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Research paper

Antiarthritic and antiinflammatory propensity of 4-methylesculetin, a coumarin derivative

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ABSTRACT

Coumarins are a group of natural compounds widely distributed in plants. Of late, coumarins and their derivatives have grabbed much attention from the pharmacological and pharmaceutical arena due to their broad range of therapeutical qualities. A coumarin derivative 4-methylesculetin (4-ME) has known to possess effective antioxidant and radical-scavenging properties. Recently they have also shown to down regulate nuclear factor-kappa B (NF-κB) and protein kinase B (Akt) that play a vital role in inflammation and apoptosis. In view of this, the present study investigated the anti-arthritic potentiality of 4-ME by assessing its ability to inhibit cartilage and bone degeneration, inflammation and associated oxidative stress. Arthritis being a debilitating joint disease, results in the deterioration of extracellular matrix (ECM) of cartilage and synovium. Participation of both enzymatic and non-enzymatic factors in disease perpetuation is well documented. The present study demonstrated the mitigation of augmented serum levels of hyaluronidase and matrix metalloproteinases (MMP-13, MMP-3 and MMP-9) responsible for cartilage degeneration by 4-ME. It also protected bone resorption by reducing the elevated levels of bone-joint exoglycosidases, cathepsin-D and tartrate resistant acid phosphatases. Further, 4-ME significantly ameliorated the upregulated non-enzymatic inflammatory markers like TNF-α, IL-1β, IL-6, COX-2 and PGE_2. Besides, 4-ME effectively stabilized the arthritis-induced oxidative stress by restoring the levels of reactive oxygen species, lipid and hydro peroxides and antioxidant enzymes such as superoxide dismutase, catalase and glutathione-S-transferase. Thus, the study suggests that 4-ME could be an effective agent to treat arthritis and associated secondary complications like oxidative stress.

1. Introduction

Coumarins are the natural phenolic compounds found mainly in citrus fruits, tomatoes, vegetables and green tea. Some naturally occurring therapeutically important coumarin derivatives include umbelliferone (7-hydroxycoumarin), esculetin (6,7-dihydroxycoumarin), herniarin (7-methoxycoumarin), psoralen and imperatorin. Of late, due to wide range of pharmacological abilities such as anti-oxidant, anti-thrombotic, anti-inflammatory, anti-viral and anti-tumor properties, coumarins and their derivatives have attracted much insight from the phyto-therapeutic arena. Besides, coumarins and their derivatives were also reported to be potent inhibitors of nuclear factor-κB (NF-κB) activation via extracellular signal-regulated kinases/mitogen-activated protein kinase (ERK/MAPK) and phosphatidylinositide 3-kinases/protein kinase B (PI3K/Akt) pathways and are also proven to be effective free radical scavengers [1,2]. The NF-κB family proteins are involved in the regulation of many biological processes and determine the outcome of the host immune responses. Lately, quite a few studies suggest that the hyperactivation of the NF-κB pathway is often implicated in the development and progression of a host of pathophysiological events including autoimmune disorders, arthritis, cancer, cardiovascular and neurodegenerative disorders. A coumarin derivative 4-methylesculetins (4-ME) has been proven to be an effective anti-oxidant, anti-inflammatory and free radical scavenger [1–3]. Recently, it has been also demonstrated that 4-ME down regulate NF-κB and Akt *in vivo* [4]. In addition, 4-ME has been claimed to be less toxic and a potent

Abbreviation: MMPs, matrix metalloproteinases; HAases, hyaluronidases; ROS, reactive oxygen species; DCF, dihydrodichlorofluorescin diacetate; HVA, homovanillic acid.

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therapeutic molecule than any other coumarin derivatives. Recently, 4-ME was demonstrated as non-genotoxic *in vivo* [5].

Based on these foregrounds, the present study investigated the anti-arthritic potentiality of 4-ME. Arthritis being a debilitating joint disease, results in the deterioration of extracellular matrix (ECM) of cartilage and synovium. The increasing incidences of obesity and oxidative stress have greatly contributed toward a growing arthritic population across the world and thus arthritis is considered as the major socio-economic threat to the middle aged population [6]. The vigorous action of matrix metalloproteinases (MMPs), hyaluronidases (HAases) and aggrecanases during arthritis ends up in degenerated articular cartilage that constitutes collagen-II, hyaluronan (HA), aggrecan and other proteoglycans, as well as subchondral bone [7]. In addition, the non-enzymatic factors like reactive oxygen species (ROS) and inflammatory mediators like tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, IL-6, cyclooxygenase-2 (COX-2) and prostaglandin E_2 (PGE_2) largely contribute in the degeneration of cartilage and bone by further stimulating synovial fibroblast to secrete inflammatory cytokines and ECM degrading enzymes [8]. As a result, bone metabolic enzymes such as exoglycosidases, cathepsin D and phosphatases were also activated at the joints ensuing bone resorption and cartilage degeneration. Furthermore, HAase- and MMPs-arbitrated end products like HA oligosaccharides and fibronectin fragments drive the NF-κB-mediated up regulation of pro-inflammatory and ECM deteriorative agents [9,10]. Besides, arthritis-associated oxidative stress worsens the condition, which is considered as the major secondary complication of arthritis that damages the vital organs. However, the severe secondary complications linked to the currently involved drugs in particular, non-steroidal anti-inflammatory drugs (NSAIDS) have been a major threat in the treatment strategy of arthritis. In consequence, plant therapeutics targeting NF-κB, pro-inflammatory mediators and ECM degrading enzymes have grabbed the limelight. In view of this, the present study evaluated 4-ME for its anti-arthritic abilities. The strategy of the study was to assess the four key features of arthritis progression that include cartilage degeneration, bone resorption, inflammation and oxidative stress.

