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Clitoria ternatea flower petals: Effect on TNFR1 neutralization via downregulation of synovial matrix metalloproteases

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ABSTRACT

Ethnopharmacological relevance: *Clitoria ternatea* Linn. (*C. ternatea*) is a traditionally used herb in arthritis, and its anti-arthritic activity has been attributed to polyphenols (e.g. quercetins) from its flower petal.
Aim of the study: The present study was designed to investigate whether *C. ternatea* or quercetin-3ß-D-glucoside (QG) support the antibody mediated TNFα-receptor 1 (TNFR1) neutralization to ameliorate arthritis in mice.
Materials and methods: Development of collagen-induced arthritis (CIA) in male Swiss mice (20–22 g, 3–4 weeks of age) was followed by estimation of synovial polymorphonuclear cell (PMN) accumulation (in terms of myeloperoxidase activity), synovial and systemic release of cytokines, chemokines and C-reactive protein (CRP) by enzyme-linked immunosorbent assay (ELISA), biochemical estimation of synovial free radical generation and antioxidant status, as well as immunoblot assessment of synovial TNFR1, toll-like receptor 2(TLR2), cyclooxygenase-2(COX-2) and inducible nitric oxide synthase (iNOS) expression; and zymographic analysis of synovial matrix-metalloprotease-2 (MMP-2) activity.
Results: CIA was induced from day 2 post-secondary immunizations as evidenced from arthritic scores and joint swelling in parallel to increased inflammatory and oxidative stress parameters in synovial joints. Long term supplementation with extract from *Clitoria ternatea* flower petals CTE (50 mg/kg) and QG (2.5 mg/kg) upto 24 days post booster immunization augmented anti-arthritic potential of TNFR1 neutralization with anti-TNFR1 antibody (10 µg per mice) in terms of reduced MPO activity, decrease in release of pro-inflammatory cytokines, chemokines, reactive oxygen species (ROS)/ reactive nitrogen species (RNS) production in parallel to significant ($p < 0.05$) reduction in TNFR1, TLR2, iNOS, COX-2 and MMP-2 expression.
Conclusion: CTE and QG possess potential anti-arthritic activity which targets synovial MMP-2 in arthritic joints and TNFR1 targeting followed by CTE or QG treatment might become a combinatorial approach in future therapeutic research in treatment of arthritis.

1. Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease into joints, with 0.8% greater incidence in women all over the world, and heredity, microbial infection, endocrine regulation, environment and several other factors were considered to contribute (Subramoniam et al., 2013). The major pathological change associated with RA is synoviositis followed by joint degradation, which is characterized by ROS/RNS generation due to abnormal regulation of NADPH oxidase (NOX), nitric oxide synthase (NOS), and 5-lipoxygenases (5-LOX)

Abbreviations: ASK-1, Apoptosis signal regulating kinase-1; CAT, Catalase; CFA, Complete Freund's adjuvant; CIA, Collagen induced arthritis; COX-2, Cyclooxygenase-2; CPCSEA, Committee for the purpose of control and supervision of experimental animals; CRP, C-reactive protein; CTE, *Clitoria ternatea* extract; DAG, Diacyl glycerol; DTNB, 5, 5′ dithiobis 2-nitrobenzoic acid; ELISA, Enzyme linked immunosorbent assay; GSH, Reduced glutathione; IAEC, Institutional Animal Ethics Committee; ICAM, Intercellular adhesion molecule-1;; IFA, Incomplete Freund's adjuvant; IFN-γ, Interferon gamma; IKK, IκB kinase; IL, Interleukins; iNOS, inducible isoform of nitric oxide synthase; IP_3, Inositol tri-phosphate; JNK, Jun-N terminus kinase; LPO, Lipid peroxides; Mac-1, Macrophage-1 antigen; MAPK, Mitogen activated protein kinase; MCP-1, Monocyte chemotactic protein-1; MPO, Myeloperoxidase; mTOR, mammalian Target of Rapamycin; NAC, N-acetyl cysteine; NADPH, Nicotinamide adenine dinucleotide phosphate; NF-κB, Nuclear factor -κB; NO, Nitric oxide; NOX, NADPH oxidase; NSAIDS, Non-steroidal anti-inflammatory drugs; PI3K, Phosphoinositol-3 kinase; PMN, Polymorphonuclear neutrophil; QG, Quercetin-3β-D-glucoside; RIP, Receptor interacting protein; RIPA, Radio immuno assay precipitation buffer; RNS, Reactive nitrogen species; ROS, Reactive oxygen species; SDS-PAGE, Sodium dodecyl sulfate Polyacrylamide gel electrophoresis; SOD, Superoxide dismutase; sTNF, Soluble Tumor necrosis factor-α; TBARS, Thiobarbituric acid reactive substrates; TCA, Trichloro-acetic acid; TNFR, Tumor necrosis factor-α receptor; TNFα, Tumor necrosis factor-α; TRAF, TNF receptor associated factor

