# "ANTIINFLAMMATORY POTENTIAL OF SELECTED MEDICINAL PLANTS FOR THE MANAGEMENT OF OSTEOARTHRITIS"

**A THESIS SUBMITTED TO**
**BHARATI VIDYAPEETH DEEMED UNIVERSITY, PUNE**
**FOR THE AWARD OF DEGREE OF**
**DOCTOR OF PHILOSOPHY IN BIOTECHNOLOGY**
**UNDER THE FACULTY OF SCIENCE**

**SUBMITTED BY**
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**OCTOBER-2015**

# CERTIFICATE

**This is to certify that the work incorporated in the thesis entitled "Antiinflammatory potential of selected medicinal plants for the management of osteoarthritis" for the degree of 'Doctor of Philosophy' in the subject of Biotechnology under the faculty of Life Sciences has been carried out by Ms. Prerna Raina in the department of Cell and Translational Research Laboratory, Interactive Research School for Health Affairs, Bharati Vidyapeeth Deemed University, Katraj, Pune during the period from 2010 to 2015 under the guidance of Dr. Ruchika Kaul- Ghanekar.**

**Place: Pune** **(Signature of Head of the Institute with seal)**

**Date:** **Dr. A.C Mishra**

# CERTIFICATE OF GUIDE

**This is to certify that the work incorporated in the thesis entitled "Antiinflammatory potential of selected medicinal plants for the management of osteoarthritis" submitted by Ms. Prerna Raina for the degree of 'Doctor of Philosophy' in the subject of Biotechnology under the faculty of Life Sciences has been carried out in the Department of Cell and Translational Research Laboratory, Interactive Research School for Health Affairs, Bharati Vidyapeeth Deemed University, Katraj, Pune during the period from 2010 to 2015 under the guidance of Dr. Ruchika Kaul-Ghanekar.**

**Place: Pune**
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# DECLARATION BY THE CANDIDATE

**I hereby declare that the thesis entitled "Anti-inflammatory potential of selected medicinal plants for the management of osteoarthritis " submitted by me to the Bharati Vidyapeeth University, Pune for the degree of Doctor of Philosophy (Ph .D) in Biotechnology under the faculty of Life Sciences is orginal piece of work carried out by me under the supervision of Dr. Ruchika Kaul-Ghanekar. I further declare that it has not been submitted to this or any other university or Institution for the award of any degree or Diploma.**

**I also confirm that all the material which I have borrowed from other sources and incorporated in this thesis is duly acknowledged. If any material is not duly acknowledged and found incorporated in this thesis, it is entirely my responsibility. I am fully aware of the implications of any such act which might have been committed by me advertently or inadvertenly.**

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**Date: /** **Prerna Raina**

