STUDY 3.1

Labda-(17),12,14trien-19-oic acid contained in fruits of Cupressus sempervirens suppresses benign prostatic hyperplasia in rat and in vitro human models through inhibition of androgen and STAT-3 signaling.
Introduction

Benign prostatic hyperplasia (BPH) is a ubiquitous condition in aging males, such that the histological evidence of BPH increases from approximately 50% at age 60 to ~90% at age 85 (Committee, 2003). BPH is a major cause of lower urinary tract symptoms (LUTS) in males that lead to significant erosion of the quality of life (QoL). Alpha blockers, 5-alpha reductase inhibitors and phytotherapy are the three main categories of pharmacological interventions currently available for BPH. However, while α-adrenergic blockers effectively relieve BPH symptoms, they do not suppress the prostatic size and hence the risk of acute urinary retention (AUR) and surgery. On the other hand, 5α-reductase inhibitors reduce these risks to some extent but provide slow symptomatic relief and require long treatment periods, which are often accompanied with undesirable side-effects. Phytotherapy is increasingly being preferred by BPH patients because of its minimal side-effects and safety in long term use. In men with BPH, evidences suggest that several plant based therapies effectively improve urologic symptoms and flow measures (Dreikorn, 2000), however, the scientific basis of such claims are very limited. Clinical studies have shown that a standardized plant extract of *Serenoa repens* is equipotent to tamsulosin (a synthetic α-blocker) and finasteride (a synthetic 5α-reductase inhibitor) in improving IPSS, QoL and peak flow rate, but is much safer in terms of its effects on ejaculatory disorders, libido and sexual potency (Carraro *et al.*, 1996). This indicates that new leads for safer anti-BPH drugs can be identified from plant source.

*Cupressus sempervirens* (Italian Cypress) is an ornamental plant mainly distributed in North America, Mediterranean and subtropical Asia (including India). Extracts of its cones and fruits have astringent and styptic uses in folklore medicine (Siddiqui *et al.*, 2010), including antiseptic, aromatherapeutic, balsamic and anti-inflammatory activities (Ibrahim *et al.*, 2007; Mothana *et al.*, 2009). Italian Cypress is also known to improve bladder tone, exert diuretic effect and manage urinary incontinence and enuresis (Tisserand R *et al.*, 1995; Valnet J *et al.*, 1980; Holmes P *et al.*, 1980; Damian
Histologically, BPH develops in the periurethral or transitional zone of the prostate through an increase in the stromal component of the gland, which is followed by hyperplasia of epithelial cells (Committee, 2003). Since the vascular supply of prostate is available only to stroma and not to epithelium, the role of blood components becomes important in pathogenesis and management of BPH. Transurethral resection of prostate (TURP) uses an endoscope to surgically remove hyperplasic periurethral tissue to clear the urinary obstruction. The excised prostatic tissue is extremely useful for in vitro/ex vivo studies on pathogenesis and management of BPH. While the dynamically proliferating stromal cells isolated from these BPH tissues have served as a useful in vitro model for primary screening of new anti-BPH agents (Corvin et al., 1998; Boulbes et al., 2006) (Corvin et al., 1998; Boulbes et al., 2006), tissue explants have served as ex-vivo systems for further validation of activity (Kumar et al., 2012). In the present study, we have made an attempt to identify the major compound(s) of *Cupressus sempervirens* (CS) that suppress BPH, using *in vitro* and *in vivo* models.
Materials and methods

Biochemicals and Reagents
Citral, collagenase-I, DNase-I, MTT and Bcl-2/Bax antibodies were purchased from Sigma-Aldrich; TGF-beta, PCNA and IGF-1 antibodies were purchased from Santa Cruz Biotechnology, USA and STAT-3 antibody was purchased from Cell Signaling Technologies, USA. TUNEL assay kit was procured from Promega. All culture media and other reagents were from Sigma-Aldrich, USA.

Plant material
C. sempervirens fruits were collected from Almora, Uttrakhand India. The collection and authentication was done by Dr. Kamal R. Arya of Botany Division at the CSIR-Central Drug Research Institute, Lucknow, India. The voucher specimen (No. 24421) is stored in the herbarium of the institute.

Extraction of C. sempervirens and isolation of compounds
The dried and powdered fruits of C. sempervirens were processed for the extraction and isolation of fractions/compounds as per the procedure detailed earlier (Rawat et al., 2010). Briefly, dried and powdered fruit (8.0 kg) of C. sempervirens was percolated three times successively with 95% ethanol at room temperature. The combined extract was filtered and concentrated under reduced pressure at 40°C to obtain a dark brown ethanolic extract (~900 g). The ethanol extract (CS-EtOH) (800 g) was triturated with hexane (200 g) and the hexane insoluble portion was dissolved in water, which was successively extracted with chloroform (5.0L × 5) and n-butanol (5.0L × 5) to yield chloroform (120 g), n-butanol (300 g) and water (180 g) fractions. Chloroform fraction (CS-EtOH-Chlor) was subjected to column chromatography on silica gels of 100-200 mesh and eluted with increasing polarity hexane gradient system of EtOAc (0 to 100%) and finally MeOH. 50 fractions were collected and combined on the basis of their TLC
profiles to give 19 fractions, from which 8 compounds were isolated (Rawat et al., 2010).

