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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

salt, 5 mM diethyl dithiocarbamate and 0.01% sodium azide. The homogenate was centrifuged at 10,000 *g* for 15 min, 4 °C. The supernatant was filtered through cheese cloth. The filtered supernatant was again centrifuged at 33,000 rpm for 1 h 15 min, 4 °C by using ultracentrifuge (Himac, CP-100-α HITACHI) to obtain microsomal pellet. This microsomal pellet was solubilised in a solubilising buffer containing 0.05 M Tris–HCl (pH 8), 0.1 mM EDTA disodium salt, 0.1 mM diethyl dithiocarbamate, 0.1% Triton X100 and 0.01% sodium azide. The solubilized microsomal fraction thus obtained was centrifuged at 4000 rpm for 1 h at 4 °C. The supernatant obtained was stored in small aliquots at −80 °C, and used as COX-1 enzyme source for further experiments.

2.4. Isolation of COX-2 enzyme

Isolation of COX-2 was carried out according to the method of Reddy et al. [24] with slight modifications. *Spodoptera frugiperda*-9 (Sf-9) cells were used for human recombinant COX-2 expression. Sf-9 cells were maintained at 28 °C in Grace's insect culture medium and at 80% confluency the cells were infected with recombinant Baculovirus containing human COX-2. After 72 h of infection, the cells were collected by centrifugation at 3000 *g* for 5 min at 4 °C. The pellet was suspended in minimum volume of Tris–HCl buffer (50 mM, pH 8.0) containing 5 mM EDTA, 300 mM sucrose, 5 mM diethyl-dithiocarbamate (DDC), 1 μg/ml pepstatin, 1 mM phenol and sonicated for 3 min on ice. The cell lysate was centrifuged at 100,000 *g* for 1 h 15 min at 4 °C by using ultracentrifuge (Himac, CP-100 α HITACHI) and the microsomal pellet obtained was suspended in Tris–HCl buffer (2.5 mM, pH 8.0) containing 0.5% glycerol, 0.8% Tween-20 and 1 mM phenol. This solubilized microsomal fraction, after centrifugation, was stored in small aliquots at −80 °C and used as COX-2 enzyme source for further studies.

2.5. COX-1 and COX-2 assays

Enzymatic activities of both COX-1 and COX-2 were measured according to the method of Copeland et al. [25] with slight modifications using a chromogenic assay based on the oxidation of TMPD during the reduction of PGG2 to PGH2 [26,27]. The assay mixture contained 100 mM Tris–HCl buffer (pH 8.0), 5 mM hematin, 5 mM EDTA, enzyme (COX-1 or COX-2) and the test compound or extract. The mixture was pre-incubated at 25 °C for 5 min and then the reaction was initiated by the addition of substrate, arachidonic acid and TMPD, and the total volume of the reaction mixture was made up to 1 ml. The enzyme activity was determined by estimating the rate of TMPD oxidation for the first 60 s of the reaction by following the increase in absorbance at 610 nm. A low rate of non-enzymatic oxidation, observed in the absence of COX-1 and COX-2 was subtracted from the experimental value while calculating the percent inhibition.

2.6. Purification and assay of 5-LOX

5-LOX from potato tubers was purified and assayed as per the method of Reddanna et al. [28]. Enzyme activity was measured using polarographic method with a Clark's oxygen electrode on Strathkelvin Instruments (Model 782, RC-300). Reaction mixture contained 50–100 μl of enzyme and 10 μl of 40 mM substrate (arachidonic acid) and the final volume was made to 3 ml with 100 mM phosphate buffer (pH 6.3). Since lipoxygenases are oxygen-consuming enzymes and decrease the concentration of oxygen in the reaction mixture, the rate of decrease in oxygen was taken as a measure of enzyme activity. Reaction was allowed to proceed at 25 °C and the maximum slope generated was taken for calculating enzyme activity. The activity was expressed as units/mg protein, where one unit is defined as one micro mole of oxygen consumed per minute. Assay standardization was done with NDGA (nordihydroguaiaretic acid), a selective LOX inhibitor.

2.7. Cell culture and treatment

RAW 264.7 cell line, procured from National Centre for Cell Science (NCCS), Pune, India, was cultured in RPMI 1640 media supplied with 10% heat inactivated FBS and 100 U/ml penicillin and 100 μg/μl streptomycin. Cells were maintained and grown at 37 °C in a humidified atmosphere with 5% CO_2. Cells were propagated by splitting and changing the media twice a week.

