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Research Article

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Investigation of *in vitro* anti-inflammatory and COX-2 inhibitory activities of *Rosmarinus officinalis*

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ABSTRACT

The present investigation was aimed to study in vitro anti-inflammatory and COX-2 inhibitory activity of Rosmarinus officinalis. For this study, 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 vitroCOX-2 inhibitory activity was evaluated by enzyme immunoassay. Among the evaluated extracts, methanolic extract at a concentration of 500 µg/ml was found to show significant membrane stabilization of 84.27% and 74.59% of remarkable COX-2 inhibition. The results observed thus, suggest that the methanolic extract of dried leaves of Rosmarinus officinalis possesses promising in vitro anti-inflammatory and COX-2 inhibitory activities.

Key words: *in vitro* anti-inflammatory, COX-2, membrane stabilization, enzyme immunoassay.

INTRODUCTION

Inflammation may be defined as a normal protective response to tissue injury which is caused by physical trauma, noxious chemicals or microbiological agents [1]. During inflammatory process various mediators like prostaglandins, leulotrienes, nitric oxide, reactive oxygen species (ROS), cytokines like TNF – α, IL - 1β and IL – 6 are released which mediate the inflammatory process and tissue damage [2]. Tissue damage may be due to production of ROS which cause damage of macromolecules as well as lipid peroxidation of membranes [3]. The key enzyme involved in synthesis of Prostaglandins from arachidonic acid is cyclooxygenase. It includes two isoforms called COX-1 and COX-2. Both these are structurally identical and differ in their intracellular locations, substrate and inhibitor selectivity [4]. Expression of COX-1 is constitutive in most tissues and is responsible for maintenance of integrity of gastrointestinal tissue, to regulate blood flow to the kidneys and normal platelet functioning. COX-2 is inducible due to pathological process like inflammation and in bearing cancers. It is primarily responsible for PGE_2 production at inflammation sites [5, 6]. To treat the inflammatory conditions many steroidal and nonsteroidal anti-inflammatory drugs are being employed. The traditional NASIDs act by directly inhibiting COX-1 and COX-2 enzymes whereas, recent coxibs act by inhibiting COX-2 enzyme. The traditional NSAIDs are known to produce severe adverse effects like gastrointestinal disturbances including stomach ulceration and bleeding due to inhibition of COX-1 and redistribution of body fat [7]. COX-2 inhibitors may show renal failure due to inhibition of prostaglandin production in the kidney [8]. Due to significant side effects of these modern anti-inflammatory agents, anti-inflammatory agents derived from natural sources are more preferable due to lower risk of side effects [9]. *Rosmarinus officinalis* commonly called rosemary is a household plant belonging to family Lamiaceae. In folk medicine leaves have been used for anti-inflammatory activity [10, 11]. Important constituents of rosemary include caffeic acid and it's derivatives like rosmarinic acid [12], beside flavonoids, phenols, volatile oil and terpenoids [10,

13]. Various studies reported the anti-inflammatory response of *Rosmarinus officinalis.* Rosemary scavenges nitric oxide which is an inducer of COX-2 enzyme thus, causing anti-inflammatory and antioxidant effect [14, 15]. In a study three extracts n-hexane, $CHCl_3$, CH_3OH of leaves of rosemary showed antiinflamamtory effect [16].Crude ethanolic extract upon administration suppressed proinflammatory mediators like Nitric oxide, PGE2, Malonaldehyde, IL-1B cytokine in lipopolysaccharide induced RAW 264.7 cells, thus inhibiting COX-2 activity and causing oxidative burst [17]. The inhibition of COX-2 is stronger than COX-1 [18].The anti-inflamamtory and anti-carcinogenic effects of rosmarinic acid due to antagonist of AP-1 dependent activation of expression of COX-2 gene [19]. Rosmanol another constituent, potently inhibited iNOS and COX-2 protein and gene expression. Ursolic acid, obtained from rosemary upon topical application show inhibitory effect on TPA induced ear inflammation [20]. The main anti-inflammatory effects may be due to triterpenes, ursolic acid, micromeric acid and oleanolic acid [16, 21]. Though various studies reported the anti-inflammatory responses of *Rosmarinus officinalis*, no attempt was made to study the specific COX-2 inhibitory activity by *invitro* methods. Hence an attempt was made to study the specific anti-inflammatory and COX-2 inhibitory activity by *invitro* methods in order to justify the prominence of the active constituents present in *Rosmarinus officinalis* towards anti-inflammatory response as well as specific COX-2 inhibitory effect in order to find scope to discover the active constituents with novel properties and with fewer side effects in comparison to modern anti-inflammatory drugs with significant side effects.

EXPERIMENTAL SECTION

Material and Methods

Collection and extraction of plant material

For the present investigation, Rosemary leaves, were obtained from Yucca Enterprises, Mumbai. The leaves were thoroughly checked for any foreign matter and shade dried. After complete drying the drug was powdered by using a laboratory grinder and sieved. 50 g of powder was extracted by successively soxhlation with petroleum ether (60 - 80 ^{o} C), chloroform, ethyl acetate, methanol and water for 8 hours. The solvent extracts obtained were further concentrated in vacuo by using rotary vacuum evaporator and then, dried in a desiccator.