Characterization of compounds

Optical rotations were measured on a Perkin-Elmer model 241 digital polarimeter. UV spectra were obtained on a Perkin-Elmer λ-15UV spectrophotometer. IR spectra were recorded on a Perkin-Elmer RX-1 spectrophotometer using KBr pellets. $^1$H and $^{13}$C NMR spectra were recorded on a Bruker DRX 300MHz NMR-spectrometer. ESMS on an Advantage Max LCQ Thermo-Finnigan mass spectrometer and FAB-MS were carried out on a JEOL SX 102/DA-6000 mass spectrometer. CC was performed using silica gel (230–400mesh). TLC was carried out on precoated silica gel plates 60 F254 or RP-18 F254 plates (Merck). Spots were visualized either by UV light or by spraying H$_2$SO$_4$–MeOH or anisaldehyde–H$_2$SO$_4$ and vanillin-H$_2$SO$_4$ reagents.

Primary culture of human BPH-derived stromal cells

Prostatic tissues of BPH patients undergoing Trans Urethral Resection of Prostate (TURP) at the King George’s Medical University, Lucknow, India, were obtained with their informed consent and used in this study. Stromal cells were isolated from these tissues and maintained in primary cultures by following the standard protocol (Padayatty et al., 1997). Briefly, tissues were washed three times in medium A [mixture of DMEM/Ham’s F-12 and Waymouth MD 752/1 (1:1 v/v) supplemented with antibiotic-antimycotic solution (Sigma-Aldrich)], minced and digested with collagenase (5 mg/mL) in medium A (enriched with 2% fetal calf serum) in a water bath at 37° for 4 h. The tissue was passed 6–7 times through an 18 G needle and centrifuged at 1000 g for 3 min. The pellets were washed and resuspended in the culture medium-A containing 10% FCS. The resulting cells were vimentin positive. Cells from passages 4–6 were used in these studies. BPH stromal cells were grown to confluence in DMEM/Ham’s F-12 media with 10% FCS and antibiotic/antimycotic solution.
MTT assays for cell proliferation

BPH stromal cells were treated separately at different concentrations of test agents for 48 h in multi-well plates. The extracts/compounds were dissolved in dimethyl sulfoxide (DMSO) and diluted with culture medium to the required concentrations. Final DMSO concentration was ≤0.05% in experimental wells and 0.05% in the control (0 µg/ml) wells. The number of viable cells at the end of treatment (indicating cell proliferation) were estimated by using the 3-(4, 5-dimethylthiazol-2-yl) 2, 5-diphenyltetrazolium bromide (MTT) assay. The formazan crystals formed in the viable cells in MTT assay were solubilized in DMSO and the optical density was read in a micro plate reader at a wave length of 540 nm.

Profiling mRNA of crucial genes in human prostatic (BPH stromal/androgen sensitive LNCaP epithelial) cells using qPCR

Total RNA of BPH stromal cells treated with labda-8(17),12,14-trien-19-oic acid (B) at 20, 30 and 40 µM, and androgen sensitive prostate cancer epithelial LNCaP cells treated with testosterone (10nM) and B (10 µM) for 48 hrs, was extracted using the Tri-reagent (Invitrogen) by following the manufacturer's instructions. The quantity and quality of the purified RNA was evaluated by spectrophotometry. cDNA was prepared from 2.0 µg of total RNA using Revert Aid H-Minus first strand cDNA synthesis kit (Fermentas Life Sciences, Glen Burnie, MD, USA). Synthesis was performed for 1 h at 42°C (for reverse transcription) and the thermocycling for each reaction was done in a final volume of 20 µl containing 1 µl of cDNA sample, 0.5 µM of each primer, 2X ready-to-use reaction mix (ABi SYBR green master mix) including Taq DNA polymerase, reaction buffer and deoxyribonucleotide triphosphate mix. After 10 s of initial denaturation at 95°C, the following cycling conditions (45 cycles) were used: denaturation at 95°C for 20 s, annealing at 59°C for 30 s and elongation at 72°C for 30 s. The detection of the PCR reaction based on fluorescence monitoring (Light Cycler 480, Roche) was employed. Quantitative results were obtained by the cycle threshold value where a signal rose above background level. Expression of the investigated genes
was compared to the steady expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The primer sequences used have been detailed in the table 3.1.1 below.