2.8. RAW 264.7 cell proliferation assay

RAW 264.7 cell proliferation was assessed by using the MTT assay [29]. Briefly, 5×10^3 RAW 264.7 cells were seeded in 96 well plate and grown for 16 h at 37 °C, 5% CO_2 and pre-treated with LPS at 1 μg/mL for 3 h. After 3 h of incubation, plant extracts were added to the cells at three different concentrations (100 μg/mL, 10 μg/mL and 1 μg/mL). This procedure was also repeated with the standards viz., celecoxib, indomethacin, and (NDGA). Wells untreated served as control for LPS whereas cells treated only with LPS were used as control for the cells treated with LPS and extracts or LPS and standards. After 48 h incubation, the cell supernatant was carefully aspirated and 20 μl of MTT (5 mg/ml in PBS) was added in each well and was incubated for an additional 3 h at 37 °C. Tetrazolium crystals were solubilized by adding 50 μl of DMSO to each well and agitated for 5 min on a rocker. Absorbance was read at 570 nm in BioTek Synergy Mx multimode reader. Percent growth of cells was calculated using control as reference.

2.9. Lymphocyte proliferation assay (LPA)

Lymphocytes were isolated from fresh blood donated by a healthy human volunteer, after obtaining informed consent, by Ficoll Histopaque (Sigma) method [30]. Lymphocytes were washed with PBS and resuspended in complete media DMEM along with addition of 50 μM β-mercaptoethanol. Cells were seeded at the density of 5×10^3 cells in each well in 96 well plates and grown for 16 h at 37 °C and 5% CO_2. Cells were treated with the plant extracts of three different concentrations (100 μg/mL, 10 μg/mL and 1 μg/mL) along with the standards viz., 25 μM celecoxib, 10 μM indomethacin and 10 μM NDGA in duplicates and incubated for 24 h. Cells treated with Con-A (4 μg/mL) served as positive control and without Con-A served as control. After 24 h, 20 μl of MTT (5 mg/ml in PBS) was added in each well and incubated for an additional 3 h at 37 °C. After incubation 50 μl of DMSO was added in each well to dissolve the formazan crystals. Absorbance was read at 570 nm in BioTek Synergy Mx multimode reader. The percent growth of lymphocytes was calculated using control as reference.

2.10. Analysis of pro- and anti-inflammatory cytokines

Analysis of pro- and anti-inflammatory markers IL-1β, IL-2, IL-6 and MIP1-α were performed using LPS stimulated RAW 264.7 cells by ELISA using commercially available kits (R & D Systems, MN, USA). In a six well plate, 2×10^5 RAW 264.7 cells per well were seeded and were allowed to grow for 16 h. They were then incubated with plant extracts at their IC_{50} concentration (from COX-2 enzyme assay studies) for 1 h. The cells were then activated with LPS (1 μg/mL) for 5 h and the supernatants were collected for further cytokine analysis.

2.11. Carrageenan-induced paw edema animal model

Male BALB/c mice (22–25 g) were purchased from the National Institute of Nutrition (NIN), Hyderabad, India. The mice were housed in a controlled environment and provided with standard rodent chow and water for a week. The laboratory temperature was 24 ± 1 °C, and relative humidity was 40–80%. All animal experiments were performed in accordance with the guidelines of CPCSEA with IAEC/UH/151/2016/05/PR/P8/Mice BALB/c/m-64.

To test inhibitory effects of most promising *Bilva* extracts from Gujarat and Odisha region on acute inflammation in an animal model, paw edema was induced by subcutaneous injection of 25 μL of carrageenan (1%) into the left hind paw [31]. The mice were randomly divided into groups (n = 6), and were then intraperitoneally injected with 50 μL DMSO or with extracts (50 mg/kg bw) or celecoxib (20 mg/kg bw). After 1 h, paw inflammation was induced by injecting 25 μL of 1% carrageenan or an equal volume of 0.9% saline into the intra-plantar space of the left hind paw. Just before injection of carrageenan, the thickness of the left hind paw was measured using a digital calliper, as a baseline reference [32]. The thickness of the paw was then measured at indicated times after carrageenan or saline injection. Five consecutive measurements were performed. Edema was calculated as the average difference in paw thickness (mm) compared with the data for the saline group and the corresponding baseline reference.

2.12. Statistical analysis

Data were expressed as mean ± S.D of three individual experiments, and a $p \leq 0.05$ was considered as statistically significant.

3. Results

3.1. Effect of Bilva extracts on COX-1, COX-2 and 5-LOX enzyme based assay

The inhibition of COX-1 and COX-2 enzyme activities by the *Bilva* extracts was analyzed in cell-free system using purified COX-1 and COX-2 enzymes respectively. The extracts that showed potent inhibition of COX-1 enzyme were not considered for further study anticipating their gastric side effects. Extracts that showed COX-2 inhibition were selected for further studies on their pro- or anti-inflammatory effects. Extracts inhibiting both COX-2 and 5-LOX were considered as most potent anti-inflammatory agents. The percent inhibition was calculated using reference control. IC_{50} values were calculated for the extracts that showed more than 50% inhibition at 100 μg/mL concentration and the data are presented in Tables 1–4.

