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Anti-TNF-α and anti-arthritic effect of patuletin: A rare flavonoid from *Tagetes patula*

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ABSTRACT

Rheumatoid arthritis (RA) poses a serious health problem as a chronic autoimmune joint disease with significant mortality and morbidity. Proinflammatory cytokines TNF-α and IL-1β, reactive oxygen species (ROS), and activated $CD4^+$ T-cells play key roles in the progression of arthritis. The aim of the study is to evaluate the *in vitro* and *in vivo* immunomodulatory and anti-arthritic effect of flavonoid patuletin, isolated from *Tagetes patula*. ELISA was applied for quantification of TNF-α and IL-1β. Intracellular and extracellular ROS production from phagocytes was measured by the chemiluminescence technique. Proliferation of T-cells was observed using a liquid scintillation counter. Cytotoxicity was assessed by a MTT assay. The serological and histological analysis studies were performed using a rodent model of adjuvant-induced arthritis (AIA). Expression of p38 and NF-κB after treatment of compound was observed by western blotting. Patuletin showed potent inhibitory effects on TNF-α *in vitro* as well as inhibited the production of both cytokines *in vivo*. It also showed potent suppression of proliferation of T-cells and significantly inhibited the extracellular and intracellular ROS production. Patuletin revealed significant anti-inflammatory and anti-arthritic activities in the rodent model of adjuvant-induced arthritis (AIA). Histologically, it causes mild bone destruction compared to the arthritic control group, thus representing its anti-arthritic potential. Based on these studies, patuletin could be considered as a potential immunosuppressive and anti-arthritic lead candidate.

1. Introduction

Rheumatoid arthritis (RA) is a very common chronic inflammatory joint disease worldwide. It causes irreversible joint destruction and functional impairment. Approximately 1% of the adult population, predominantly females, are affected with the severity of this disease. Although the precise etiology is still remains unclear, pathogenic mechanism involves intense inflammation in synovial joints so that the normally delicate synovial membrane becomes infiltrated with mononuclear phagocytes, lymphocytes, and neutrophils. The course of RA is variable, but usually patients develop difficulties in mobility due to progressive dysfunction of cartilage and bones around the joints [1–5].

Activated $CD4^+$ T helper cells stimulate synovial fibroblasts, monocytes and macrophages. They are the main cells involved in the early stages of inflammation; releasing proinflammatory cytokines tumor necrosis factor (TNF)-α, and interleukin (IL)-1β, resulting in the secretion of degradative enzymes, namely the matrix metalloproteinases (MMPs), and cause the activation of the whole cascade of inflammation that eventually leads to cartilage erosions and destruction of bones and soft tissues. According to literature, TNF-α is the main cytokine in the progression of inflammatory process and bone destruction. Increased synovial fluid concentration of this cytokine correlates directly with gradual bone and cartilage destruction in RA. Currently, the blockage of cytokines by various means like blocking of TNF-α and IL-1β in RA by monoclonal antibodies or receptor antagonist increases possibilities for the treatment of chronic inflammatory and autoimmune diseases [6–12].

Evidences support that inflammatory cells including macrophages, neutrophils, lymphocytes and endothelial cells produce ROS that are

Abbreviations: RA, rheumatoid arthritis; TNF, tumor necrosis factor; NO, nitric oxide; IL, interleukin; ROS, reactive oxygen species; AIA, adjuvant induced arthritis; MMP, matrix metalloproteinase; PMNs, polymorphonuclear cells; PO, peroxide; RF, rheumatoid factor; SD, Sprague Dawley; NF-κB, nuclear factor kappa B; AP-1, activator protein 1; iNOS, inducible nitric oxide synthase; COX, cyclooxygenase; MAPK, mitogen associated protein kinase; JNK, c-Jun N-terminal kinases; ERK, extracellular signal regulated kinases; ATF, activating transcription factor; NFAT, nuclear factor of activated T-cells; TCR, T-cell receptor.

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involved both directly and indirectly in the progression of pathogenesis of inflammatory synovitis and oxidative damage [13–15]. Although nitric oxide (NO) plays the central role in various physiological processes, its increased production is pathological. At the site of synovial inflammation it is found to mediate the production of cytokines, signal transduction molecules and mitochondrial functions *etc.* and plays an important role in the pathogenesis of RA [16–17]. Antioxidant defense system includes enzymes like glutathione peroxidase, superoxide dismutase and catalase, which are known to have a protective role against the ROS. But overproduction of ROS reduces the level of endogenous antioxidants thereby leading to oxidative stress induced tissue damage in RA patients [18–20].

Tagetes patula Linn. (French marigold) belongs to the family compositae is widely known for its phytochemical and medicinal properties. Traditionally, the plant is used to treat cough, colic, constipation, diarrhea, rheumatism and eye problems. The plant is known to possess antimicrobial, antiseptic, blood purifying, and diuretic properties. The whole plant of *T. patula* is taken internally in the form of a powder or as a decoction. The flowers of *T. patula* are edible and used in refreshing drinks. Chemically, different parts of *T. patula* contain carotenes, terpenes, steroids, flavonoids and thiophenes [21–26]. Flavonoids of different classes are known to exhibit several pharmacological and biochemical properties and also have a regulatory role on different hormones and have great therapeutic potential due to their wide biological actions [27]. Patuletin is one of the major flavonoids found in the *T. patula*. It was first isolated by Rao and Seshadri in 1941 from the petals of *T. patula* and represented as 3,5,7,3′,4′-pentahydroxy-6-methoxy flavone. It belongs to a biologically active group of phenolic compounds "flavonols" which are widely distributed in nature. It is a non-toxic flavonoid that can be extracted easily in bulk quantity mainly from the flowers. It has also been reported from other *Tagetes* species. Patuletin is known to possess various biological activities, which include radical scavenging, anti-inflammatory, antimicrobial, analgesic, antispasmodic, hypotensive, nematicidal and cholagogic properties. Moreover, molecular recognition ability of patuletin coated gold nanoparticle has also been reported [23–30].