### Table: 3.1.1. Primer Sequences

<table>
<thead>
<tr>
<th>GENE</th>
<th>Forward (5’-3’)</th>
<th>Reverse (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>STAT3</td>
<td>CCCTGGGATTGAGAGCTCAAGA</td>
<td>AAGCGGCTATATCGCTGGTC</td>
</tr>
<tr>
<td>JAK2</td>
<td>GGTGAAATGCTGATATTCTGTT</td>
<td>AGGCCACAGAAAATCGCTGTC</td>
</tr>
<tr>
<td>SOCS</td>
<td>GCTGGATTGGATGCGATTCT</td>
<td>AGGCCACAGAAAATCGCTGTC</td>
</tr>
<tr>
<td>PIAS</td>
<td>GGAGCTGGGCGAGATTTAAG</td>
<td>AGCACCTGGAGCTAGACAC</td>
</tr>
<tr>
<td>TGF-alpha</td>
<td>TTGCTGCCACTCAGAACAG</td>
<td>ATCTGCCACAGTCCACCCCTG</td>
</tr>
<tr>
<td>IL6</td>
<td>GATGAGTACAAGGATCTGATCC</td>
<td>CTGCACGACTGGTTCTGT</td>
</tr>
<tr>
<td>PSA</td>
<td>GTGCTTGTGGCCTCCTCGT</td>
<td>CAGCAAGATGCACGCTCTTGT</td>
</tr>
<tr>
<td>TMPRSS2</td>
<td>CGCTGGCCTACTCTGGAA</td>
<td>CTGGAGAGTCGACTCTATCC</td>
</tr>
<tr>
<td>GAPDH</td>
<td>AGCCACATCGCTAGACAC</td>
<td>GCCCAAATACGACCAATCC</td>
</tr>
</tbody>
</table>

### Rat BPH-model and treatment with *C. sempervirens* extracts

Adult mature male rats (10-12 months old) were randomly divided into different groups of five animals each. BPH was induced by treatment with citral, a mild estrogen and known to cause prostatic hyperplasia in the rats (Servadio et al., 1986; Geldof et al., 1992; Engelstein et al., 1996). Animals were randomly divided into four groups, viz. Group I (vehicle control); Group II (Citral 100mg/kg); Group III (Citral 100mg/kg + CS-EtOH 10mg/kg) and Group IV (Citral 100mg/kg + CS-EtOH-Chlor 10mg/kg). Group II, III, and IV were administered with Citral at a dose of 100mg/kg once daily for 28 days while animals in Group III and IV were co-administered ethanolic CS-extract (CS-EtOH) and its labda-8(17),12,14-trien-19-oic acid enriched chloroform...
fraction (CS-EtOH-Chlor), respectively at 10.0 mg/Kg, treatment beginning from the 8th day of citral treatment and continuing once daily for remaining 21 days of treatment. All treatments were oral. At the end of experiment, animals were sacrificed according to the guidelines of Institutional Animal Ethics Committee, and the weights of testis, epididymis, seminal vesicles and prostate were recorded. Prostatic tissues were fixed in 10% formalin solution for histology and remaining tissues were frozen at –70°C for other biochemical investigations.

**Histology**

Prostate tissues fixed overnight in 10% formalin solution were processed for histology by following the standard protocol. Thin (5.0 micron) sections were stained with hematoxyline & eosin and examined under a light microscope (Eclipse 80i, Nikon Corporation, Japan).

**TUNEL staining of rat BPH tissues**

Deparaffinized tissue sections (5.0 μ thick) were rehydrated gradually through graded alcohol. Sections were fixed, permeabilized and processed for *in situ* Terminal-deoxynucleotidyl transferase dUTP Nick End Labeling (TUNEL) for DNA fragmentation using a ‘Dead End’ Fluorometric TUNEL kit (Promega, USA) by following the manufacturer’s instructions. Briefly, after a rinse in terminal deoxy-nucleotidyl transferase (TdT) buffer (30 mM Trizma base, 140 mM sodium cacodylate, 1 mM cobalt chloride, pH 7.2) mixture of rTdT and FITC conjugated dUTP were added to tissue sections and incubated for 60 min at 37°C. Sections were post rinsed in buffer for 15 min to stop the reaction. Sections were subsequently washed thrice in PBS for 5 min each, immersed in 0.5 μg/ml DAPI solution for 10 min at room temperature, gently washed in PBS for another 3x5 min and mounted. The slides were examined under a microscope (Eclipse 80i, Nikon, Japan) and images were captured digitally with the NIS-Elements F 3.0 software using a digital camera (DS-Fi1, Nikon, Japan).
Western blotting of molecular markers of BPH
Prostatic tissues were homogenized on ice in lysis buffer (50 mM Tris, 150 mM NaCl, 1.0% IGEPL®, 0.5% sodium deoxycholate, 0.1% SDS, pH 8.0) containing protease and phosphatase inhibitor cocktail (Sigma-Aldrich). Likewise, human BPH-stromal cells in culture were lysed in Lysis buffer (Sigma) by sonication. Homogenates were incubated for 20 min on ice and centrifuged (13,000g, 4°C, 15 min). The protein concentration of the supernatant was determined by Bradford method. Forty microgram protein of each sample was boiled for 10 min in denaturing sample buffer [10% glycerol, 1% SDS, 1% β-mercaptoethanol, 10 mM Tris-HCl (pH 6.8), 0.01% bromophenol blue], separated on 8–12% acrylamide gels and transferred on to Immobilon-P PVDF Membranes (Millipore). The membranes were blocked with 5% skimmed milk (in TBS, pH 7.6) for 1 hour and incubated overnight at 4°C with primary antibodies (TGF-beta, IGF-1, and PCNA 1:5,000), Bel-2, bax (1:10,000) and pSTAT-3 (1:2000) separately, and then re-probed with β-actin antibody (1:10,000, Sigma-Aldrich) and STAT-3 (1:2000) for loading correction. Subsequently, the blots were washed three times in 0.1% Tween-20 in TBS and incubated with 1:10,000 dilution of secondary antibody (HRP conjugate). After extensive washing in 0.1% Tween-20 in TBS, substrate solution was added to the membrane, incubated for 5 min and exposed at room temperature. The membranes were developed with enhanced chemiluminescence (ECL) kit, by following the manufacturer’s (Millipore, USA) instructions.