Table 1
Bilva extracts showing selective inhibition of COX-1 enzyme activity (9 out of 191 extracts showing selective inhibition of COX-1 than COX-2 are presented). COX-1: cyclooxygenase-1, COX-2: cyclooxygenase-2, GJ1: Gujarat region-1, OR2: Odisha region-2, AP: Andhra Pradesh, KA: Karnataka, MH: Maharashtra, YR: young roots, YR1: one year of YR, YR1.5: one and half year of YR, YR2: two year of YR, YR3: three year of YR, LF: leaves, PE: petroleum ether, EA: ethyl acetate.

S. No	Extract	COX-1 IC_{50} (μg/mL)	COX-2 IC_{50} (μg/mL)	COX-2/COX-1
1	GJ1/YR-1.5/PE	73.7	91.79	1.25
2	OR2/YR-2/EA	29.28	74.85	2.56
3	AP/YR-1.5/PE	75.4	95.29	1.26
4	AP/YR-3/EA	8.24	36.29	4.40
5	KA/YR-1.5/EA	7.07	77.76	10.9
6	KA/YR-2/PE	31.01	66.07	2.13
7	KA/LF/PE	7.65	92.96	12.15
8	KA/LF/EA	4.15	41.53	10.01
9	MH/LF/PE	66.66	83.01	1.24

Table 2
Bilva extracts showing specific inhibition of COX-1 enzyme activity (23 out of 191 extracts showing specific inhibition of COX-1 are presented). Cyclooxygenase-1, GJ1: Gujarat region-1, GJ2: Gujarat region-2, GJ3: Gujarat region-3, OR1: Odisha region-1, OR2: Odisha region-2, AP: Andhra Pradesh, KA: Karnataka, YR: young roots, YR1: one year of YR, YR1.5: one and half year of YR, YR2: two year of YR, YR3: three year of YR, LF: leaves, SB: stem bark, PE: petroleum ether, EA: ethyl acetate, ET: ethanol.

S. No	Extract	COX-1 IC_{50} (μg/mL)
1	GJ3/LF/ET	59.09
2	GJ1/YR-3/EA	88.87
3	GJ3/YR-1/EA	80.68
4	OR1/YR-1.5/PE	76.61
5	GJ2/YR-3/PE	59.06
6	OR1/YR-3/PE	22.85
7	OR2/YR-2/EA	44.48
8	CG/YR-1.5/PE	85.90
9	CG/YR-2/ET	80.09
10	CG/YR-3/EA	60.24
11	AP/SB/PE	77.17
12	AP/YR-1/PE	76.58
13	AP/YR-1/EA	84.77
14	AP/YR-1/ET	56.73
15	AP/YR-3/PE	9.42
16	AP/YR-3/ET	47.39
17	KA/YR-1/PE	86.54
18	KA/YR-1/ET	7.07
19	KA/YR-1.5/PE	26.36
20	KA/YR-2/EA	48.54
21	KA/LF/ET	9.42
22	KA/YR-1/EA	79.53
23	KA/YR-3/ET	77.76

Table 3
Bilva extracts showing selective inhibition of COX-2 enzyme activity (6 out of 191 extracts showing selective inhibition of COX-2 than COX-1 are presented). COX-1: cyclooxygenase-1, COX-2: cyclooxygenase-2, GJ2: Gujarat region-2, GJ3: Gujarat region-3, KA: Karnataka, SB: stem barks, YR: young roots, YR1: one year of YR, YR3: three year of YR, LF: leaves, PE: petroleum ether, EA: ethyl acetate, ET: ethanol.

S. No	Extract	COX-2 IC_{50} (μg/mL)	COX-1 IC_{50} (μg/mL)	COX-2/COX-1
1	GJ3/SB/PE	8.24	47.98	0.17
2	GJ3/LF/PE	44.5	83.63	0.53
3	GJ3/LF/EA	56.73	138.49	0.41
4	GJ2/YR1/PE	63.18	83.03	0.76
5	KA/YR-1/EA	78.94	79.53	0.99
6	KA/YR-3/ET	70.75	77.76	0.91

As shown in Tables 1–4, out of 191 extracts screened for COX-1 and COX-2 inhibition, 23 extracts showed inhibition of COX-1 only (Table 2), 30 extracts showed inhibition of COX-2 only (Table 4), 9 extracts showed selective inhibition of COX-1 (Table 1) and six extracts showed selective inhibition of COX-2 (Table 3). None of the 191 extracts showed any significant inhibition of 5-LOX even at a concentration of 100 μg/mL (data not shown). Based on the above screening of the plant extracts for direct inhibition of COX-1, COX-2 and 5-LOX, key anti-inflammatory target enzymes, further anti-inflammatory profiling studies were taken up with the 44 extracts showing COX-2 inhibition.