2. Materials and methods

2.1. Chemicals

Freund's complete adjuvant (FCA), dihydrodichlorofluorescin diacetate (DCFDA), O-phthalaldehyde (OPT) and HEPES were obtained from Sigma chemicals, USA. 4-ME was purchased from Acros organics-part of Fisher scientific, Geel, Belgium. Homovanillic acid (HVA) was from Sisco research laboratories Pvt. Ltd. Mumbai, India. Antibodies were purchased from Cayman chemicals, Ann Arbor, Michigan 48108, USA and Epitomics, Inc. Burlingame, CA, USA. PGE_2 EIA express kit was purchased from Cayman chemicals, USA. Micro-titer plates were purchased from Tarsons product Pvt. Ltd. Kolkata, India. All other chemicals were of analytical grade.

2.2. Experimental design

Adult Wistar male rats were collected from University Central Animal Facility, DOS in Zoology and housed under a controlled environment. All experiments were approved by the Animal Ethical Committee (Order No: MGZ/637/2011-12 dated 12-07-2011; UOM/IAEC/04/2011), DOS in Zoology, University of Mysore, Mysore and were in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). The experimental rats were divided into 8 groups each consisting of 7 rats. Group I – Saline control (0.9% saline); Group II – Arthritic; Group III – Arthritic rats treated with Ibuprofen (10 mg/kg); Group IV and V – Arthritic rats treated with 4-ME (25 and 50 mg/kg respectively); Group VI – Ibuprofen (10 mg/kg) treated rats with no arthritis induced; Group VII and VIII – 4-ME (25 and 50 mg/kg respectively) treated rats with no arthritis induced. 4-ME at this dose has been claimed to be non-toxic and details of toxic effects not reported other than lethal dose value. According to the EPA TSCA Section 8(b) chemical inventory data, the lethal dose of 4-ME when administered orally is above 3000 mg/kg body weight for rodents (RTECS No.: GN6384500). Arthritis was induced by subcutaneous injection (100 μL) Freund's complete adjuvant (FCA) containing 10 mg/mL heat killed *Mycobacterium tuberculosis* at the right hind palm surface of all rats except saline control group and housed for 10 days. 4-ME, ibuprofen and saline were fed through oral gavage once a day from 11th day to 25th day. Ibuprofen was used as a standard NSAID control. On the 25th day, paw joint of all animals were radio-graphed before sacrificing. Animals were then sacrificed and blood was collected through cardiac puncture. Serum was separated and stored at −20 °C. Liver and spleen tissues were obtained, homogenized and stored at −20 °C for the assessment of different biochemical parameters. The results obtained with Groups VI, VII and VIII were as similar to Group I (there was no significant difference) in all aspects. Thus, they were not described in any of the results in order to avoid the possible misreading.

2.3. Physical assessment

The paw volumes of all rats were measured every fourth day by mercury displacement method. The right hind legs were dipped in mercury up to the hairline and the amount of mercury displaced was read in centimeters using mercury edema meter and statistically expressed [11]. Similarly, the body weight of all the rats was also measured at the same interval and statistically expressed in grams. In addition, radio-graphs of control and experimental rat joint were done to evaluate the cartilage and bone damage. All radiographs were taken with X-ray film (Kodak Diagnostic Film) using MBR-1505R (Hitachi Medical Corporation, Japan). Settings for radiography were 5 mA, 40 kV and 1 s exposure. Films were placed 60 cm below the X-ray source.

2.4. Assessment of cartilage degradation

2.4.1. Determination of HAase and MMP activity

The serum HAase activity was measured by zymography. HAase activity was detected as unstained translucent bands against dark blue/violet background [12]. The serum MMP-3, MMP-9 and MMP-13 levels were estimated by western blot analysis using MMP-3 (1:1500), MMP-9 (1:150,000) and MMP-13 (1:10,000) diluted antibody respectively. Further, β-actin was used as loading control.

2.4.2. Assessment of bone degeneration

Severity of bone degeneration and resorption were assessed by determining the levels of bone metabolic enzymes in bone joint homogenate. Hexosaminidase and glucuronidase activities were measured by the determination of *p*-nitrophenol released from *p*-nitrophenyl β-*N*-acetyl glucosaminide and *p*-nitrophenyl β-glucuronide, respectively [13]. Further, acid phosphatase (ACP), tartrate resistant acid phosphatase (TRAP) and alkaline phosphatase (ALP) levels were determined using *p*-nitrophenyl phosphate as the substrate [14]. Cathepsin D (Cat-D) was measured according to the protocol of Vijayan et al., [13]. Briefly,

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