These significant properties of patuletin along with its non-toxicity [23] and potent inhibitory effect on various inflammatory parameters that are critically involved in propagation of chronic inflammation; particularly potent suppressive effect observed on production of proinflammatory cytokine TNF-α led us to evaluate its inhibitory potential in our *in vivo* studies, using a model of chronic inflammatory disease *i.e.* adjuvant- induced arthritis.

The current study describes for the first time the anti-arthritic and anti-TNF-α activity of patuletin and its significant effect on other inflammatory parameters. The correlation between *in vitro* and *in vivo* studies was observed throughout the study. Based on the results obtained, it is possible to conclude that patuletin could be taken further as a potential immunosuppressive, anti TNF-α and anti-arthritic lead candidate.

2. Materials and methods

2.1. Animals

In vivo experiments were performed using female Sprague Dawley (SD) rats weighing 150–250 g. Animals were kept at 21 ± 2 °C with 12 h light/dark cycle and were fed with standardized pelleted diet and water. Experiments were performed under the ethical guidelines of the International Association for the Study of Pain in Conscious Animals [35] and guidelines set by the scientific advisory committee, animal care, use and standards, International Center for Chemical and Biological Sciences were followed (Protocol No. 1209004).

Each treatment group contained 12 animals with randomly distributed weights.

For oxidative burst study NMRI mice weighing (25–30 g) were used. The homing conditions were maintained as same to that of rats.

2.2. Cell lines and antibodies

THP-1 cell line (human monocytic leukemia) was purchased from ECACC; 3T3 (mouse embryo, fibroblast cells), CC1 (rat, epithelial, liver cells) and MDBK (bovine kidney cells) were purchased from ATCC. Jurkat (human T lymphocyte leukemia) cells were kindly provided by Professor Daniel Hoessli from University of Geneva, Switzerland and RAW 264.7 (mouse macrophage leukemia) cells were kindly donated by Dr. Maria Lerm from Linkoping University, Sweden.

All primary antibodies including rabbit polyclonal to p38 and phosphorylated p38 phospho Y182 + T180), anti-NF-κB p65 antibody and phosphorylated anti-NF-κB antibody (phospho S536) were purchased from Abcam (Cambridge, UK). Immunopure (31460) Goat anti-rabbit IgG, peroxidase conjugated secondary antibodies were purchased from Thermoscientific (Rockford, USA).

2.3. Plant material

T. patula flowers were collected during November 2008 from gardens of University of Karachi. It was identified by plant taxonomist Dr. Rubina Dawar of Botany department, University of Karachi and the voucher specimen (KUH GH 67280) was deposited in the herbarium of the same department. Patuletin was obtained through the process as described earlier [23–26].

2.4. General method

EI mass spectrum (70 eV) was recorded on MAT-312 (Finnigan, Germany) spectrometer. ^{1}H NMR spectra were measured using deuterated solvent C_3D_6O, on Bruker Avance AV-400 operating at 400 MHz. ^{13}C NMR spectra (Broad band, DEPT) were recorded in the same solvent and on the same instruments at 100 MHz.

2.5. Isolation of patuletin from T. patula flowers

Fresh, undried and uncrushed *T. patula* flowers (9.1 kg) were extracted with different organic solvents sequentially with increasing polarity using petroleum ether (P.E), dichloromethane (D.C), and ethyl acetate (EtOAc). The insoluble matter settled down in concentrated EtOAc extracts when left for few days at room temperature, was filtered giving filtrate and insoluble yellow powdery material which was washed with P.E and D.C several times furnishing refined powdery material showing a single spot on TLC (silica gel 60F_{254} $CHCl_3$:MeOH, 9:1, R_f = 0.28; RP-18 MeOH:H_2O 6:4, R_f = 0.34). It was identified as pure flavonoid, patuletin (**1**, 15.1 g).

2.6. Characterization of patuletin (1)

State: Yellow powder EI-MS (70 eV) *m/z* (rel. int.): 332 (M^+, 93), 314 (70), 289 (100); ^{1}H NMR (δ, Hz): 3.87 (s, 3H, OCH_3), 6.59 (s,1H, C-8), 7.82 (d, *J* = 2.2 Hz, C-2′), 6.98 (d, *J* = 8.4 Hz, C-5′), 7.69 (dd, *J* = 8.4, 2.2 Hz, C-6′), 9.45 (br. s, 3-OH), 12.20 (br. s, 5-OH), 9.28 (br. s, 3′,4′-OH), OH signals disappeared upon D_2O shake ^{13}C NMR (δ) 147.14 (C-2), 136.40 (C-3), 176.80 (C-4), 153.12 (C-5), 131.72 (C-6), 157.79 (C-7), 94.53 (C-8), 152.39 (C-9), 104.58 (C-10), 123.86 (C-1′), 115.82 (C-2′), 145.75 (C-3′), 148.30 (C-4′), 116.22 (C-5′), 121.55 (C-6′) and 60.79 (OCH_3-6).

2.7. Cytokines production and quantification

THP-1 cells were maintained in RPMI-1640 (Sigma-Aldrich, Steinhiem, Germany), supplemented with 5.5 mmol/L glucose (Bio M laboratories, Dalan, Malaysia), 10% fetal bovine serum (FBS), 2 mmol/L glutamine, 1% penicillin-streptomycin (PAA Laboratories, GmbH, Pasching, Austria), 50 μmol/L mercaptoethanol (Merck, Darmstadt, Germany), 10 mmol/L HEPES (MP Biomedicals, Illkirch, France), and