3.2. Effect of Bilva extracts on LPS induced inflammation in RAW 264.7 cells

To further study the anti-inflammatory properties of *Bilva* extracts in cell based assays, inflammation was induced in RAW 264.7 cells with LPS, a known pro-inflammatory agent, and the effects of plant extracts were evaluated on the proliferation of RAW cells as per the methods described in the methodology. The extracts that did not induce cell death were considered as non-cytotoxic but

Table 4
Bilva extracts showing specific inhibition of COX-2 enzyme activity (30 out of 191 extracts showing specific inhibition of COX-2 are presented). COX-2: cyclo-oxygenase-2, GJ1: Gujarat region-1, GJ2: Gujarat region-2, GJ3: Gujarat region-3, OR1: Odisha region-1, OR2: Odisha region-2, AP: Andhra Pradesh, KA: Karnataka, CG: Chhattisgarh, YR: young roots, YR1: one year of YR, YR1.5: one and half year of YR, YR2: two year of YR, YR3: three year of YR, LF: leaves, SB: stem bark, MR: mature roots, RB: roots barks, PE: petroleum ether, EA: ethyl acetate, ET: ethanol.

S. No	Extract	COX-2 IC_{50} (μg/mL)
1	GJ2/YR2/PE	66.1
2	GJ1/YR-3/PE	94.7
3	GJ2/YR-3/EA	80.68
4	GJ3/YR-1.5/EA	83.01
5	OR1/YR-1.5/EA	50.31
6	OR2/LF/PE	53.22
7	OR2/LF/EA	76.02
8	OR1/YR-2/EA	59.65
9	OR1/YR-1/PE	95.88
10	OR1/YR-1/EA	58.5
11	OR2/YR-1/PE	64.33
12	OR2/YR-1/EA	59.06
13	OR1/YR-3/EA	78.35
14	CG/SB/PE	52.64
15	CG/MR/EA	89.46
16	CG/RB/PE	56.73
17	CG/RB/EA	64.92
18	CG/LF/PE	44.48
19	CG/LF/EA	70.75
20	CG/YR-2/EA	72.52
21	CG/YR-3/PE	83.6
22	CG/YR-1.5/EA	52.64
23	AP/LF/EA	72.5
24	AP/LF/ET	54.42
25	AP/YR-2/EA	94.11
26	AP/YR-1.5/EA	87.69
27	KA/YR-3/PE	94.7
28	MH/YR-1/EA	76.58
29	MH/YR-2/EA	70.16
30	MH/LF/EA	90.02

anti-inflammatory in nature. Cell proliferation assay was done for all the 191 extracts of *Bilva* plant and the data on the extracts showing inhibition of proliferation with IC_{50} values were given in the supplementary data (Supplementary-2). Among COX-2 inhibitors tested for the proliferation, three specific COX-2 inhibitor extracts from Chhattisgarh (Table 4: CG/SB/PE, CG/RB/EA and CG/YR-3/PE) showed potent cytotoxic activity on RAW cells stimulated with LPS. Though these three extracts showed inhibition of COX-2, they may not be better anti-inflammatory candidates as they were showing cytotoxic effect on LPS stimulated macrophages at the concentrations studied. This may be due to the contribution of factors other than COX-2 inhibitors present in these extracts. Therefore, they were not considered for further studies.

3.3. Lymphocyte proliferation assay

Immunomodulatory effects of these extracts were evaluated by lymphocyte proliferation assay by culturing human lymphocytes in the presence or absence of each extract as described in the methodology. Though the lymphocyte proliferation assay for all 191 extracts of *Bilva* was carried out (Supplementary-2), the data for LPA shown in (Fig. 1), represent those extracts that have shown IC_{50} value of COX-2 inhibition. Con A, a plant lectin was used as a positive control in this study, which was known to induce proliferation of lymphocytes. Extracts that showed significantly higher proliferation of lymphocytes at 100 μg/mL concentration compared with Con A (positive control) were considered as mitogenic or carcinogenic and those extracts showing proliferation between Con A and Control as immune-modulators. Out of the 44 COX-2 inhibiting extracts, 6 extracts were immune-modulators and 2 have shown mitogenic effect (Fig. 1).

3.4. Cytokine analysis

Bilva extracts that showed inhibition of COX-2 without mitogenic activity on lymphocyte proliferation and pro-inflammatory activity on LPS induced macrophages were further studied for their effects on pro-inflammatory (IL-1β, IL-6, MIP1-α) and anti-inflammatory (IL-2) cytokines in LPS induced mouse macrophage cell line, RAW 264.7, as described in the methodology. The extracts exerting either one fold increase in anti-inflammatory (IL-2) cytokine production or two fold decrease in the pro-inflammatory cytokines compared to LPS treatment, could be considered as mediating their anti-inflammatory effect through modulation of cytokine analysis. The extracts showing up regulation of IL-2 or down regulation of pro-inflammatory cytokines were shown in Fig. 2. These results indicate that the extracts of young roots, compared with mature stem and root barks, exhibit potent anti-inflammatory effects by decreasing pro-inflammatory cytokines (IL-1β, IL-6, MIP-1α) and increasing the anti-inflammatory (IL-2) cytokines in LPS treated RAW cells. The extracts of young roots and stem barks from CG region showed decrease in expression of one or other pro-inflammatory cytokines and a less significant increase in IL-2 expression. Though the extracts of all the young roots from Gujarat region showed anti-inflammatory effect, the 2 and 3 years old young roots were found to be more potent in anti-inflammatory effects compared with 1.5 year old young roots which showed an increase in IL-2 expression. The young roots of *Bilva* from Odisha, showed significant decrease in the levels of the IL1-β compared with-IL-6 and MIP1-α and showed significantly higher levels of IL-2 expression which explains their anti-inflammatory effects. The extracts of young roots of *Bilva* from Andhra Pradesh and Karnataka are more potent in reducing the levels of pro-inflammatory cytokines.