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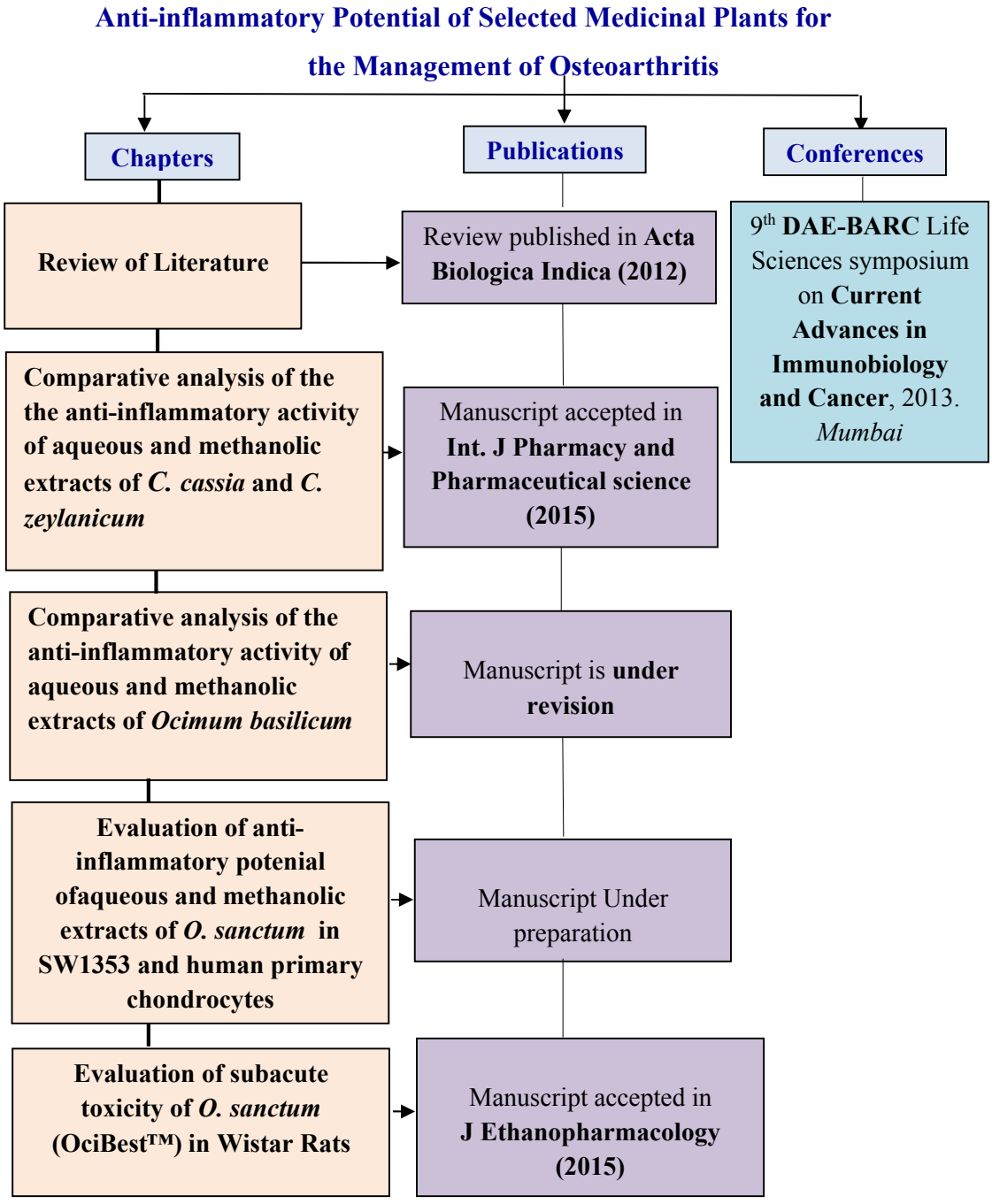
**Prerna Raina**

# Thesis at a Glance



# Index

# SECTION-1

# ORGANIZATION OF THE THESIS

## 1.1. Introduction and Review of literature.

Under this heading, information regarding osteoarthritis, its pathophysiology and current treatment modalities available for the management of OA has been mentioned. This is followed by importance of Complementary and Alternative Medicine (CAM) with a special emphasis on the significance of herbal medicines that are being explored for the management of OA.

## 1.2. Comparing the anti-inflammatory activity of aqueous and methanolic extracts of *C. cassia* and *C. zeylanicum* in RAW264.7, SW1353 and human primary chondrocytes.

The anti-inflammatory potential of the aerial parts of aqueous and methanolic extracts of Cinnamon (*Cinnamomum cassia* and *Cinnamomum zeylanicum*) has been compared in different cell lines such as RAW264.7, SW1353 and human primary chondrocytes. The chapter has discussed as to how the methanolic extract of Cinnamomum (*cassia, zeylanicum*) reduced the levels of $PGE_2$, LTB4 and MMPs more effectively than the aqueous extract.

## 1.3. Comparing the anti-inflammatory activity of aqueous and methanolic extracts of *Ocimum basilicum* in RAW264.7, SW1353 and human primary chondrocytes.

The anti-inflammatory potential of the aerial parts of aqueous and methanolic extracts of *O. basilicum* has been compared in different cell lines such as RAW264.7, SW1353 and human primary chondrocytes. The chapter has discussed

as to how the aqueous extract of *O. basilicum* reduced the levels of $PGE_2$, LTB4 and MMPs more effectively than the methanolic extract.

### 1.4. Comparing the effect of aqueous and methanolic extracts of *O. sanctum* on IL-1β induced $PGE_2$ and LTB4 levels in human chondrosarcoma (SW1353) cell line

The anti-inflammatory potential of the aerial parts of aqueous and methanolic extracts of *O. sanctum* has been compared in different cell lines such as SW1353 and human primary chondrocytes. The chapter has discussed as to how aqueous and methanolic extracts were found to be equally effective in terms of $PGE_2$ and LTB4 inhibition. *Boswellia serrata* was used as a positive control. Interestingly, *O. sanctum* showed more significant decrease in $PGE_2$ levels in human chondrosarcoma cell line SW1353 compared to the positive control *B. serrata*.

