SECTION-6

MATERIALS AND METHODS
6. Materials and Methods

6.1. In vitro assays

6.1.1. Cell lines and chemicals

RAW264.7 and SW1353 cell lines were purchased from American Type Culture Collection (ATCC, USA), DMEM, L-15 media, Ham's F12, FBS, penicillin and streptomycin, lipopolysaccharide (LPS), IL-1β, dexamethasone, 1400W dihydrochloride and (3-4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA), L-glutamine was purchased from Himedia Corporation, Mumbai, India). Antibodies for NF-kB p65, COX-2 and tubulin were purchased from Santa Cruz Biotechnology, Inc., CA, USA). MMP kit was purchased from Cisbio, PGE$_2$ and LTB4 kits were purchased from Cayman and tissue culture plasticware was purchased from BD Biosciences (San Diego, CA, USA).

6.1.2. Cell Culture

RAW264.7 and SW1353 cell lines were purchased from American Type Culture Collection (ATCC, USA). RAW264.7 and SW1353 were maintained in DMEM and L-15 media containing 2 mM L-glutamine, respectively, (Himedia Corporation, Mumbai, India) supplemented with 10% FBS (Sigma, St. Louis, MO, USA), 20Units/ml penicillin and 20 µg/ml streptomycin (Gibco BRL, USA).

6.1.3. Plant material

The bark of cinnamon (Cassia and zeylanicum) as well as aerial parts of Ocimum (O. basilicum and O. sanctum) were procured from Natural
Remedies, Pvt. Ltd. Bangalore. The plant material was collected from cultivatable sources by Natural remedies. The material was identified by National Institute of Science Communication and Information Resources (NISCAR), New Delhi and Dr. P. Santhan, in-house taxonomist, Pharmacognosy department, R&D centre, Natural Remedies Pvt. Ltd, Bangalore, India. A voucher specimen no. 206 for *C. cassia*, 215 for *C. zeylanicum*, 208 for *O. basilicum* and 106 for *O. sanctum* were deposited in Natural Remedies Pvt. Ltd., library.

### 6.1.4. Preparation of extracts

#### 6.1.4.1. Method of preparation of aqueous and methanolic extracts

For the preparation of methanolic extracts, the coarsely powdered raw material (50 g) was extracted with methanol (~200 ml) under reflux at 70°C for 1 h and the solvent was filtered. The remaining raw material was refluxed by adding 150 ml methanol for 1 h, repeated twice and again filtered. The liquid filtrate was combined and concentrated using rota vapour under vacuum to a thick paste at temperature NMT 60°C and 10.0 g of crude extract was obtained. For the preparation of aqueous extracts, coarsely powdered raw material (50 g) was mixed with water and extracted at 85 to 90°C (3 times each with 200 ml water for 1 h each wash) and filtered each time. The combined liquid filtrates were concentrated using rota vapour under vacuum to a thick paste at temperature NMT 60°C and 15.0 g of crude water extract was obtained. The combined liquid filtrates were concentrated using rota vapour under vacuum to a thick paste at temperature NMT 60°C and 15.0 g of crude water extract was obtained.
6.1.4.2. Method of preparation of *O. sanctum* LOTs (combination of aqueous and methanolic extracts)

The coarse ground whole plants of *O. sanctum* (300 kg) were charged into a stainless steel jacketed extractor fitted with a reflux condenser. Methanol (1200 L) was added to the extractor and the contents were refluxed for 3 h by providing steam in the jacket. The liquid extract was drained from the extractor into a separate vessel and fresh methanol (1000 L) was added to the extractor containing the marc. The extraction procedure as above was carried out two times and the liquid extracts from each extraction step was separately subjected to distillation under vacuum (at <55 °C) until a thick paste with a total solid content of 40–50% (w/w) was obtained. Thick paste obtained from the three extraction steps was mixed and dried under vacuum (<65 °C) to get lumps of the extract that were milled and sieved (# 40) to get a uniform-powdered extract (around 27 kg). Methanol was stripped off from the marc by passing the steam and heating at 80°C. After removal of methanol, demineralised water (1200 L) was added in the extractor containing marc and the contents were refluxed for 3 h by providing steam in the jacket. The extraction of marc with water was carried out totally three times. The liquid aqueous extracts were drained from the extractor, combined and passed into a concentrator and were subjected to distillation under vacuum (at <75°C) until the total solid content in the liquid reached about 15–20% (w/v). The concentrated liquid was then spray dried to get water extract of *O. sanctum* (around 45 kg). The alcohol and water extracts were then analysed for the content of active constituents and blended to get final extract with the required levels of active constituents.

