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A novel coumarin derivative, 8-methoxy chromen-2-one alleviates collagen induced arthritis by down regulating nitric oxide, NFκB and proinflammatory cytokines

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ABSTRACT

Ruta graveolens (Rue) is a well-known medicinal plant having anti-inflammatory and other healing properties. This contains many active phytochemicals such as coumarins which possess anti-inflammatory and anti-cancer activities. The present study was carried out to evaluate the therapeutic potential of a newly isolated coumarin derivative from rue plant, 8-methoxy-chromen-2-one (MCO) in the collagen induced arthritic (CIA) rat model. MCO showed inhibition of cytokines and NF-κB in LPS stimulated J774 cells which prompted its possible use in animal. In CIA, arthritic index and arthritic score reduced markedly within 15 days of MCO treatment at doses of 2 mg and 20 mg per kg body weight. Alleviation of joint damage in CIA animals on treatment with MCO was evident from radiographic and histological data. Behavioral studies by open field tests also showed convalescence in the MCO treated CIA rats. Further, escalated plasma levels of pro-inflammatory cytokines TNF-α, IL-1β and IL-6, and also nitric oxide reduced significantly with the treatment. All these results indicate the therapeutic efficacy of MCO and its possible use as an anti-arthritic drug.

1. Introduction

Ruta graveolens L. is a medicinal as well as culinary plant native to the Mediterranean region of northern Africa and southern Europe. Commonly known as rue, it is used for the treatment of rheumatism, eye ailments, dermatitis, etc. for example, rue oil and infusions act as emmenagogues and antispasmodics [1]. The plant has also been commonly used as condiment for many food items such as coffee, tea, soup, cheese, and butter. Other uses of rue include flavoring of meats, fish, salads, sauces, and cheese dishes [2]. More than 120 compounds of different classes such as alkaloids, coumarin derivatives, flavonoids, and volatile oils have been identified in this plant [3]. These classes of compounds have gained particular interest in medicinal chemistry due to their spasmolytic, analgesic, antiphlogistic, anti-helminthic and anti-inflammatory activities. Whole plant extract using 50% methanol in water was found to inhibit inflammation and oxidative stress in adjuvant and carrageenan induced arthritis in rats [4]. It was also observed to inhibit both the protein and mRNA expression of iNOS and IL-1β in LPS challenged macrophages [5].

The active compound MCO was isolated from 50% hydromethanolic extract of this plant by ethyl ether fractionation followed by reverse phase HPLC through bioactivity guided isolation techniques. The compound MCO was further identified and characterized using ESI-MS, MALDI, FT-IR and NMR as 8-methoxy-chromen-2-one or MCO.

Among various inflammatory diseases, rheumatoid arthritis (RA) is known to be a systemic autoimmune disease characterized by chronic inflammation of multiple synovial joints with concomitant destruction of joint tissues. Currently prescribed non-steroidal anti-inflammatory drugs (NSAIDs) and disease modifying anti-rheumatic drugs (DMARDs) have several discouraging side effects. As the rue plant is already used in folklore medicine for treatment of inflammatory conditions and our in vitro observations further pointed towards anti-inflammatory property of this plant. We decided to find out the active components of the plant hence we isolated and purified the active compound MCO and tested it in an animal model of inflammation such as collagen induced arthritic (CIA) rat. CIA is a T-cell dependent animal model of RA which is widely used to evaluate potential therapeutic compounds [6]. Similar to RA, the up-regulation of pro-inflammatory cytokines and nitric oxide (NO) are also associated with CIA. There are many clinical, pathological and histopathological features which are mutually shared by RA and CIA. In the present study, the therapeutic potency of the active compound MCO was evaluated in the CIA rat model for its use as a possible lead compound for RA treatment.

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2. Materials and methods

2.1. Chemicals and reagents used

Methanol, diethyl ether, acetonitrile, ethanol, glacial acetic acid, dimethyl sulphoxide (DMSO), silica, toluene, ethyl acetate, formic acid, hydrogen peroxide and sulphuric acid of HPLC and/or analytical grade were purchased from Merck. Trifluoroacetic acid, complete Freund's adjuvant, bovine serum albumin and Tween-20 were purchased from Sigma Aldrich. Other reagents were of analytical grade.

2.2. Ethics statement

Animal experiments were performed in accordance to the protocols approved by the Animal Ethics Committee of the Institute of Genomics and Integrative Biology (registration number: 9/1999/CPCSEA).

2.3. Plant extraction and isolation of MCO

R. graveolens L. plant was procured from Homeopathic Pharmacopeia Laboratory (HPL) herbal garden and authenticated by Dr. H.B. Singh, Head, Raw Materials Herbarium and Museum, National Institute of Science Communication and Information Resources (NISCAIR) (NISCAIR is a central government Institute responsible for recognition and validation of plants, and plant products in Delhi, India). A specimen was deposited in the herbarium (specimen voucher number: 1557/155).

The plant extraction and compound isolation was done using bioactivity guided method as described by Raghav et al. [7] with some modifications. Briefly, the dried whole plant material was ground and soaked in 50% methanol in water, exhaustively extracted thrice at room temperature and evaporated under reduced pressure (using Rotavapor, Buchi). The extract was then filtered and lyophilized using freeze drier (Vertis, USA). The dried extract was resuspended in deionized water and stirred to get uniform suspension. Active material was exhaustively extracted using diethyl ether. The dried sticky mass recovered from the ether fraction was dissolved in DMSO, loaded on to the silica column and was eluted with 30% acetonitrile. The eluted fractions were freeze dried and tested for homogeneity through thin layer chromatography (Merck KGaA, Germany), with the mobile phase prepared in a specific proportion (toluene:ethyl acetate:formic acid: 50:40:10). Pooled fractions were lyophilized and dissolved in acetonitrile for further purification using high performance liquid chromatography (HPLC) with pre-equilibrated analytical Sunfire™ C_{18} column (5 μm; 4.6 × 150 mm, photodiode array detector, Waters system, pump 600e) at room temperature. The peak showing biological activity (NO inhibition) was subjected to rerun in RP-HPLC column with gradient mobile phase to confirm its purity.