### 1.5. Evaluation of anti-inflammatory potenial of combination of aqueous and methanolic extracts of *O. sanctum* (LOT001, LOT002 and LOT02) in SW1353 and human primary chondrocytes

The chapter has discussed as to how different combinations of *O. sanctum* (LOT001, LOT002 and LOT02) methanolic and water extracts were tested for their anti-inflammatory activity at in vitro. It was found that LOT02 showed a significant decrease in the $PGE_2$ and $LTB_4$ levels at lower concentrations. LOT02 significantly decreased the expression of IL-1β, TNF-α and COX-2 proteins in SW1353 compared to IL-1β stimulated control cells. In chondrocytes, LOT02 (named as OciBest™), significantly reduced the hyaluronidase and proteoglycan activity, thereby indicating that it exhibited chondroprotective activity.

### 1.6. Evaluation of subacute toxicity of LOT02 (OciBest™) in Wistar rats

*O. sanctum* LOT02 (OciBest™) was further evaluated for subacute toxicity in Wistar rats. The chapter has discussed the important findings wherein no treatment-related adverse effects were found in the rat's upto 1000 mg/kg/day dose. OciBest™ did not induce any adverse effects as was evident after analyzing clinical, pathological, biochemical, hematological, urine and histopathological parameters.

# SECTION-2

# INTRODUCTION

## 2. Introduction

Osteoarthritis (OA) is a heterogeneous group of skeletal disorder that is characterized by common structural and functional changes in overall joint tissues. It involves cartilage loss, synovium inflammation and bone sclerosis (Henrotin *et al*., 2014). The etiology of OA is multifactorial that has been attributed to be a result of complex interplay of different biochemical factors (Toriyabe *et al*., 2004). Even though OA is the most common type of arthritis encountered worldwide, the development of effective disease-modifying treatments have lagged behind compared to the other types of arthritis. The current modalities for treating arthritis are symptomatic and fail to recover the cartilage degradation and joint destruction. The treatment options include the use of non-steroidal anti-inflammatory drugs (NSAIDs) that inhibit COX enzymes and give a temporary relief to the patient. However, their consumption is usually associated with various side effects such as gastrointestinal (GI) ulcers, increased risk of cardiovascular events and renal problems (Cho *et al*., 2015). Thus, alternative methods of treatment are being constantly explored along with the conventional therapies. Various complementary medicines such as glucosamine and chondroitin sulphate that exhibit chondroprotective properties are being currently used in the management of OA along with the conventional drugs, however, their therapeutic efficacy remains controversial (Bottegoni *et al*., 2014). Thus, it has become important to identify novel chondroprotective agents that would not only help in the management of pathophysiology of OA but would also be free from any side effects.

The present study aimed at comparing the anti-inflammatory activity of aqueous and methanolic extracts from different medicinal plants and identifying the most active effective variety or a blend of the potent varieties, which could be proposed in the management of OA related pathophysiology. The standardized extracts were obtained from Natural Remedies Pvt. Ltd. Bangalore. The anti-inflammatory activity of standardized extracts (aqueous and methanolic) from two different species of Cinnamon [*Cinnamomum*

*zeylanicum* (True cinnamon/Ceylon cinnamon) and *Cinnamomum cassia* (Cassia, Chinese cinnamon)] and Ocimum [*Ocimum basilicum and Ocimum sanctum*] was compared in murine macrophage cell line (RAW264.7), human chondrocytic cell line (SW1353) and human primary chondrocytes (isolated in-house). The preliminary screening of the eight different extracts was done on the basis of their NO scavenging and inhibition of $PGE_2$, LTB4 and MMP activity.

We found that the methanolic extracts of *C. cassia* (CC) and *C. zeylanicum* (CZ) were more effective than the aqueous extracts in terms of $PGE_2$, LTB4 and MMP inhibition in all the tested cell lines. However, on comparing the two species of cinnamon, CZ was found to be more potent than CC. In case of *O. basilicum* (OB), in RAW 264.7, the aqueous extract ($OB_W$) decreased NO and $PGE_2$ production more effectively compared to the methanolic extract ($OB_M$)**.** Interestingly, the decrease in NO was accompanied by a corresponding decrease in iNOS protein expression. $OB_W$ decreased total NFκB and COX-2 proteins significantly compared to $OB_M$. Similarly, in SW1353 and chondrocytes, $OB_w$ decreased $PGE_2$ and LTB4 production appreciably compared to $OB_M$. In chondrocytes, $OB_W$ reduced the production of MMP-2, MMP-9 and MMP-13 significantly, than $OB_M$. All these data suggested that compared to the methanolic extract, the aqueous extract of *O. basilicum* could be explored for its potential applications in the management of inflammatory conditions associated with OA.