3.5. Bilva extracts effect on carrageenan-mediated paw edema in BALB/c mice

Edema was calculated as the average difference of paw thickness (mm) in treated groups compared with that in saline-treated groups (normal saline-induced edema) and the corresponds to baseline reference. The intraplantar injection of 25 μL of 1% carrageenan caused an increase in the thickness of mouse paw. The paw edema peaked at 3 h after induction, and the thickness were 1.48 ± 0.04 mm in the control group and 0.73 ± 0.03 mm in celecoxib group. In the *Bilva* extract treatment groups (OR1/YR1/PE, GJ1/YR3/PE and GJ2/YR2/PE at 50 mg/kg body weight), the mean peak thicknesses were 1.15 ± 0.05 mm, 1.08 ± 0.08 mm and 1.12 ± 0.07 mm, respectively, three of which indicated significantly attenuated paw edema compared with the control group (Fig. 3).

4. Discussion

Different parts of *Bilva* plant are being used for various therapeutic purposes such as asthma, allergy, diabetes, healing wounds [33,34], swollen joints etc. Inflammation is the underlying process in all of these disease conditions and the protective effect of Bilva plant parts might be attributed to their anti-inflammatory properties.

Further, the study intends to identify the alternative plant parts to the conventional use of mature roots or root bark or stem bark for treatment of inflammatory conditions as envisaged in Ayurveda. Such substitution of plant parts through a systematic investigation