6.1.5. Isolation of human chondrocytes

Human cartilage sample was obtained from the patient undergoing knee replacement surgery after approval from Bharati Vidyapeeth Deemed
University institutional ethics committee (Ref: BVDU/ MC/ 55) and proper consent from the patient. Chondrocytes were prepared by the enzymatic digestion of cartilage with 0.25% collagen and plated (1 × 10^6 cells/ml) in 35 mm primaria coated culture dishes. The cells were cultured in DMEM: Hams F12 containing 2 mM L-glutamine, 10% FBS, 100 Units/ml penicillin and 100 µg/ml streptomycin and grown in 5% CO2 incubator at 37°C.

6.1.6. Cell viability Assay

RAW264.7, SW1353 and human primary chondrocytes were seeded at a density of 5x10^5 cells/ml in 96-well plates. The cells were treated with different concentrations (0-100 µg/ml) of extracts for 24 h. Cell viability was determined by MTT assay.

6.1.7. Nitric oxide (NO) Assay

RAW264.7 cells were seeded at a density of 5x10^5 cells/ml in 96 well plate and allowed for 24 h to adhere. The cells were pre-treated with different concentrations (0-100 µg/ml) of extracts for 1h followed by stimulation with 1 µg/ml of LPS for 18 h. The amount of nitrite released was measured by Griess reaction.

6.1.8. PGE2 Assay

RAW264.7 cells, SW1353 and human primary chondrocytes were seeded at a density of 5x10^5 cells/ml in 96 well plate and allowed to adhere for 24 h. The RAW264.7 cells were pre-treated with different concentration of extracts as described above. SW1353 and human chondrocytes were starved for 18 h in L-15 media containing 0.25% FBS and 1:1 DMEM/Hams F-12 respectively, prior to treatment with the test samples. The cells were pre-treated with the extracts followed by stimulation with 10 ng/ml of IL-1β for
18 h. PGE$_2$ concentration was determined in the cell supernatants by using PGE$_2$ EIA-Monoclonal based kits (Cayman Co., Ann Arbor, Mich., USA).

6.1.9. LTB4 assay

SW1353 and human chondrocytes were starved for 18 h and pre-treated with the extracts as described above. LTB4 levels were determined in the supernatant by using LTB4 EIA-Monoclonal based kits, (Cayman Co., Ann Arbor, Mich., USA)

6.1.10. MMP assay

Human chondrocytes were starved for 18 h and pre-treated with the extracts as described above. MMPs (2, 9, 13) were quantified in the supernatant by using commercial SensoLyte® 520 Generic MMP Activity Kit (Cysbio Anaspec Eurogentec group, USA).

6.1.11. Intracellular NO assay

SW1353 were seeded at a density of 5x10$^5$cells/ml in 96 well plate and allowed for 24h to adhere. The cells were starved for 18 h in L-15 media containing 0.25% FBS prior to treatment with the test samples. The cells were pre-treated with the extracts followed by stimulation with 10 ng/ml of IL-1β for 18 h. The amount of nitrite released was measured by using DAF-FM dye.

6.1.12. Hyaluronidase assay

Hyaluronidase was assayed by a highly sensitive spectrophotometric method, based on precipitation of HA with cetylpyridinium chloride, which is used for high throughput screening for hyaluronidase inhibitors (Tung et al 1994).
Enzyme (800 U/ml) and HA substrate (0.40 mg/ml) were incubated at 37°C for 1 h. Enzyme activity was measured by monitoring the percentage of undigested HA substrate in the cetylpyridinium chloride precipitate at absorbance 415 nm (A415 nm) after the enzyme reaction.

6.1.13. DMMB Assay for analyzing the release of Proteoglycans

Total GAG content in supernatants of IL-1β stimulated chondrocytes was measured by the dimethylmethylene blue dye binding assay using chondroitin sulphate (CS) as a standard.