Human BPH explant culture and treatment ex vivo
BPH tissues obtained during TURP were also used for explants culture (with informed consent of patients) by following the method of Le Torterec et al. (Le Tortorec et al., 2008). Immediately following surgery, BPH tissues were placed in Medium 199 (Sigma-Aldrich) at 4°C and processed within 2 h. Prostate tissue was diced into ~3.0 mm³ fragments, and transferred into a 12-well tissue culture plate (4-6 pieces/well) containing 1 ml of media (Medium 199 supplemented with 10% FCS and antibiotics). The cultures were maintained for 7 days in a humidified atmosphere of
5% CO$_2$ at 37°C and the medium was replaced every second day. The explants were treated separately on the 8th day of culture with extracts/compound at 10 μg/ml concentration each for another 7 days and thereafter fixed in 10% buffered formaldehyde for histology.

Data analysis
All in vitro and ex vivo experiments were repeated three times whereas the in vivo data represents average of 5 animals. The results were analyzed by one way analysis of variance (ANOVA) using the Prism Graph Pad software. P values less than 0.05 were considered as significant. For Fourier transform image analysis of human BPH tissues, the analog microscopic images (RGB) were digitized and converted to Grayscale [0 (black) to 255 (white), total 256 pixels]. The digitized images were then analyzed using the MATLAB (version 6.5, release 13), on an Intel 2.86 GHz quad core processor that was programmed for the digital images on the basis of the frequency of pixel values. The statistical analysis was made in term of the mean, median, mode & standard deviation of the pixel data. The Gaussian plots were positively and negatively skewed on the basis of the mean & median values. The power spectrum of the pixels by the Fourier analysis of the images has been calculated. Interpretations of information from all the four techniques, i.e., histogram, Gaussian plot, standard deviation and Fourier analyses were similar.
Results

Antiproliferative activity against human BPH stromal cells

The crude ethanolic fraction of *C. sempervirens* (CS-EtOH) significantly inhibited the proliferation of human BPH stromal cells with an IC$_{50}$ of 21.0±1.53 µg/ml, *in vitro*. In a parallel assay, ethanolic fruit extract of *Dodecadenia grandifolia* was used as negative control and it exhibited negligible anti-proliferative activity against BPH stromal cells with an IC$_{50}$ of >500 µg/ml (data not included).

Figure 3.1.1. Antiproliferative activity of the fruit extracts of *Cupressus sempervirens* against human BPH-stromal cells. Effective concentration-50 (EC$_{50}$ µg/ml) of parent ethanolic extract (CS-EtOH) and its chloroform (CS-EtOH-Chlor) and butanol (CS-EtOH-Butanol) soluble fractions.

Thereafter, different fractions (hexane, butanol, chloroform, water etc.) isolated from ethanolic extract of *C. sempervirens* were tested and the activity was essentially localized in the chloroform soluble fraction (CS-EtOH-Chlor), which exhibited an antiproliferative IC$_{50}$ of 21.3±2.33 µg/ml against BPH stromal cells, *in vitro*. Negligible antiproliferative activity was detected in the butanol fraction (CS-EtOH-Butanol; IC$_{50}$ = 109.0±4.93 µg/ml) [Figure 3.1.1], while other fractions were largely inactive.
Antiproliferative activity of chloroform soluble phyto-compounds of *Cupressus sempervirens*

A total of 8 major phyto-compounds (A–H) were isolated and characterized from the chloroform fraction of *C. sempervirens*, as per the procedures detailed (Rawat *et al.*, 2010).

![Bar graph showing EC50 (µM) of eight compounds (A-H) isolated and purified from CS-EtOH-Chlor.](image)

*Figure 3.1.2. Antiproliferative activity of the pure diterpenes of *Cupressus sempervirens* against human BPH-stromal cells. EC50 (µM) of eight compounds (A-H) isolated and purified from CS-EtOH-Chlor.*

All these compounds were diterpenes that exhibited moderate to potent anti-proliferative activity against human BPH-stromal cells *in vitro* [Figure 3.1.2]. Compound B (labda-8(17),12,14-trien-19-oic acid; communic acid) was found to be the most potent, with an anti-proliferative IC50 of 11.3±0.71 µg/ml (37.5±2.36 µM) against human BPH stromal cells [Figure 3.1.2].
Figure 3.1.3. Molecular structures of isolated diterpenes (A-H) and Structure Activity Relationship (SAR) explaining the activity pattern of the isolated compounds in BPH stromal cells.