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(Aggarwal et al., 2011). Erosion of the joint is brought about by matrix metalloproteases (MMPs) released chiefly by fibroblasts and to lesser extent by chondrocytes, macrophages as well as osteoblasts, and mature osteoclasts in arthritic joints (Shiozawa et al., 2011). MMP-2 and MMP-9 particularly plays crucial role in cartilage destruction and bone erosion, and are therefore suggested as potential therapeutic targets in rheumatoid arthritis (Bartok and Firestein, 2010).

Considering the disadvantages of many conventional medicines and other therapeutic approaches the modern research is still focussed on development of plant based remedies in RA. *Clitoria ternatea* Linn (Family: Fabaceae, commonly known as butterfly pea plant in English) is an evergreen twinning herb which is found in adequate amounts in regions of South-eastern Asia. Roots of *C. ternatea* were reported to possess potential anti-arthritic effect besides its immunosuppressive and antioxidant actions (Mukherjee et al., 2008). However, the use of flower petals as anti-arthritic ailment has not been reported. Quercetins and their different derivatives are basically flavonoid class of polyphenols found in adequate amounts in fruits, flowers and other parts of a wide variety of medicinal plants. Flower petals of *C. ternatea* Linn is a rich source of a huge variety of polyphenols.

Three flavonol glycosides, kaempferol-3β-glucoside, quercetin-3β-glucoside, and myricetin-3β-glucoside were isolated from the petals of *C. ternatea*; however quercetin-3β-D-glucosides were identified by NMR studies, and LC/MS studies in highest quantities; particularly in methanolic extracts of the *C. ternatea* flower petals (Kazuma et al., 2003; Slimestad et al., 1995). Derivatives of quercetins, particularly the glycoside conjugated forms are abundant in nature (Aziz et al., 1998). Bioactivities of quercetin glucosides include long lasting anti-inflammatory action via inhibition of iNOS, COX and LOX enzyme activities (Williams et al., 2004; Lawrence et al., 2002), decreased NF-κB-DNA binding (Chen et al., 2005; Ruiz et al., 2007). The hydroalcoholic extracts of blue flower petal possess potential anti-oxidant and anti-inflammatory activities, and its disease modifying activities has been correlated to its flavonoid components (Neda et al., 2013; Chayaratanasin et al., 2015), as well as the in vitro anti-arthritic potential of *C. ternatea* (Bharathee et al., 2014) has also been reported.

Amongst a complex network of many different components involved in RA, TNFα and IL-1β plays pivotal role in its pathogenesis as evidenced from earlier studies depicting the effect of neutralization of TNF, IL-1 or their functional receptors (Williams et al., 2000; Fischer et al., 2015). Despite of successful clinical trial, long-term treatment with TNF blockers is accompanied by a higher risk of tuberculosis reactivation and serious infections, whereas the effect of TNF blockers on incidence and/or manifestation of malignancies is sometimes discussed controversially (Desai and Furst, 2006; Wallis, 2008; Rosenblum and Amital, 2011), which explores the rationale underlying targeting of TNF receptors instead of TNF itself. This might explain why selective targeting of TNFR type 1 would be desirable in clinical trials against chronic inflammatory diseases.

Elevated TNFα was shown to increase TLR2 expression dramatically at transcriptional level via NF-κB pathway in murine fibroblasts (Bonnard et al., 2000), whereas TNFR1 knockdown in murine staphylococcal infection have partially suppressed TLR2 expression in pre-osteoblastic cells (Chen et al., 2016). Toll like receptor (TLR-)s like TLR-4 and TLR2 have found to play crucial role in pathogenesis of RA probably via increasing NF-κB mediated transcription of inflammatory genes and increased ROS/RNS production (Lucas and Michael, 2013), and it is also described that elimination of ROS/RNS by many naturally occurring substances such as quercetin, pycnogenol, and some other flavonoids (polyphenolic compounds present in dietary plants) have attenuated expression of TLR4 and TLR2 (Lucas and Michael, 2013). Therefore TNFR1-TLR2-NF-κB mediated joint inflammation via increased oxidative stress and synovial MMPs (Fig. 1) might be susceptible to natural antioxidant mediated amelioration.