Semi-preparative Sunfire™ C_{18} (10 μm; 10 × 250 mm) column was used for collecting sufficient material and the isocratic mobile phase containing 30% acetonitrile, in deionized water with 0.05% TFA was used for the columns. The purified compound designated as MCO was identified and characterized using electron spray ionization-mass spectroscopy (ESI-MS), mass spectrum of compound MCO showed the $[M–H]^+$ at m/z 174.9669 corresponding to the formula $C_{10}H_8O_3$.

2.4. Maintenance of cell line and their use to test MCO

Murine macrophage cell line J774 was procured from National Centre for Cell Science (NCCS), Pune and were cultured in RPMI-1640 medium (HiMedia) containing 2 g/l of sodium bicarbonate. RPMI medium was supplemented with antibiotic–antimycotic solution (HiMedia) and 10% heat inactivated fetal bovine serum (Biological Industries, Israel) and maintained at 37 °C in a humidified CO_2 incubator. Before starting any experiment, the culture was allowed to grow to 80% confluence.

The J774 cells were challenged with lipopolysaccharide (LPS; Sigma) of *Escherichia coli* (serotype, 055:B5) at a concentration of 1 μg/ml for inducing measurable concentration of nitrite with minimum cytotoxicity. The inhibitory effect of MCO on nitric oxide (NO) production by macrophage was preliminarily studied by the addition of different concentrations of the former (5, 10 and 20 μg/ml) with LPS (1 μg/ml) stimulation for 24 h. The cell free culture supernatant after 24 h of incubation was used to estimate NO using Griess nitrite assay [8] and RT-PCR as described below. The cells were also subjected to electrophoretic mobility shift assay (EMSA) for NF-κB. In all the above cases, unstimulated J774 cells were treated as negative control.

2.5. Gel retardation assay (EMSA) for NF-κB activation

A double stranded DNA probe for the consensus sequence of NF-κB (Gel shift assay system, Promega) was used for gel shift analysis after end labeling of the probes with [γ-^{32}P] ATP and T4 polynucleotide kinase by the vendor recommended protocol. The reaction mixtures contained 2 μl of 5× binding buffer containing 20% glycerol, 5 mM $MgCl_2$, 250 mM NaCl, 2.5 mM EDTA, 2.5 mM DTT, 0.25 mg/ml poly-dI–dC and 50 mM Tris–HCl (pH 7.5), 2 μg of nuclear extracts and sterile water in a total 10 μl volume. Reactions were initiated by the addition of 1 μl probe (10^6 cpm) for 30 min at room temperature. The specificity of protein binding to the DNA was confirmed by competition reactions, in which a 20-fold molar excess of unlabelled oligonucleotides was added to each reaction mixture before the addition of radio-labeled probe. Samples were loaded onto 4% native polyacrylamide gel and ran at 140 V. The gels were removed, covered by saran wrap followed by autoradiography using Fuji FLA-2000 Phosphorimager.

2.6. Experimental design, induction of CIA and compound administration in rats

Female Wistar rats, aged 6–8 weeks (170–200 g) were procured from Vallabhbhai Patel Chest Institute, Delhi, India. Rats were maintained at a controlled temperature of 25 °C ± 2 and humidity of 42% ± 5 on a 12-hour light/dark cycle and were fed with standard rodent chow (Nutrilab rodent feed, Provini) and water ad libitum.

Animals were randomly divided into 5 groups with 5 animals in each (n = 5) and acclimatized for 7 days before the initiation of the experiments. One of the groups served as 'control' whereas, the other 4 groups were subjected to CIA induction. Immunization of rats was carried out by injecting porcine immunization grade native type II collagen (CII) (Chondrex, USA). Collagen was dissolved in sterile filtered acetic acid (0.05 M) at a concentration of (2 mg/ml) by keeping it overnight at 4 °C with mild shaking and emulsified in CFA (Sigma) at a ratio of 1:1 to the final concentration of 1 mg/ml for injection.

Multiple intradermal injections of 200 μg emulsified collagen were given proximal to the base of the tail and four other sites at the back of each rat on day 0 (primary immunization) and a booster injection on day 7 of the same dosage as the primary immunization. Rats were examined throughout the experiment for evidence of arthritis or non-articular lesions. The onset of clinical arthritis was observed within 12 to 16 days after the primary immunization. CIA was allowed to develop till day 20, and then two of these 4 groups were treated daily with MCO (2 and 20 mg/kg body weight) and designated as 'CIA + MCO' group; the 'CIA + indomethacin' group was treated daily with indomethacin (1 mg/kg). The collagen immunized group, which was given isotonic PBS as vehicle only, was designated as 'CIA + vehicle' group. 'Control' was the naïve animals' group with no collagen immunization but was injected with vehicle only. All the groups were given the respective dosages daily by intraperitoneal (i.p) injections for 20 days, from day 21 until day 41. Animals were euthanized on day 42 by intraperitoneal injection of sodium thiopental.

The dose selection was done on the basis of some preliminary experiments; four groups of CIA animals were treated with different doses of MCO (2, 5, 10 and 20 mg/kg body weights). Morphological parameters such as arthritic score, arthritic index, and behavioral