The BPH activity obtained for the diterpenes shown in the Figure 3.1.2. First, all the active compounds have a decalin basic unit in their structures (Compound B, H, A, C). These results suggest that the presence of decalin ring is mandatory for an acceptable anti BPH activity in these compounds. However the lack of activity obtained for compounds E, F, D, G clearly indicates that the presence of a decalin ring would be a structural requirement but not by itself sufficient for the BPH activity. In an attempt to find other potentially reactive sites in the compounds, we have proposed a biogenetic pathway of these terpenes. When the compounds were tested against BPH activity, the highest activity was found for the compound B with EC<sub>50</sub> values of 37.5 µM, along with C (59.7 µM), A (77.3 µM) and H (87.5 µM). These results led us to speculate that two moieties, decalin ring system and double bonded...
conjugated system, would be necessary to produce the anti BPH activity for the
diterpenes. To more clearly define the binding sites of these molecules, we have
evaluated eight compound of this series.

All the compounds can be categorized into two groups on their chemical similarities. Compounds \( F, D, G \) and \( B \) are substituted open C-ring diterpenoids, among them Communic acid (\( B \)) showed a remarkable potent activity. Within the other compounds activity decreased with the substitution in alkyl chain, as can be observed by comparing the effect of acetyl group (\( D \)), hydroxyl group (\( G \)) and carboxylic group (\( F \)). It should be noted that principal structural difference among these compounds is the conjugated double bond in the alkyl chain present on C-9 decalin ring position for compound \( B \). Compounds \( E, H, A \) and \( C \) are diterpenoids having substituted C-ring. In these compounds, we speculate that cyclization into C-ring retain the activity but interestingly, the relative positions of hydroxyl groups exhibited potent activity. The compound \( C \) in which aromatic ring was hydroxylated had as much potent activity as those in which 18-CH\(_3\) was hydroxylated. Thus positions of hydroxyl groups could be necessary for this activity. Activity dramatically decreased with the presence of a carbonyl group in the B-ring as in the compound \( E \). On the contrary, isopropyl moieties impart no effect on the activity.

Structure-activity relationship also revealed that the presence of carboxylic acid group and terminal double bond (Compound \( F, D, G \)) do not affect the activity but the position of hydroxyl group and C-ring formation (Compound \( E, A, H, C \)) slightly changed the activity. Finally, since diterpenoids having decalin system with conjugated double bonds in alkyl chain have been reported as anti BPH active agents [Figure 3.1.3].

**Effect of labda-8(17), 12,14-trien-19-oic acid on JAK-2/STAT-3 signaling pathway and allied proteins at transcription level**

Labda-8(17),12,14-trien-19-oic acid at 20 - 40 \( \mu \)M upregulated transcripts of the negative regulators of JAK/STAT signaling pathway, viz. SOCS (Suppressor of
Cytokine Signaling) and PIAS (Protein Inhibitor of Activated STAT), in a concentration dependent manner (Figure 4). However, the mRNA levels of IL-6, Janus Kinase-2 (JAK-2) and TGF-α were reduced non-significantly [Figure 3.1.4].

**Figure 3.1.4. Transcript profile of interleukin-6 (IL-6), Suppressor Of Cytokine Signaling (SOCS), Janus Kinase-2 (Jak-2), Protein Inhibitor of Activated STAT (PIAS) and Transforming Growth Factor-α (TGF-α) genes in human BPH-stromal cells treated with the most active diterpene (compound-B, trans communic acid) at 0, 20, 30 and 40 µM for 48 hours. Significant difference from control (0 µM) is indicated as *P<0.05; ***P<0.001.**

**Effect of labda-8(17),12,14-trien-19-oic acid on expression and phosphorylation of STAT-3**

The effect of labda-8(17),12,14-trien-19-oic acid on JAK/STAT-3 (JAK/STAT-3) signaling of actively proliferating human BPH stromal cells was evaluated by estimating the Tyr<sup>705</sup> phosphorylation (activation) of STAT-3 by Janus kinases (JAK), using western blotting.
Investigation of novel synthetic molecules and natural products for prostatic hyperplasia

Figure 3.1.5. Western blot (A) and densitometric analysis (B) for expression of Stat-3 protein in BPH-stromal cells treated with compound-B. Significant difference from control (0 µM) is indicated as **P < 0.01; ***P < 0.001.

The phosphorylation of Stat-3 in BPH stromal cells was visibly affected by labda-8(17),12,14-trien-19-oic acid, especially at concentrations 30 and 40 µg/ml. However, the expression of non-phosphorylated Stat-3 protein did not change very significantly during this treatment and served as loading standard [Figure 3.1.5].

Effect of labda-8(17),12,14-trien-19-oic acid on Androgen responsive genes in androgen dependent LNCaP cells

Labda-8(17),12,14-trien-19-oic acid was found significantly active in decreasing the expression of androgen responsive genes KLK3/PSA and TMPRSS2(Nelson et al., 2002) in androgen sensitive LNCaP prostate cancer epithelial cell line.
Investigation of novel synthetic molecules and natural products for prostatic hyperplasia

**Figure 3.1.6.** Transcript profile of androgen sensitive PSA and TMPRSS2 genes in androgen responsive prostatic epithelial LNCaP cells treated with testosterone (10 nM) and compound B (10 µM) for 48 hours. Significant difference from control is indicated as **P<0.01; ***P<0.001.

The expressions of both KLK3/PSA and TMPRSS2 genes increased significantly (p<0.01) in cells incubated with testosterone (10 nM) while it decreased significantly (p<0.01 and p<0.001, respectively) in cells treated with the test compound (10 µM). The test agent potently countered the effect of testosterone and significantly reduced KLK3/PSA and TMPRSS2 (p<0.01 and p<0.001, respectively) at 10 µM in LNCaP cells co-incubated with 10 nM testosterone [Figure 3.1.6].

