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Original Research Article (Experimental)

Anti-inflammatory profile of *Aegle marmelos* (L) *Correa* (*Bilva*) with special reference to young roots grown in different parts of India

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ABSTRACT

Background: *Aegle marmelos* (*Bilva*) is being used in Ayurveda for the treatment of several inflammatory disorders. The plant is a member of a fixed dose combination of Dashamoola in Ayurveda. However, the usage of roots/root bark or stems is associated with sustainability concerns.
Objectives: The present study is aimed to compare the anti-inflammatory properties of different extracts of young roots (year wise) and mature parts of *Bilva* plants collected from different geographical locations in India, so as to identify a sustainable source for Ayurvedic formulation.
Materials and methods: A total of 191 extracts (petroleum ether, ethyl acetate, ethanol and aqueous) of roots, stems and leaves of *A. marmelos* (collected from Gujarat, Maharashtra, Odisha, Chhattisgarh, Karnataka and Andhra Pradesh region) were tested for anti-inflammatory effects *in vitro* on isolated target enzymes cyclooxygenase-1 (COX-1), cyclooxygenase-2 (COX-2) and 5-lipoxygenase (5-LOX), lymphocyte proliferation assay (LPA), cytokine profiling in LPS induced mouse macrophage (RAW 264.7) cell line and *in vivo* carrageenan induced paw edema in mice.
Results: Of 191 extracts, 44 extracts showed COX-2 inhibition and 38 extracts showed COX-1 inhibition, while none showed 5-LOX inhibition. Cytokine analysis of the 44 extracts showing inhibition of COX-2 suggested that only 17 extracts modulated the cytokines by increasing the anti-inflammatory cytokine IL-2 and reducing the pro-inflammatory cytokines like IL-1β, MIP1-α and IL-6. The young (2 and 3 years) roots of *Bilva* plants from Gujarat and young (1 yr) roots from Odisha showed the most potent anti-inflammatory activity by suppressing the pro-inflammatory cytokines and inducing anti-inflammatory cytokines. These three extracts have also shown *in vivo* anti-inflammatory activity comparable to that in adult stem and root barks.
Conclusion: The present study reveals that young roots of *Bilva* plants from Gujarat and Odisha region could form a sustainable source for use in Ayurvedic formulations with anti-inflammatory activities. The present study also indicates that the region in which the plants are grown and the age of the plants play an important role in exhibiting the anti-inflammatory effect.

1. Introduction

Aegle marmelos (L.) Correa is a member of *Dashamoola* (10 root drugs) group. This combination is widely used in generic Ayurvedic formulations such as *Dasmularishta*, *Dasamoola Kashayam*, and *Dasamulakatutrayadi Kashayam*. The plant grows wild in dry forest in outer Himalayas and Shivaliks. *Bilva* is a medium to large sized deciduous glabrous armed tree with axillary and 2.5 cm long

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alternate trifoliate leaves, short flowers and has globular fruit. This plant is attributed with enormous therapeutic value in traditional method of treatment. While its fruits and leaves are used in Ayurveda for specific indications, the roots/root bark are specifically suggested for use in anti-inflammatory combination of *Dashamoola*.

The crude extracts of *Bilva* are reported widely to act as anti-diabetic [1,2], anti-inflammatory and analgesic [3], antiulcer, anti-microbial [4], antihyperglycemic and antidyslipidemic [5], antidiarrhoeal [6], oral hypoglycaemic [7], antifungal [8], gastric mucosal protective, antioxidant [9], anticancer [10], antiviral [11], radioprotective [12], cardioprotective [13], antiasthmatic [14], and antispermatogenic [15] agents. Recent studies demonstrate the curative effects of the ethanolic extract of *Bilva* plants against 2,4,6-trinitrobenzene sulfonic acid (TNBS) – induced colitis in rats through its anti-bacterial and anti-oxidant [16] properties. Thus there is extensive data on the use of leaves, bark, roots, fruits and seeds of *Bilva* in Ayurveda for prevention and treatment of variety of inflammatory diseases.