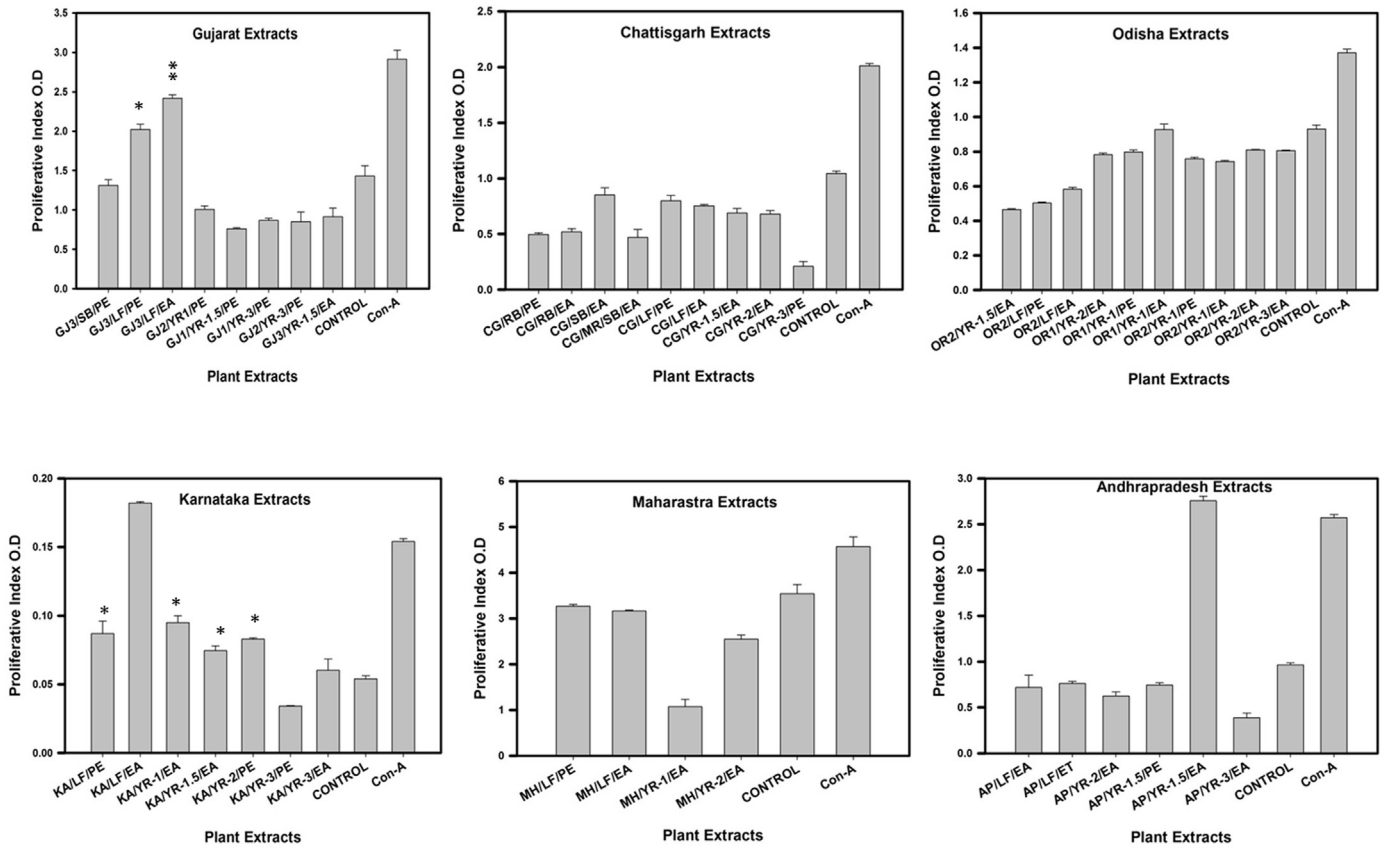

Fig. 1. Effect of *Bilva* extracts on human lymphocytes proliferation. Out of the 44 COX-2 inhibiting extracts, 6 extracts were immune-modulators and 2(KA/LF/EA & AP/YR-1.5/EA) have shown mitogenic effect. The data represented are mean ± S.D of three independent experiments. $^{**}p < 0.01$; $^{*}p < 0.05$ compared between cells treated with Con A in the presence of extract *vs* absence of extract. GJ: Gujarat, KA: Karnataka, MH: Maharashtra, OR: Odisha, CG: Chhattisgarh, AP: Andhra Pradesh, YR: young roots, YR1: one year of YR, YR1.5: one and half year of YR, YR2: two years of YR, YR3: Three years of YR, LF: Leaf, SB: Stem Bark, PE: Petroleum ether, EA: Ethyl Acetate, ET: Ethanol.

was warranted by sustainability concerns associated with conventional practices.

This study presents data on the anti-inflammatory profiles of young roots of *A. marmelos* (*Bilva*), which were shown to be comparable or sometimes much higher to those of adult stem/root barks, suggesting it as the potential alternative and sustainable source for Ayurvedic drugs of *Dashmoola*/*Brihat panchamoola*. The anti-inflammatory effects of the extracts were analysed at three levels *in vitro*: by inhibition of key inflammatory target enzymes such as COX-1, COX-2, 5-LOX; immunomodulatory effects based on LPA and by modulation of cytokine analysis in LPS stimulated mouse macrophages. A total of 191 extracts that include root bark, stem bark and leaves from adult mature plants, and young roots of different aged plants (1, 1.5, 2 and 3 years), were employed in the present study. The current anti-inflammatory therapy mostly comprises the use of NSAIDs [35,36] including aspirin, ibuprofen, and indomethacin, which non-selectively inhibit both COX-1 and COX-2, blocking the synthesis of all prostaglandins [37]. Of all the *Bilva* extracts analysed in the present study, 9 selective COX-1 and 23 specific COX-1 inhibitors along with 6 selective COX-2 and 30 specific COX-2 inhibitors were identified by enzyme based assays (Tables 1–4). Young roots from Karnataka, Gujarat, Orissa, Andhra Pradesh and Maharashtra and leaves from Karnataka showed selective COX-1 inhibition. All the extracts which showed selective inhibition of COX-1 were not considered for further study as the selective inhibition of COX-1 is known to result in undesirable side effects. In addition to the studies on isolated target enzymes, further studies were taken up on cell based assays involving mouse macrophage cell line, RAW 264.7 and human lymphocytes.

Of the total 191 samples analysed, only six extracts showed mitogenic activity in lymphocyte proliferation assay using freshly isolated lymphocytes from healthy donor blood. As two of these six extracts showed specific COX-2 inhibition, they may not form good candidates for anti-inflammatory applications in view of their potential mitogenic activity. Among the 191 samples tested for LPS-treated macrophage cell proliferation assay, only 21 samples showed significant inhibition of proliferation and among these three are COX-2 inhibitors.

Cytokines are small secretory proteins that are produced mainly by macrophages and lymphocytes during inflammation. Cytokines secreted by activated macrophages, mediate effective immune response that links innate and adaptive immunity and also influence macrophage's micro-environment [38,39]. These small proteins mediate varieties of functions ranging from local to systemic inflammation, chemotaxis and tissue repair. Hence in the present study we have evaluated pro-and anti-inflammatory cytokines in the extracts of *Bilva* showing COX-2 inhibition but with no cytotoxic effects on macrophages. Out of 44 COX-2 inhibiting extracts, 17 were selected for further cytokine profiling, pro- and anti-inflammatory cytokines, in LPS-induced mouse macrophage cell line. The extract that inhibits all the pro-inflammatory cytokines and at the same time induces anti-inflammatory cytokine IL-2, could be considered as potential source for anti-inflammatory therapy. The young roots from Odisha are potent inducers of IL-2 and have shown good inhibition of IL-1β but comparatively low inhibition of IL-6 and MIP1-α. Among these one year old root of Bilva (OR1/YR-1/PE) exhibited the highest anti-inflammatory effect by inducing anti-inflammatory cytokine IL-2 and inhibiting