In this study anti-arthritic effect of methanolic extract from *C. ternatea* flower petal (CTE) and purified quercetin-3β-D-glucoside was evaluated using multiple oral doses in the clinical regimen at every alternative day following induction of arthritis (CIA) in mice. To better characterize the beneficial effect CTE and QG were also applied after anti-TNFR1 antibody treatment in CIA mice; and clinical score of arthritis, and joint swelling were evaluated, followed by assessment of neutrophil infiltration (MPO activity), estimation of synovial and systemic levels of TNFα, interleukins (IL)−1β, −6, −8-, 10, and −12p40, interferon (IFN)-γ, monocyte chemoattractant protein (MCP)−1, and C-reactive protein (CRP), along with measures of synovial free radical generation ($\bullet O_2^-$ and NO production), redox balance (reduced glutathione [GSH], lipid peroxides [LPO]), and activities of superoxide dismutase (SOD) and catalase (CAT) enzymes. Synovial of TNFR1, TLR2, iNOS, and COX-2 expressions as well as MMP-2 activity were also determined.

2. Materials and methods

2.1. Preparation of Clitoria ternatea extract

Whole plants of *C. ternatea* were collected from south eastern wild region of West Bengal. Taxonomical identification of the collected plant materials was done by the Central National Herbarium (CNH), Botanical Survey of India (Ministry of Environment, Forest & Climate Change), Government of India, Shibpur, Howrah and the voucher specimen was deposited, Voucher No. CU/RA/003 (Ref. No. CNH/2017/Tech.II/19).

Plant materials were dried under shade, and ten grams of finely ground *C. ternatea* flower petals were soaked into 30 ml methanol at 30 °C for 12 h with shaking. The methanol was then allowed to evaporate completely under sterile conditions; this extraction protocol of the original materials was repeated thrice. Each final residue was then dissolved into 10 ml methanol and filtered through Whatman No.1 filter paper. Each filtrate was then centrifuged at 2000 rpm for 10 min. Supernatant was collected and air-dried to completeness under sterile conditions. The final yield was 6.5% for the flower petals of *C. ternatea*.

2.2. Animals

Male Swiss-Albino mice (20–22 g, 3–4 weeks of age) were procured from Chittaranjan National Cancer Institute (CNCI), Kolkata, India for use in this study. All animals were housed in separate polystyrene cages in pathogen-free facilities maintained at 25 [± 2] °C, with 50–60% relative humidity, and 12 h light: dark cycle. All mice had ad libitum access to normal laboratory diet that consisted of 22.5% wheat flour, 60% roasted Bengal-gram flour, 5% skimmed milk powder, 4% casein, 4% refined groundnut oil, 4% salt mixture and 0.5% vitamin mixture, as recommended for mice, by the National Center for Laboratory Animal Sciences, National Institute of Nutrition, India and filtered tap water. All experiments involving animals were conducted according to the protocols approved by Department of Animal Ethical Committee, Department of Physiology, University of Calcutta [as it is affiliated to the University of Calcutta is called Institutional Animal Ethics Committee (IAEC)], under the supervision of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines (Registration No. IAEC/IV/Proposal/BB-2/2014, dated August 26, 2014), Ministry of Environment and Forest, Government of India. Mice were randomly allocated into seven groups (n = 6/group): control (Con) group, collagen-induced arthritis (CIA) group, collagen-induced arthritis+CTE (CIA+CTE) group, collagen-induced arthritis+quercetin-3β-D-glucoside (CIA+QG) group, collagen-induced arthritis+anti-TNFR1 antibody (CIAab) group, collagen-induced arthritis+ anti-TNFR1 antibody + CTE (CIAab+CTE) group and collagen-induced arthritis+ anti-TNFR1 antibody + quercetin-3β-D-glucoside (CIAab+QG) group.

CTE at a dose of 50 mg/kg was used for animal experiments in this