**In vivo activity of C. sempervirens fruit extract in rat BPH model**

Aged rats that were given citral (100 mg/Kg) for four weeks to induce BPH had apparently bigger prostate (175.0 ± 13.8 mg/100 g body weight) than vehicle-treated control (151.0 ± 11.1 mg/100 g) rats. [Figure 3.1.7]
Figure 3.1.7. Weight of prostate (per 100 gm of body weight) after treatment with vehicle, citral, citral+CS-EtOH and Citral + CS-EtOH-Chlor. Significant difference from Control and Citral is indicated as *P < 0.05; **P < 0.01.

However, when citral-treated rats were co-administered crude ethanolic extract of *C. sempervirens* at a dose of 10 mg/Kg for 3 weeks (treatment starting one week after initiation of citral treatment and continued up to the end of experiment) a significant reduction in prostate weight was evident (104.0 ± 6.2 mg/100 g; P<0.01). A similar reduction was also seen when the labda-8(17),12,14-trien-19-oic acid enriched chloroform fraction of CS was used.
Prostatic histology revealed that the 4-week citral treatment of aged rats induced extensive epithelial hyperplasia with a sizable increase in stromal component of the prostate [Figure 3.1.8 b,B]. Notably, the prostatic histology of citral-treated rats was quite similar to that of human BPH (McNeal John E., 2007). However, no such hyperplasia was evident in rats that were co-administered CS ethanolic extract at a dose of 10 mg/Kg for 3 weeks [Figure 3.1.8 c,C]. The prostatic histology of these rats was fairly comparable to that of control, vehicle-treated rats [Figure 3.1.8 a,A]. On the other hand, when the labda-8(17),12,14-trien-19-oic acid enriched CS-fraction was given to citral-treated rats (10 mg/Kg X 21 days) a better management of hyperplasia was evident. The prostatic histology of this group of rats appeared marginally superior
to vehicle-treated control rats [Figure 3.1.8 d,D]. We did not use pure labda-8(17),12,14-trien-19-oic acid in this experiment due to non-availability of enough quantity for in vivo studies.

**C. sempervires** * induces stromal-cell apoptosis in rat prostate*

Prostatic tissue sections of citral-treated rats were fluorescently stained with 4’,6-diamidino-2-phenylindole (DAPI) and FITC-Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) to detect the presence of apoptotic cells. As seen in Figure 3.1.9, DAPI stained the DNA of epithelial cells more brightly than that of stromal cells. In contrast, TUNEL (FITC) staining was limited only to the stromal cells.

![Figure 3.1.9](image)

*Figure 3.1.9. Representative TUNEL (green) and DAPI (blue) labeled histological sections of prostate of rats that were given citral [A & B]; citral + CS-EtOH [C] and citral + CS-EtOH-Chlor [D]. Positive control sections [B] were treated additionally with DNAse for 10 min before fluorescent labeling. The protocol for citral and CS treatments was same as detailed in legend for Figure 3.1.8.*

Both ethanolic extract [Figure 3.1.9C] and its labda-8(17),12,14-trien-19-oic acid enriched (chloroform-soluble) fraction [Figure 3.1.9D] were convincingly effective in
instigating TUNEL labelling (apoptosis) of prostatic stromal cells while the vehicle itself was ineffective [Figure 3.1.9A]. On the other hand, prostatic sections treated with DNAse (positive control) had TUNEL labelling predominantly in the epithelial cells [Figure 3.1.9B].

Effect of *C. sempervirens* on molecular markers of prostatic hyperplasia

The anti-apoptotic B-cell lymphoma protein-2 (Bcl-2) increased markedly in prostate of rats with BPH (citral treated) but fell sharply after co-administration of *C. sempervirens* (CS-EtOH or CS-EtOH-Chlor). In contrast, the pro-apoptotic Bcl-2 associated protein-X (Bax) remained low in prostatic tissues of control and BPH rats but increased prominently in those treated with CS extracts [Figure 3.1.10].

![Western blot and densitometric analysis](image)

*Figure 3.1.10. Western blot (A) and densitometric analysis (B) for protein levels of bcl-2, bax, TGF-β, IGF-1 and PCNA in prostate of rats treated orally with vehicle*
(control), citral (BPH), citral + CS-EtOH (CS-EtOH) and citral + CS-EtOH-Chlor (CS-EtOH-Chlor). Citral was given at 100 mg/Kg and plant extracts were given at 10 mg/kg, once daily. CS treatments were started 1-week after beginning of citral treatment and both the treatments continued for another 3-weeks. The total duration of treatments in all groups was 4-weeks. Significant difference from Control and Citral is indicated as **P < 0.01; ***P < 0.001.

The prostate of animals in treatment groups had lower expression of growth factors IGF-1 and TGF-β (Insulin like Growth Factor-1 and Transforming Growth Factor-β) than vehicle-control and BPH groups. The proliferating cell nuclear antigen (PCNA) increased sharply in prostate of rats with induction of BPH but was reduced to control levels with co-administration of CS-extracts [Figure 3.1.10].