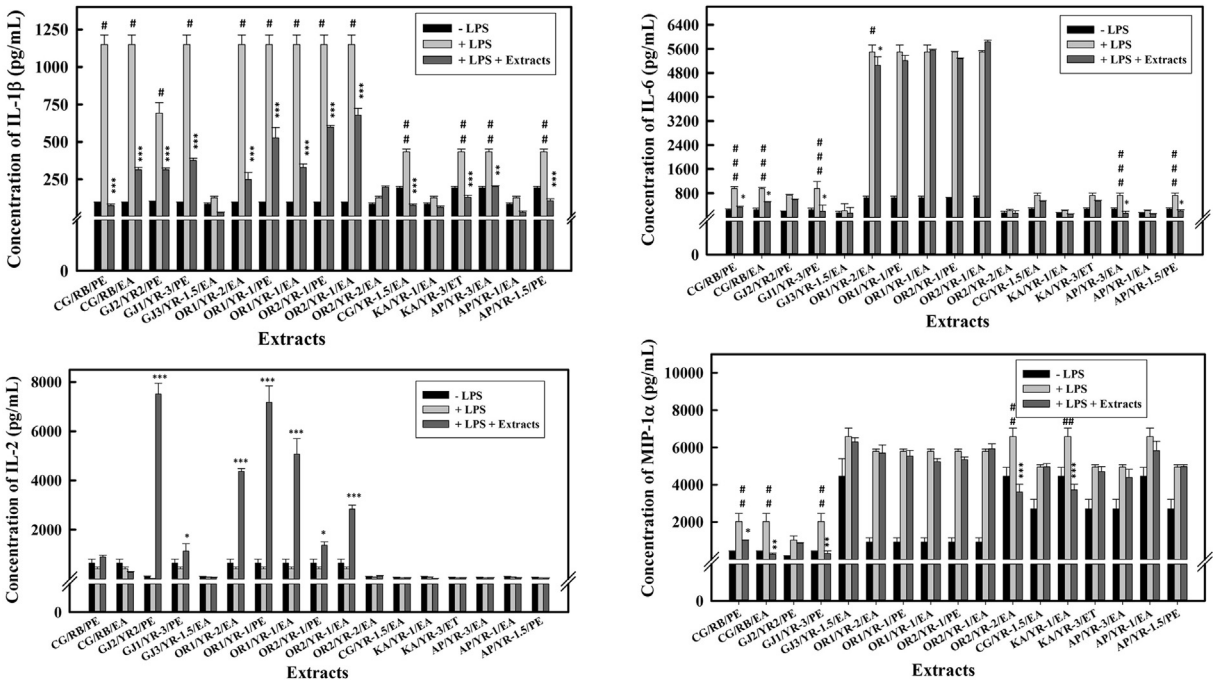

Fig. 2. Effect of *Bilva* extracts on LPS-mediated pro and anti-inflammatory cytokines (IL-1β, IL-6, IL-2 and MIP1-α) secretion in mouse macrophage cells. The extracts of young roots and stem barks from CG region and young roots 2 and 3 years old from GJ region were found to be more potent in anti-inflammatory effects compared with 1.5 year old young roots which showed an increase in IL-2 expression. The young roots of *Bilva* from OD, showed significant decrease in the levels of the IL1-β and showed significantly higher levels of IL-2 expression. Whereas, young roots of *Bilva* from AP and KA are more potent in reducing the levels of pro-inflammatory cytokines. In over all, the three extracts GJ1/YR3/PE, GJ2/YR2/PE and OR1/YR1/PE have shown inhibition of pro-inflammatory cytokines and induction of anti-inflammatory cytokine. The data represented are mean ± S.D of three independent experiments. $^{\#}p < 0.001$; $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.05$ for comparison between +LPS *vs.* −LPS. $^{***}p < 0.001$; $^{**}p < 0.01$; $^{*}p < 0.05$ compared between cells treated with LPS in the presence of extract *vs* absence of extract. GJ: Gujarat, KA: Karnataka, MH: Maharashtra, OR: Odisha, CG: Chhattisgarh, AP: Andhra Pradesh, YR: young roots, YR1: one year of YR, YR1.5: one and half year of YR, YR2: two years of YR, YR3: Three years of YR, LF: Leaf, SB: Stem Bark, PE: Petroleum ether, EA: Ethyl Acetate, ET: Ethanol.