6.1.14. Western blotting

The cells were seeded at a density of 4x10^5 cells/well in 6-well plates and allowed to adhere for 24 h. The cells were pre-treated for 1 h with different concentrations (0–100μg/ml) of extracts followed by stimulation with 1μg/ml of LPS for 18 h. The cells were trypsinized and total protein was isolated. Briefly, the cell pellet was resuspended in 60 ml lysis buffer containing 50 mM Tris (pH 7.4), 5 mM EDTA, 0.5% NP40, 50 mM NaF, 1 mM DTT, 0.1 mM PMSF, 0.5mg/ml leupeptin (Pro-pure Amersco, Solon, USA), 1mg/ml pepstatin (Amresco, Solon, USA), 150 mM NaCl, 0.5mg/ml aprotinin (Amersco, Solon, USA). The cells were incubated on ice for 45 min with intermittent mixing. The extract was centrifuged for 20 min at 4°C at 12,000 rpm. The protein was estimated by using Bradford reagent (Biorad Laboratories Inc, CA, USA). Thirty micrograms of total protein was loaded onto a 10% SDS-polyacrylamide gel and electro-transferred to Amersham Hybond-P PVDF membrane (GE Healthcare, UK) in sodium phosphate buffer (pH 6.8). The membrane was blocked in 5% BSA in TST and incubated at 4°C overnight with primary antibody against iNOS, COX-2, NFκB or tubulin at a 1:500 dilution. The membrane was washed in TST and incubated with secondary IgG HRP conjugate at 1:5000 dilution. Proteins
were visualized with a chemiluminescence kit (Amersham ECL Advance western blotting detection kit, GE Healthcare, UK) and densitometric analysis was performed on scanned immunoblot images using the Image J gel analysis tool and normalized with respect to tubulin as an internal control.

6.1.15. Statistical analysis

All the results were obtained from three independent experiments, each performed in triplicates and the values have been presented as mean±SD. Differences among means were tested for statistical significance using one-way analysis of variance (ANOVA). The analyses were carried out using Graph-pad prism 5 software (San Diego, CA, USA). *p<0.05; **p<0.01; ***p<0.001 were considered to be statistically significant. For multiple comparison Tukey test was used.

6.2. In vivo studies

6.2.1. Subacute toxicity study

6.2.1.1. Experimental Animals

Wistar rats (7–8 weeks) of either sex (males: 120 -169 g and females: 119-152 g) were housed in polypropylene cages with stainless steel grill tops and provided with bedding of clean paddy husk. Temperature was maintained between 25±20°C with relative humidity of 44–56%, with light and dark cycles of 12 h, respectively, for one week before and during the experiments. The animal experiment was conducted taking into consideration the Committee for the Purpose of Control and Supervision of Experiments on Animals guidelines and after approval by the Institutional Animal Ethics Committee (IAEC No. May_2012-09_045 on dated May 12, 2012).
6.2.1.2. Experimental groups

The repeated doses for oral toxicity studies were carried out in rats according to the OECD test guideline 407. Rats were divided randomly into 6 groups of 5 animals each (5 males and 5 females). Group I served as a vehicle control and received only distilled water. Group II, III and IV received OSE orally at the doses of 250, 500, 1000 mg/kg, respectively, everyday for 28 days. Group V and VI served as control recovery and high dose reversible groups, respectively. Group II received only distilled water and Group VI received 1000 mg/kg dose of OSE orally for 28 days. The test item was administered orally by gavage, as a single dose at similar times each day. For all dose groups, volume (10 ml/kg) was adjusted and rounded up to single decimal point as per the body weight for an individual animal throughout the treatment period. During this period, all the animals were observed for signs of toxicity and mortality throughout the experimental period. The changes in body weight, food consumption and clinical signs were also observed and recorded. At the end of the treatment and recovery periods, evaluation of clinical pathology parameters (haematology, coagulation, biochemistry and urine analysis), behaviour, and motor activity were conducted. The animals were sacrificed with an overdose of ether and other body organs were taken out for detailed weight and histopathological changes. All the parameters in the present study were outsourced for analysis to Sa-Ford, Navi Mumbai-410 208, India.

6.2.1.3. Motor Activity and Behavioural Observations

Animals were subjected to examination motor activity distance travelled (DT), resting time (RT), stereotypic time, ambulatory time, burst of stereotypic movements (BSM), horizontal count, ambulatory count, horizontal break, clock wise rotation and counter clock-wise rotation measurements using an automated animal activity measuring system.
Animals were also examined for sensory reactivity measurements (response, touch response, click response, pupil response, tail pinch response and air righting reflex); fore limb and hind limb grip strength; hind limb foot splay records and sensory reactivity, during last week of treatment and recovery period.