We further compared the efficacy of aqueous ($OS_W$) and methanolic ($OS_M$) extracts of *O. sanctum* (OS) in modulating the expression of proinflammatory molecules. Both $OS_W$ and $OS_M$ effectively modulated IL-1β induced $PGE_2$ and LTB4 levels, compared to the positive control *B. serrata*.

Since all the tested herbals could not be taken further, we thus focussed only on *O. sanctum* because of its strong traditional background, easy availability and potent anti-inflammatory activity was selected for mechanistic and *in vivo* studies.

The aqueous extract of *O. sanctum* has been known to contain primary metabolites [amino acid, nucleotides, carbohydrates (sugar, starch) and lipids (fats, essential oils, waxes terpenoids and oleoresin)] whereas the alcoholic extract has been known to contain secondary metabolites (as total phenols, tannins, steroids and alkaloids) in large proportions (Chandrasekaran *et al*., 2013; Jamal, 2011). Thus, in our further studies the aqueous and methanolic extracts of *O. sanctum* were mixed together in different proportions and evaluated for their activity in modulating the pro-inflammatory molecules. We wanted to find whether at lower concentrations compared to the individual extracts of $OS_M$ and $OS_W$.

The aqueous and methanolic extracts of *O. sanctum* were mixed in the ratios of 1:1 (LOT001), 1:2 (LOT002) and 1:4 (LOT02). Among all other LOTs, LOT02 induced an enhanced decrease in $PGE_2$ and $LTB_4$ levels at lower concentrations. LOT02 significantly decreased the expression of IL-1β, TNF-α and COX-2 proteins in SW1353 compared to IL-1β stimulated control cells. LOT02 (OciBest$^{TM}$) was further evaluated for its anti-inflammatory activity in human primary chondrocytes. We found that OciBest$^{TM}$ significantly reduced the IL-1β induced $PGE_2$ and $LTB_4$ levels. It was found to reduce the hyaluronidase activity and proteoglycan loss from the chondrocytes. These results suggested that OciBest$^{TM}$ modulated the inflammatory molecules involved in cartilage degradation and could thus be used as a chondroprotective agent for the management of OA. OciBest$^{TM}$ was further found to be non-toxic, based upon subacute toxicity studies in Wistar rats, suggesting its safety for future clinical applications.

# SECTION-3

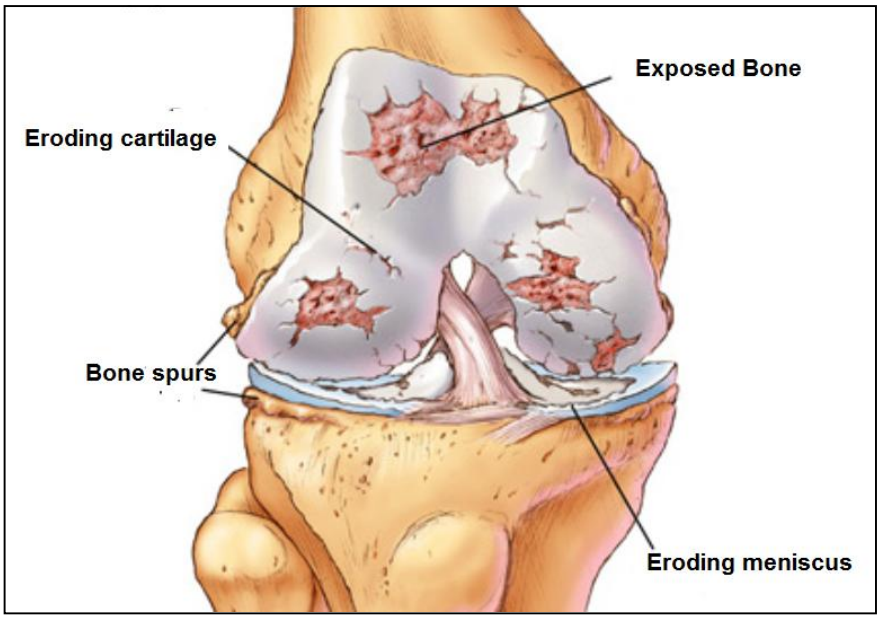
# RATIONALE OF THE STUDY

## 3. Rationale of the study

Recent developments in the use of herbal medicines for the prevention and/or treatment of a wide number of diseases has aroused a lot of interest in this field. A large number of herbs and plants have been traditionally used in managing various inflammatory disorders. However, their efficacy and mechanistic action in the management of osteoarthritis has not been evaluated in detail. Based on the available literature, we selected two medicinal plants, Cinnamon (*C. zeylanicum* and *C. cassia*) and Ocimum (*O. basilicum and O. sanctum*) that have been reported to exhibit anti-inflammatory potential, but their efficacy and safety in the management of osteoarthritis has not been evaluated. Thus, we hypothesized that the selected plants would modulate the expression of important catabolic mediators associated with pathophysiology of osteoarthritis. Based on this hypothesis, we compared the selected plant materials for their anti-inflammatory potential against RAW264.7, SW1353 and human chondrocytes to select the variety that would exhibit higher efficacy at lower therapeutic doses.