**Effect of CS on human BPH explants**

Periurethral BPH tissues (removed during TURP from BPH patients) were maintained in defined medium as explant cultures and treated with either vehicle (BPH-control), or 10.0 µg/ml of CS-EtOH or labda-8(17),12,14-trien-19-oic acid enriched (CS-EtOH-Chlor) fraction or pure labda-8(17),12,14-trien-19-oic acid, for 7 days. The histological investigations were made microscopically and the degree of differentiation was quantified by Fourier Transform Spectral Analysis. The Gaussian plots of this analysis are included in Figure 3.1.11. As evident from microscopic pictures of stained sections, BPH control tissues were full of stroma with very little differentiation.
Figure 3.1.11. Hematoxylin/eosin stained section of human BPH tissues maintained as explants cultures in vitro for 7 days and thereafter treated with vehicle (BPH), CS-EtOH, CS-EtOH-Chlor or compound-B at 10 µg/ml for another 7 days [A]. Gaussian plots for Fourier Transform Spectral Analysis of digitized microscopic images indicate level of differentiation [B].

Treatment with CS extracts and pure labda-8(17),12,14-trien-19-oic acid decreased the stromal component and differentiated the tissues into distinct stromal and acinar compartments [Figure 3.1.11], which was substantiated by an increase in AUC (area under curve) of the Gaussian plots [Figure 3.1.11].
Discussion

BPH originates in the transition zone surrounding the urethra and is predominantly characterized by stromal hyperplasia. Transurethral resection of prostate (TURP) is a technique that clears the bladder outlet obstruction caused by BPH through removal of tissues from the hyperplastic peri-urethral zone. The removed tissue layers provide excellent experimental material for pre-clinical research. The stromal cells isolated from these BPH tissues undergo rapid proliferation and have been used for in vitro evaluation of anti-BPH drugs (Padayatty et al., 1997). In the present study, the eight major chloroform soluble compounds (all diterpenoids) exhibited anti-proliferative activity against BPH stromal cells with EC$_{50}$ ranging between 37 to 195 µM. These compounds belonged to the labdane family, which are bio-synthesized by enzymatic oxidation and transformation of the copalyl diphosphate precursors formed by successive hydride shift and proton eliminations of geranyl geranyl diphosphate. The eight chloroform soluble compounds identified could further be categorized into two groups of four compounds each on the basis of their structural prototypes. In the first group, compounds F, G, D and B had substituted open C-ring system, and among them labda-8(17),12,14-trien-19-oic acid (B) showed remarkably potent activity. The activity hierarchy of the other 3 compounds was in accordance with substitutions in alkyl chain, in the order of acetyl group (D) > hydroxyl group (G) > carboxylic group (F). The conjugated double-bonded alkyl chain present on C-9 decalin ring position of compound B contributed to its potent anti-proliferative effects against BPH stromal cells in vitro. In the second group, compounds E, A, H and C were diterpenoids with substituted C-ring. In these compounds, the cyclization of C-ring largely retained the activity but the presence of a carbonyl group in the B-ring (E) reduced the activity significantly, though the presence of isopropyl moiety in these structures did not have any notable effect. The structure-activity relationship is available as online resource.

At least two natural diterpenes have recently been found very promising against prostate cancer and have been studied in some detail, but none has been studied against
BPH. Carnosol, a dietary diterpene isolated from culinary herbs, inhibited prostate cancer cell proliferation \textit{in vitro} (IC\textsubscript{50} ~40 µM) as well as \textit{in vivo}, apparently through dual antagonistic activity against androgen and estrogen receptors (Johnson \textit{et al.}, 2010). Similarly, 6-hydroxy-5,6-dehydrosugiol (HDHS), a natural diterpene from Japanese cedar (\textit{Cryptomeria japonica}), exhibited potent anti-proliferative and pro-apoptotic activity against prostate cancer cells and the primary mechanism of action was through inhibition of androgen receptor (Lin \textit{et al.}, 2008). While Carnosol is structurally similar to the diterpene A, HDHS is an analog of diterpene E (sugiol) isolated from \textit{C. sempervirens}. Though both these structures have an aromatic C-ring (similar to the aromatic ring of estradiol), they were found to be lesser active against BPH stromal cells than labda-8(17),12,14-trien-19-oic acid [trans-communic acid, B] which has an open C-ring structure. It is important to note here that trans-communic acid possesses 5α-reductase inhibitory activity (Ko, J.H, 2006) like finasteride (the standard BPH drug). Hence suppression of androgen action in target cells could likely be one of the anti-BPH mechanisms of this compound. Accordingly, labda-8(17),12,14-trien-19-oic acid [B] was tested for inhibition of androgen action in androgen sensitive LNCaP cells. It decreased expression of androgen responsive genes in absence and presence of testosterone, indicating potent androgen-antagonistic effects. Significant inhibition of these two androgen sensitive markers for epithelial proliferation of prostate (Nelson \textit{et al.}, 2002) in presence of testosterone indicates that the action of labda-8(17),12,14-trien-19-oic acid could apparently be mediated via inhibition of 5-α reductase. However, the androgen receptor (AR) is also reported to be activated in absence of androgens by JAK/STAT pathway (Ueda \textit{et al.}, 2002) via IL-6, which is elevated in BPH-stromal cells (Penna \textit{et al.}, 2009). Consequently we investigated the role of STAT-3 signaling in the anti-proliferative action of labda-8(17),12,14-trien-19-oic acid [B] isolated from CS. In BPH-stromal cells the compound down-regulated STAT-3 signaling mainly at the level of post-translational activation (phosphorylation) \textit{in vitro}. Though IL-6 and TGF-α, the known activators of STAT-3 (via phosphorylation of tyrosine at position 705), were not reduced significantly, the negative regulators of JAK/STAT pathway viz. SOCS (Suppressor of cytokine signaling) and PIAS
(protein inhibitor of activated STAT) were significantly upregulated at transcription level by the labda-8(17),12,14-trien-19-oic acid in BPH cells. Activated STATs stimulate expression of SOCS, which in turn binds to phosphorylated JAK to turn off the pathway by a negative feedback loop (Rawlings et al., 2004). On the other hand, PIAS binds activated STAT dimers and prevents their binding to DNA (Greenhalgh and Hilton, 2001). Thus STAT-3 signaling is negatively regulated by labda-8(17),12,14-trien-19-oic acid to prevent proliferation of BPH stromal cells.