Chemical and reagents

Diclofenac sodium was obtained 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 antirabbit 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.

Evaluation of In Vitro Anti-inflammatory activity

*In vitro*anti-inflammatory activity of the extracts was performed by the method described by Mongeli *et al* 1997 [22]. By this method, stabilization of human red blood cells by the test extract was studied. Anti-inflammatory response was measured directly by observing the percentage of stabilization, as HRBC membrane is similar to that of lysosomal membrane. Thus, stabilization of lysosomal membrane prevents the release of lysosomal enzymes which are responsible for inflammation.

Preparation of HRBCs (human red blood cells)

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

Effect of plant extracts on HRBC system

The reaction mixture (4.5 ml) consisted of 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. The HRBC membrane stabilizing standard drug used was diclofenac sodium.

Evaluation of in vitro COX-2 inhibitory activity

In vitro COX-2 inhibition was evaluated by the method described by Pradelles*et al*, 1985 [23].

The ability of the test compound to inhibit COX-2 (human recombinant) was determined by using enzymes immunoassay (EIA) kit (Catalogue No.560131, Cayman Chemical, Ann Arbor, MI, USA) according the Manufacturer's instructions. Cyclooxygenase catalyzes the first step in the biosynthesis of the arachidonic acid (AA) to PGH_2. $PGF_{2\alpha}$ produced from the PGH_2 by reduction with stannous chloride, was measured by enzymes immunoassay (EIA). 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. 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 spectrophotometerically (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:

Absorbance α [Bound PG tracer] α 1/PGs.

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

Statistical analysis

Data were expressed as mean ± SEM, where, n = 3, $p \leq 0.05$ was considered to be statistically significant.

RESULTS AND DISCUSSION

By performing *in vitro* anti-inflammatory studies by HRBC membrane stabilization method the percentage of membrane stabilization for methanolic extracts of rosemary at a concentration of 500 µg/ml was significant when compared to other extracts studied. The percentage protection of methanolic extracts at a concentration of 500 µg/ml was comparable to 500 µg of standard drug diclofenac. The percentage of membrane stabilization of various extracts studied, represented in table 1. In order to determine the capability of the extracts in inhibiting COX-2 enzyme**,** the test extracts werefurther studied by*in vitro* method using COXcatalysed prostaglandin biosynthesis assay for COX-2 inhibitory capacity. Among all the herbal extracts studied for *in vitro* COX-2 inhibitory effect, the methanolic extract at a concentration of 500 µg/ml was found to show promising COX-2 inhibitory response in comparative with other extracts. Results pertaining to extracts studied for COX-2 anti-inflammatory activity are shown in table 2.The study has revealed that methanolic extracts at a concentration of 500 µg/ml are capable of stabilizing the red blood cell membrane and exerting anti-inflammatory activity. As the membrane of red blood cells is similar in structure, to lysosomal membrane, the capacity of the test extracts to stabilize the human red blood cells may be directly correlated to stabilization of lysosomal membranes. Thus, the anti-inflammatory activity of the methanolic extract may stem from a stabilization of lysosomal membranes. The possible mechanism of anti-inflammatory response was further detected by studying the COX-2 anti-inflammatory activity of the extracts by enzyme immunoassay which revealed the significant COX-2 inhibitory activity of methanolic extracts of *Rosmarinus officinalis* giving scope to study the active constituents involved in inhibition of COX-2 enzyme.

"Table 1: Percentage of membrane stabilization of various extracts"

Extract	% Protection Concentration (µg/ml)	
	300	500
PEE	44.31±0.04	47.57±0.01
CE	51.26±0.03	53.56±0.02
EAE	59.14±0.02	62.52±0.02
ME	81.56±0.01	84.27±0.02
AE	52.75±0.05	56.43±0.03
Standard	84.73±0.01	88.45±0.01

Values expressed as mean ± SEM, P ≤ 0.05

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

Table 2: Percentage COX-2 inhibition of various extracts"

Extract	% COX-2 inhibition Concentration (µg/ml)	
	300	500
PEE	40.26±0.02	42.9 ±0.02
CE	55.82±0.02	57.32±0.05
EAE	51.44±0.04	53.53±0.03
ME	68.49±0.01	74.59±0.03
AE	56.26±0.02	61.33±0.04

Values expressed as mean ± SEM, P ≤ 0.05

CONCLUSION

Among all the herbal extracts studied for *in vitro* anti-inflammatory activity and specificCOX-2 inhibitory effect, methanolic extract of *Rosmarinus officinalis*was found to show membrane stabilization of HRBCs andCOX-2 inhibition to significant extent. If the active constituents are isolated from the crude methanolic extract and further studied, novel COX-2 inhibitors with fewer side effects may be developed. Hence, the results of this preliminary study urge for the detailed study of the active constituents involved in the COX-2 inhibitor activity.

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