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Evaluation of *Cissus quadrangularis* extracts as an inhibitor of COX, 5-LOX, and proinflammatory mediators

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ABSTRACT

Ethnopharmacological relevance: Cissus quadrangularis is an ancient medicinal plant. It is an active ingredient of one Ayurvedic formula called "Laksha Gogglu". Its stem is used in food preparation in India. Traditionally it is used to treat various diseases like asthma, indigestion, ear diseases, irregular menstruation, skin diseases, piles, fractured bones, etc.
Aim of the study: This study aimed to evaluate the ability of the plant extracts to inhibit cycloxygenase (COX-1), cycloxygenase (COX-2), and 5-lipoxygenase (5-LOX) enzyme activity. Western blot analysis was also carried out in the quest to determine the effect of active acetone fraction of *Cissus quadrangularis* (AFCQ) on proinflammatory mediators as acetone extract is found to be the most effective in this study.
Materials and methods: The differential extract of the stem were tested for enzyme inhibition of COX and 5-LOX using spectroscopic and polarigraphic method. Effective acetone extract was partially purified by silica column, one of the active fraction showed dual inhibition against COX and 5-LOX. Western blotting shows downregulation of proinflammatory mediators as well as upregulation of phase-II enzymes.
Results: AFCQ extract showed COX and 5-LOX inhibition with IC_{50} values of 7 μg/ml, 0.4 μg/ml, and 20 μg/ml for COX-1, COX-2 and 5-LOX respectively. It also showed anti-inflammatory activity on RAW 264.7 cell line with IC_{50} value 65 μg/ml. In addition to this it is showing inhibition of proinflammatory mediators like iNOS and TNFα, along with translocation of Nrf-2 and upregulation of HO-1.
Conclusion: AFCQ is a COX and 5-LOX inhibitor isolated from the stems of *Cissus quadrangularis*. It is also effectively downregulate the iNOS, TNFα, and upregulation of HO-1.
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1. Introduction

Cissus quadrangularis is an ancient medicinal plant that has been used in many traditional medicines. Traditionally it is used to treat diverse diseases like rheumatic diseases, allergies, skin diseases, piles, fracture, and bone diseases (Udupa et al., 1965; Deka et al., 1994). It is an active ingredient of Ayurvedic formula called "Laksha Gogglu" which is used for alleviating pain, reducing inflammation and promoting the healing of wounds or simple fracture (Kulkarni, 2001). Stem of *Cissus quadrangularis* is very important part of the plant and used as raw drug in Indian folk medicine (Panda, 1990). Leaves of *Cissus quadrangularis* is used to treat anorexia whereas dried powder of stem with honey is taken orally to reduce pain and inflammation. The stem and leaf of the *Cissus quadrangularis* are used in food preparation in India. Numerous studies demonstrated that methanolic and petroleum ether extract of the whole plant are used in treating osteoporosis and fracture (Deka et al., 1994; Shirwaikar et al., 2003; Sharpa et al., 2007). It possesses antioxidant (Jainu and Shyamaladevi, 2005), anti-inflammatory, antibacterial (Kashikar and George, 2006; Thakur et al., 2009), antiviral (Balasubramaniana et al., 2010), and antiulcerogenic (Jainu and Shyamaladevi, 2004, 2006; Jainu et al., 2010) properties. It has also solved the problems of weight loss and various metabolic syndromes (Oben et al., 2006, 2007). Phytochemical studies of *Cissus quadrangularis* have shown the presence of several phytochemical constituents, such as ascorbic acid, flavonoids, and triterpenoids (Potu et al., 2010).

Inflammatory and pathogenic condition activates the enzymes cycloxygenase (COX) and 5-lipoxygenase (5-LOX). COX and 5-LOX are the key enzymes in the synthesis of prostanoid and eicosonoids from poly unsaturated fatty acids (PUFAs), which are involved in various inflammatory and allergic disorders (Martel-Pelletier et al., 2003; Daniel et al., 2004). Nuclear factor E2 p45-related factor 2 (Nrf-2) is a member of leucine zipper transcription family

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responsible for upregulation of genes of phase-II enzymes in response to oxidative stress and LPS treatment (Annukka et al., 2008). Heme oxygenase-1 (HO-1) is the phase-II enzyme catalyzes the degradation reaction of heme to convert biliverdin further to bilirubin. Deficiency of HO-1 isozyme reveals severe damage of iron metabolism, which leads to liver, and kidney oxidative damage and inflammation. Consequently HO-1 expression increases cell resistance to oxidative injury (Daniel et al., 2004). Inducible nitric oxide synthase (iNOS) and tumour necrosis factor alpha (TNFα) are the proinflammatory mediators. The concentration of iNOS is elevated in injury and inflammation, while TNFα is mainly formed through monocytes and activated macrophages. It is pleotropic proinflammatory cytokine with many actions like fever, macrophage activation, etc., that are central to the pathogenesis.

In the present study, we have examined the in vitro anti-inflammatory activity of different extracts of *Cissus quadrangularis* on COX, 5-LOX and proinflammatory mediators. Inhibition of COX-1 and COX-2 were evaluated by using chromogenic assay and inhibition of 5-LOX was evaluated by using polarigraphic method. Whole plant of C*issus quadrangularis* was extracted with different organic solvents and the acetone extract gave the potential inhibition of COX and 5-LOX. Consequently number of partially purified fraction of acetone extract we have screened to examine the inhibition of COX and 5-LOX. Single active acetone fraction of C*issus quadrangularis* have shown potent inhibition hence called AFCQ. AFCQ was further used to evaluate the eventual mechanism essentially its anti-inflammatory effect in LPS induced inflammation. We found that AFCQ have potentially inhibited COX-2, iNOS, and TNFα expression in LPS treated murine macrophages RAW 264.7 cells. Upregulation of HO-1 expression connected with activation and expression of Nrf-2 might be related in the anti-inflammatory activity of AFCQ.