A decline in testicular endocrine function of aged Sprague Dawley rats has been reported (Karpas et al., 1983; Wu et al., 2009), with androgen levels falling by nearly 40% (Roselli et al., 1986) and estrogen levels remaining almost unaffected (Winters and Takahashi, 1983). Despite this androgen-estrogen imbalance, aged rats normally do not develop prolific hyperplasia of the prostate like humans. Nevertheless, treatment of these rats with a weakly estrogenic compound (citral) induced profuse hyperplasia and produced typical BPH histology of the prostate (resembling that of humans) involving both epithelial and stromal compartments. Similar effects of citral on rat prostate have been reported earlier (Servadio et al., 1986). However it is important to note that citral does not induce hyperplasia in castrated rats (Geldof et al., 1992), which indicates the mandatory role of androgens in BPH of rat, as in humans. The labda-8(17),12,14-trien-19-oic acid enriched (chloroform-soluble) fraction of C. sempervirens could suppress this hyperplasia very effectively and the action was apparently mediated through the stromal component since apoptosis (TUNEL labeling) was limited only to the stromal compartment. Decreased expression of the anti-apoptotic B-cell lymphoma protein-2 (Bcl-2) and increased expression of its associated protein-x (Bax, which promotes apoptosis) further support induction of apoptosis in treated rat prostates. These effects become crucial in light of the fact that clinical symptoms of BPH are not necessarily related to the prostatic size, but rather to the proportion and composition of its volume occupied by stromal tissue (Bartsch et al., 1979; Deering et al., 1994; Marks et al., 1994). Additionally, increased levels of TGF-β in BPH tissue (Lee et al., 1999) can induce smooth muscle cell phenotype in BPH stromal cells (Peehl and Sellers, 1997),
which become the predominant cell-type in aged prostatic stroma and are responsible for clinical symptoms. The anti-BPH activity of CS-extracts was also evident by a reduction in TGF-β in rat prostate, especially by its labda-8(17),12,14-trien-19-oic acid enriched chloroform soluble fraction. We could not evaluate the effect of pure labda-8(17),12,14-trien-19-oic acid [B], in the rat model because enough test material was not available for the in vivo assays. Nevertheless, the improved results obtained with the fraction enriched with this compound over the parent ethanolic extract specifically indicate the potential of diterpenes from *C. sempervirens* to curb prostatic hyperplasia.

The expression of prostatic Insulin-like growth factor-I (IGF-I), a mitogenic and antiapoptotic agent, in humans increases with age and may rise up to 3-times in BPH (Bonnet *et al*., 1993). While IGF-I transcripts have been localized only to the prostatic stroma (Barni *et al*., 1994) its specific binding sites are located in the epithelium (Fiorelli *et al*., 1991; Barni *et al*., 1994), which govern epithelial proliferation through paracrine regulation. In the rat BPH model, a significant reduction in prostatic IGF-I expression after treatment with *C. sempervirens* extracts may well promote pro-apoptotic activity (authenticated by reduced Bcl-2/Bax ratio) and repress proliferation (supported by reduced PCNA expression) in BPH. However, it is pertinent to note that most of the molecular markers that were studied in rat BPH tissues exhibited a better response with the labda-8(17),12,14-trien-19-oic acid enriched fraction than the parent ethanolic extract. The prostatic histology also supported a better activity of the former than the latter. It is thus apparent that CS diterpenes, especially labda-8(17),12,14-trien-19-oic acid constitute the major anti-BPH component of *C. sempervirens*.

The human BPH tissues (in explant cultures) responded very well to the presence of *C. sempervirens* extract as well as to pure labda-8(17),12,14-trien-19-oic acid and its enriched fraction. An apparent reduction in stromal component of treated samples was marked by better differentiation of tissue histology into stromal and epithelial compartments. To quantitate this effect we used Fourier transform spectroscopy which measured the relative light density of each pixel (total 65536 pixels) on a scale of 0
Investigation of novel synthetic molecules and natural products for prostatic hyperplasia