pro-inflammatory cytokines such as IL-1β, MIP1-α and IL-6. Previous studies have shown that the leaf extracts of *Bilva* plant showed strong anti-inflammatory effect in rats because of the presence of active ingredients such as lupeol and lupeol linoleate [40]. In the present study 2 and 3 years old young roots of *Bilva* from Gujarat region (GJ2/YR2/PE and GJ1/YR3/PE) exhibited the highest anti-inflammatory effect by inducing anti-inflammatory cytokine IL-2 and inhibiting pro-inflammatory cytokines such as IL-1β, MIP1-α and IL-6. These three extracts also showed specific COX-2 inhibition and non-mitogenic activity in Con A induced lymphocytic proliferation assay. When these three extracts were used for *in vivo* assay on carrageenan induced paw edema, they have indeed shown satisfactory anti-inflammatory effect, suggesting as potential sources for application in the treatment of inflammatory disorders. Thus the *in vivo* anti-inflammatory effects of these three extracts may be mediated by a combination of factors showing inhibition of COX-2, inhibition of pro-inflammatory cytokines and activation of anti-inflammatory cytokines, as observed in the present study. From a resource management perspective, it is technically possible to produce young

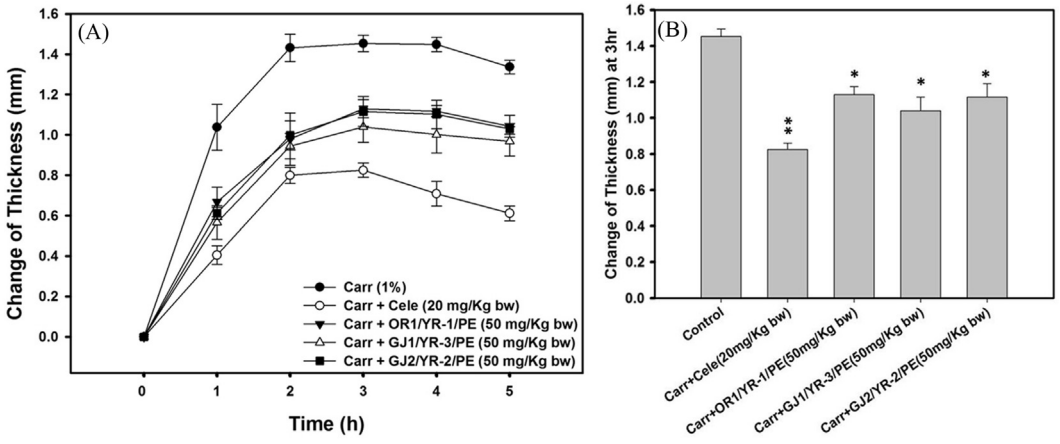

Fig. 3. Effect of *Bilva* extracts on carrageenan-induced paw edema in BALB/c mice. Animals were injected with *Bilva* extracts (50 mg/kg body weight), celecoxib (20 mg/kg) or an equal volume of the vehicle (50 μL, 10% DMSO) intraperitoneally. One hour later, paw inflammation was induced by injecting 25 μL of 1% solution of carrageenan in 0.9% saline subcutaneously into the plantar region of the left hind paw. (A) The thickness of the paw was measured in the dorsal plantar axis at the metatarsal level by digital vernier caliper at the indicated times after carrageenan injection. (B) The thickness of the paw edema at 3 h after induction was shown. All data are expressed as mean ± S.D. $^{*}p < 0.05$; $^{***}p < 0.001$ compared with the control.

roots in a sustainable manner in high-density short term plantation schemes thereby sparing the mature tree populations from the conventional practice of bark stripping.

The anti-inflammatory effect of aqueous extracts of *Bilva* root have been studied on rats [41] which might be due to the additive or synergistic effect of the chemical constituents present in the aqueous extract of *Bilva* root. Previous studies have shown that marmin, a coumarin isolated from the roots of *Bilva* showed anti-inflammatory effect against carrageenan induced inflammation [42]. Marmin, marmesin, umbelliferine and skimmianine have been identified in the bark and roots of *Bilva* which were found to contribute to the anti-inflammatory property of *Bilva* [43,44]. However, none of these studies have been tested for their effects on specific target enzymes such as COX and LOX enzymes and cytokine profile. It would be very appropriate to isolate and identify the specific compounds, from the young roots of *Bilva* from Gujarat and Odisha region, exhibiting anti-inflammatory effects, where three hits have been identified in the present study.

5. Conclusion

Through a systematic and scientific validation on the anti-inflammatory properties of *Bilva* plant parts we conclude that the young roots could potentially be replaced for adult stem and root barks and thus could form a sustainable source in all the Ayurvedic formulations. The present study also indicates that the region in which the plants are grown and the age of the plants are very important points to be considered while sourcing the plant materials. Based on these studies young roots of *Bilva* from Gujarat and Odisha region could be taken up for in depth studies in isolating and characterising the potent anti-inflammatory candidates in drug discovery programs.

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Conflict of interest

None.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jaim.2017.03.006.

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