2. Materials and methods

2.1. Plant material

Plant material was collected from Vidarbh region of Maharashtra in March 2008. The plants were authenticated at the Department of Botany, Rashtrasant Tukdoji Maharaj Nagpur University, Nagpur and voucher specimens were deposited in the herbarium. Botanical name is *Cissus quadrangularis* and voucher specimen number is 9433.

2.2. Preparation of extracts of Cissus quadrangularis

The stem of *Cissus quadrangularis* was shade dried for five to six months, powdered, and extracted successively using soxhlet apparatus with hexane, chloroform, acetone, ethyl acetate, methanol, ethanol, and water in the increasing polarity index. 250 g of dried plant powder was taken for the extraction. All extracts were concentrated to dryness using rotary evaporator. We obtained hexane extract 7.27 g, chloroform extract 5.10 g, acetone extract 2.17 g, ethyl acetate 1.7 g, methanol extract 3.53 g, ethanol extract 1.2 g, and water extract 1 g. The concentrated dry extracts were again dissolved in dimethyl sulphoxide (DMSO), mixed and vortexed for few min and then centrifuged 2000 × *g* for 10 min. Supernatant was subsequently used to check the inhibition of COX-1, COX-2, and 5-LOX.

2.3. Partial purification of acetone extract by column chromatography

Acetone extract partially purified by column chromatography. Active acetone extract passed through open silica column. We obtained around thirteen different fractions, this fractions were dried using rotary vaccum evaporator. Dried fractions again used to verify the inhibition of COX-1, COX-2, and 5-LOX. Out of thirteen fraction ninth fraction shows potent inhibition of COX and 5-LOX, hence called active fraction of *Cissus quadrangularis* (AFCQ) and used in further studies.

2.4. Reagents and chemicals

Phosphate buffered saline (PBS), RPMI 1640; fetal bovine serum (FBS), penicillin and streptomycin were purchased from Gibco BRL. TMPD (N,N,N,N-tetramethyl-*p*-phenylenediamine), Hematin, Trypsin, MTT (3-(4,5-dimethylthiazol-2-yl)-diphenyl tetrazolium bromide), β-actin antibody and lipopolysaccharide or LPS (*Escherichia coli* serotype), nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) were purchased from Sigma–Aldrich chemical company (St. Louis, USA). Antibodies for COX-2, HO-1, TNFα, Nrf-2, iNOS and alkaline phosphatase conjugated anti-mouse, anti-goat and anti-rabbit antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All other chemicals and solvents for chromatography were of HPLC grade. All other chemicals and solvents were of analytical grade purchased from authorized standard companies.

2.5. Extraction and isolation of cyclooxygenase-1 enzyme. (from Ram seminal vesicles)

Ram seminal vesicles were collected from local slaughter house and stored at −80 °C. Before starting the experiment, the ram seminal vesicles were taken out from −80 °C and kept at 4 °C overnight in refrigerator. All the process of Extraction and isolation were carried out below 7 °C.

2.5.1. Preparation of microsomes as a source of cycloxygenase-1

Preparation of microsome was carried out, according to the method of Hemler et al. (1976), with some modifications. Ram seminal vesicles were ground in a grinder. The ground material was homogenized with a waring blender in buffer containing 0.05 M Tris–HCl (pH 8), 5 mM EDTA disodium salt, 5 mM diethyl dithiocarbamate and 0.01% sodium azide. The homogenate was centrifuged at 13,000 × *g* for 15 min, 4 °C. The supernatant was filtered through cheesecloth. The filtered supernatant was again centrifuged at 42,000 rpm for 1 h 10 min, 4 °C by using ultracentrifuge (Himac, CP-100α HITACHI) to obtained microsomal pellet. This microsomal pellet was rehomogenized with a motor-driven Potter-Elvehjem homogenizer, connected to a teflon pestle in a buffer containing 0.05 M Tris–HCl (pH 8), 0.1 mM EDTA disodium salt, 0.1 mM diethyl dithiocarbamate and 0.01% sodium azide. This microsome fraction was stored as small aliquots at −80 °C, and used for further studies as enzyme source.

2.6. Extraction and isolation of cycloxygenase-2 enzyme (from Sf9 insect cell line)

2.6.1. Preparation of microsomes as a source of cyclooxygenase-2

Preparation of microsome was carried out according to the method of Reddy et al. (2000), with some modifications. Human recombinant COX-2 expressed in *Spodoptera frugiperda*. *Spodoptera frugiperda* (Sf9) cells were maintained at 28 °C in Grace's insect culture medium. Inscet Cells at 60% confluency were infected with recombinant baculovirus containing human COX-2. After 72 h of infection, the cells were collected by centrifugation at 2000 × *g* for 5 min at 4 °C. The pellet was suspended in minimum volume of Tris–HCl buffer (50 mM, pH 7.2) containing 5 mM EDTA, 300 mM sucrose, 5 mM diethyl thiocarbamate, 1 μg/ml pepstatin, 1 mM phenol and sonicated for 3 min. The cell lysate was centrifuged at 100,000 × *g* for 1 h 10 min at 4 °C by using ultracentrifuge (Himac,

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