acetate, ME: Methanolic extract, AE: Aqueous extract

Table 2: Percentage COX-2 inhibition of various extracts

% COX-2 inhibition	Extract concentration (µg/ml)	
	300	500
PEE	35.21±0.01	42.9±0.03
CE	45.82±0.04	47.32±0.04
EA	38.59±0.05	41.43±0.02
ME	35.44±0.05	39.68±0.03
AE	60.46±0.01	68.62±0.03

PEE: Petroleum ether extract, CE: Chloroform extract, EA: Ethyl acetate, ME: Methanolic extract, AE: Aqueous extract

DISCUSSION

AEs of *Matricaria recutita* at a concentration of 500 µg/ml exerted significant *in vitro* anti-inflammatory effect by stabilizing the red blood cell membrane. During inflammatory response, lysosomal enzymes are released from the lysosomal membrane. The stabilization of lysosomal membrane results in preventing the release of lysosomal enzymes, thus inhibiting the inflammatory response. The percentage inhibition of membrane stabilization of HRBCs is thus the direct measure of anti-inflammatory response due to the similarity in membranes of HRBC and lysosomes.[11] The significance in the percentage of membrane stabilization is directly correlated with the potential of the extract in inhibiting the inflammation. The involvement of COX-2 inhibition was analyzed by studying the COX-2 inhibitory activity of the chamomile extracts by EIA where AEs at a concentration of 500 µg/ml showed promising results which indicated the potential of the chamomile AE for COX-2 inhibitory activity. The EIA revealed the significant COX-2 inhibitory activity of AEs of *Matricaria recutita*. Hence, it can be stated that COX-2 inhibition may be one of the mechanisms involved in anti-inflammatory activity of *Matricaria recutita*.

CONCLUSION

Novel COX-2 inhibitors with fewer side effects in comparison to modern drugs can be developed by isolating the active constituents from the crude AEs of *Matricaria recutita* involved in the anti-inflammatory activity. Thus, this preliminary study gives a scope to study the active constituents in detail which are involved in COX-2 inhibitor activity.

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