Though inflammation is an inbuilt defence mechanism to combat or overcome the invading pathogens or autoimmune reactions, chronic inflammation however, is associated with many diseases. As a result, treating inflammatory condition is the first round of treatment strategy followed by the specific therapy for the concerned disease. Currently the non-steroidal anti-inflammatory drugs (NSAIDs) constitute one of the major groups of drugs being used to treat pain and inflammation. However, these are associated with unwanted side effects and it was reported that 34–46% of the users have gastrointestinal damage due to the inhibition of the protective COX-1 enzyme in gastric mucosa. The primary mechanism of action of NSAIDs is the inhibition of the activity of cyclooxygenase enzymes (COX-1 and COX-2) and a consequent reduction in prostaglandin levels [17]. The synthesis of prostaglandins (PGs) from arachidonic acid is initiated by the cyclooxygenases, COX-1 and COX-2. Both enzymes are membrane-bound homodimers, found predominantly on the perinuclear membranes, including the endoplasmic reticulum. COX-1 is constitutively expressed in most tissues and has important roles in tissue homeostasis, particularly in the stomach and kidneys, as well as in blood clotting. In contrast, expression of COX-2 is induced in response to inflammation [18]. The conventional NSAIDs inhibit both COX-1 and COX-2 and as a result are associated with severe side effects like gastrointestinal bleeding and damage to gastric mucosa [19]. The selective COX-2 inhibitors (COXIBs), though devoid of gastric side effects, have been linked with cardiovascular toxicity [20,21]. Given the myriad of adverse side effects of classical NSAIDs and COXIBs, there is increasing attention on developing safer anti-inflammatory drugs by exploiting the traditional system of knowledge and by employing scientific principles of inflammation for determining their efficacy and safety.

Ayurvedic treatises though recommend the use of roots of *Bilva* plant – a member of *Brihat panchamoola*, stem bark and root bark of adult plants came into vogue perhaps due to unsustainability of roots from mature plants. Currently the use of bark is also not sustainable. Hence there is need for identifying the alternative sustainable source, provided the process is developed through a methodical endeavour. The need for such an endeavour has been highlighted in a recent publication [22]. From phytochemistry perspective, uses of young roots offer logical tenability, as the biosynthesis of secondary metabolites is more active in the roots during formative years of trees. Keeping this in mind, the present study aims at screening multiple samples of young roots of *A. marmelos* and evaluating the anti-inflammatory activities of different parts including leaves of mature plant and young roots of different ages (six months to 3 years) and stem bark, root bark of adult plants collected from different regions of India. The anti-inflammatory activities were carried out using *in vitro* and *in vivo* protocols and compared with those of stem bark and root bark from mature plants.

2. Materials and methods

2.1. Chemicals

Reagents and Chemicals: Roswell Park Memorial Institute medium (RPMI-1640), Dulbecco's Modified Eagle's Medium (DMEM), antibiotics (Penicillin/Streptomycin), and concanavalin-A (Con-A) were purchased from Himedia, fetal bovine serum (FBS) from Hyclone. Ficoll Histopaque, lipopolysaccharide (LPS), λ carrageenan and methyl thiazolyl tetrazolium (MTT) from Sigma–Aldrich (St Louis, MO 63103, USA). The TMPD (N, N, N′, N′-tetramethyl p-phenylenediamine), hematin and Tween 20 were purchased from Sigma, and arachidonic acid was purchased from Nu-check Prep, Inc (MN, USA). The dimethyl sulfoxide (DMSO) used was of HPLC grade. The plant extracts were provided by Dabur Research & Developing Centre, India. All the solutions were prepared in de-ionised distilled water. All other reagents used in the studies were of standard quality.

2.2. Plant material and extracts

The samples of young roots (1 year, 1.5 yr, 2 yrs and 3 yrs age) were collected from two sets of sampling sites. In the states of Gujarat, Maharashtra and Odisha, the resource augmentation project areas, the new plantation activity was initiated correlating to the project period. In the states of Andhra Pradesh, Chhattisgarh and Karnataka, limited plantation activity was initiated for sampling purpose – using locally available mother stocks. Samples of root bark and stem bark were collected (Andhra Pradesh, Chhattisgarh and Gujarat sites) taking care that, the matured tree was not damaged. Details of different agro-climatic zones of India from where these samples were collected are listed in supplementary (Supplementary-1).

Four different extracts of each plant part were used viz., petroleum ether (PE), ethyl acetate (EA), ethanol (ET) and aqueous (AQ). Thus a total of 191 extracts were used in the present study. The collection, processing and preparation of extracts was carried out by Dabur Research & Development Centre (DRDC) and all the biochemical assays were carried out in the department of Animal Biology, University of Hyderabad. All the reference samples are preserved at the laboratories of DRDC in both crude form and as extracts. Voucher specimens were identified by Dr. S. K. Srivastava, Scientist-E, BSI, Dehradun and Sample Herbarium Sheets deposited with Northern Regional Centre, Botanical Survey of India, Dehradun (Accession no. 116125).

2.3. Extraction and isolation of COX-1 from ram seminal vesicles

Ram seminal vesicles were collected from local slaughter house and stored in deep freezer (−80 °C). One day before starting the experiment, the ram seminal vesicles were kept at 4 °C overnight in refrigerator. Process of extraction and isolation was carried out below 5 °C in cold room.

2.3.1. Preparation of microsomes as a source of COX-1

Preparation of microsomes was carried out, according to the method of Hemler and Lands [23] with minor modifications. Ram seminal vesicles were minced and homogenized with a blender in buffer containing 0.05 M Tris–HCl (pH 8), 5 mM EDTA disodium

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