6.2.1.4. Clinical Observations

Animals were subjected to a detailed clinical examination on day 8, 15, 22 and 28 day of dosing. This included home cage observations (posture and presence of convulsions), handling observations (ease of removal from the cage, handling reactivity, palpebral closure, lacrimation, eye examination, piloerection, and salivation) and open field observations (changes in gait and mobility; arousal; respiration; presence of clonic or tonic movements or stereotypic movements or bizarre behaviour; urination, defecation, vocalizations and rearing). Ophthalmological examination was carried out using direct ophthalmoscope initially, prior to dosing and last week of treatment/recovery period prior to blood collection for clinical pathology. During last week, motor activity, grip strength, foot splay, sensory reactivity and ophthalmoscopic examinations were performed on animals allocated to control (G1) and OSE treated (G2-G4) groups and extended to recovery (Group G5 and G6) animals. Before ophthalmologic examination, mydriasis was induced using 1% tropicamide.

6.2.1.5. Haematological parameters

Total erythrocyte count (RBC), haemoglobin (HGB), haematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), total leukocyte count (WBC), platelet count (PLT), prothrombin time (PT), prothrombin time (PT), activated partial thromboplastin time (APTT) were determined in control
(G1) and OSE treated (G2-G4) and recovery (Group G5 and G6) group animals.

6.2.1.6. Clinical Biochemistry

The serum was carefully aspirated with a Pasteur pipette into sample bottles for the various biochemical assays. The serum clinical biochemistry parameters that were analysed included albumin/globulin ratio (A:G), alanine amino transferase (ALT), albumin (ALB), aspartate amino transferase (AST), alkaline phosphatase (ALP), blood urea nitrogen (BUN), chloride (Cl) mmol/l, cholesterol (CHO), creatinine (Crea), globulin (GLB), glucose (GLU), gamma-glutamyl transpeptidase (GGT), potassium (K), sodium (Na), total bilirubin (T.BIL), total prOSEin (T.PRO), triglycerides (TRIG), calcium (CAL), phosphorus (PHOS) and urea.

6.2.1.7. Urinalysis

Urine samples from the rats of main and recovery groups were collected in graduated tubes attached at the bottom of metabolic cages. The parameters that were analyzed from the urine samples included colour, appearance, volume specific gravity, pH, prOSEin, glucose, bilirubin, blood / blood cell, leucocytes, urobilinogen, nitrite, ketone, microscopical parameters [presence of epithelial cells, red blood cells, pus cells (white blood cells), casts, crystals and other sediments (e.g. sperms etc.).

6.2.1.8. Organ weights and histology

The rats were dissected and different organs were excised and weighed for recording absolute organ weights. The relative organ weights were calculated against terminal body weights for every individual animal taken just prior to necropsy. The specimens for histopathology were fixed in 10% neutral
buffered formalin, except for organs like eye(s) and testes; which were initially fixed in modified Davidson’s solution for 24 hr and then transferred to 10% neutral buffered formalin (NBF) for preservation. The specimens (3-4μm in thickness) of liver, kidney, heart, spleen, aorta, caecum, colon, duodenum, eyes with optic nerve, ileum, jejunum, , mammary glands, mesenteric and mandibular lymph node, oesophagus, ovary with oviduct, pancreas, peyer’s patches, pituitary, prostate, seminal vesicle with coagulating gland, rectum, salivary glands, sciatic nerve, skeletal muscle, skin, spinal cord (cervical, mid-thoracic and lumbar), spleen, sternum with marrow, stomach, testis, thymus, thyroid with parathyroid’s, trachea, urinary bladder, uterus with cervix, vagina were trimmed of any adherent tissue and stained with hematoxylin and eosin stain following the standard laboratory procedures. The stained sections were examined under microscope for any cellular damage or change in morphology.

6.2.1.9. Statistical analysis

Raw data was analysed using Sigma Plot 11.0 statistical software (Supplied by Cranes Software International Ltd. Bangalore). All body weight data were checked for normality using Shapiro-Wilk test and for homogeneity of variance using equal variance test. Data showing significance in their variances were subjected to Dunnett and t-test.