# SECTION-4

# REVIEW OF LITERATURE




**The overview given in the chapter has been published in Acta Biologica Indica (2012)**

## 4.1. Osteoarthritis -an overview

Osteoarthritis (OA) is a chronic, painful and progressive debilitating disease that affects the elderly population. It mainly leads to thinning of joint cartilage in the knees, hips, spine and/ or hands (Higashiyama *et al.,* 2010). Cartilage is the slippery tissue that covers the ends of bones in a joint. Healthy cartilage allows bones to glide over each other and helps to absorb the shock of the movements (Higashiyama *et al.,* 2010). In osteoarthritis, the top layer of cartilage breaks down and wears away that results into rubbing of the underneath bones, in effect causing pain, swelling, and loss of motion of the joint (Abramson and Krasnokutsky, 2006). Over the time, the joint may lose its normal shape and bone spur may grow on the edges of the joint. Bits of bone or cartilage could break off and float inside the joint space, which results into more pain and damage (Henrotin *et al*., 2014). OA is one of the most prevalent causes of disability in the aging population of the developing countries and occurs mostly in older people. However, younger people sometimes get osteoarthritis, primarily due to joint injuries.

### 4.2. Statistics of OA in India

According to the World Health Organisation (WHO), India has been projected to have endemic of osteoarthritis with about 80% of the 65+ population suffering from wear and tear of joints (Litwic *et al.,* 2013). About 40% of these people have been proposed to suffer from severe osteoarthritis, which would disable them from daily activities (Bhatia *et al*., 2013). By 2020, the number of 65+ population in India has been predicted to be about 177 million, which was earlier reported to be 100 million in 2010 (BBC News, retrieved in 2011; United States Census Bureau database, retrieved in 2011). The incidence of clinically significant knee osteoarthritis above the age of 55 years has been reported to be very high in India (25 to 30 % in women and 15-20 % in men) (Litwic *et al.,* 2013; Chopra *et al*., 2001; Jasrotia *et al*., 2003).

due to various reasons such as obesity, lack of balanced diet and regular exercise; and increased incidence of smoking (Zhang *et al.,* 2015; Marks, 2015).

Osteoarthritis is most common in women because post-menopause, they have weaker bones compared to men of their age. As a result of this, women develop osteoporosis, also known as porous bones (Multanen *et al.,* 2015). Although the incidence of OA is high, awareness about the disease is very low compared to other diseases such as diabetes, HIV and cancer. Thus, one should be made aware about this disease, which would help the sufferers to take preventive steps that could change the course of the disease and improve their quality of life.

### 4.3. OA related pathophysiology

OA results from the pathological imbalance between destructive and reparative processes, ultimately leading to the destruction of articular cartilage and subchondral bone (Abramson and Krasnokutsky, 2006). Despite its frequency in the population, the etiopathogenesis of OA remains poorly understood with few therapeutic options available. In OA, the regions that are mainly affected include cartilage, synovium and the bone (Abramson and Krasnokutsky, 2006). OA was traditionally believed to be a non-inflammatory type of arthritis, however, inflammatory mediators of pain have now been shown to be associated with it (Bonnet and Walsh, 2005). In response to various stimuli such as trauma, inflammation, age, obesity, mechanical stress, a cascade of molecular events lead to articular cartilage degradation. These events include downregulation of anti-inflammatory cytokines (IL-4, -10 and -13); tissue inhibitors of matrix metalloproteinases (TIMPs) and growth factors (IGF-1, TGF-β, bFGF and BMPs); upregulation of pro-inflammatory cytokines such as interleukin-1β (IL-1β), tumor necrosis factor alpha (TNF-α), IL-6, -8, -11, -17, -18) (Goldring, 2000; Papachristou *et al.,* 2008); and production of matrix metalloproteinases, collagenases, aggrecanases, nitric

oxide (NO), prostaglandins (PGE2) and cyclooxygenase-2 (COX-2) (Figure 1).

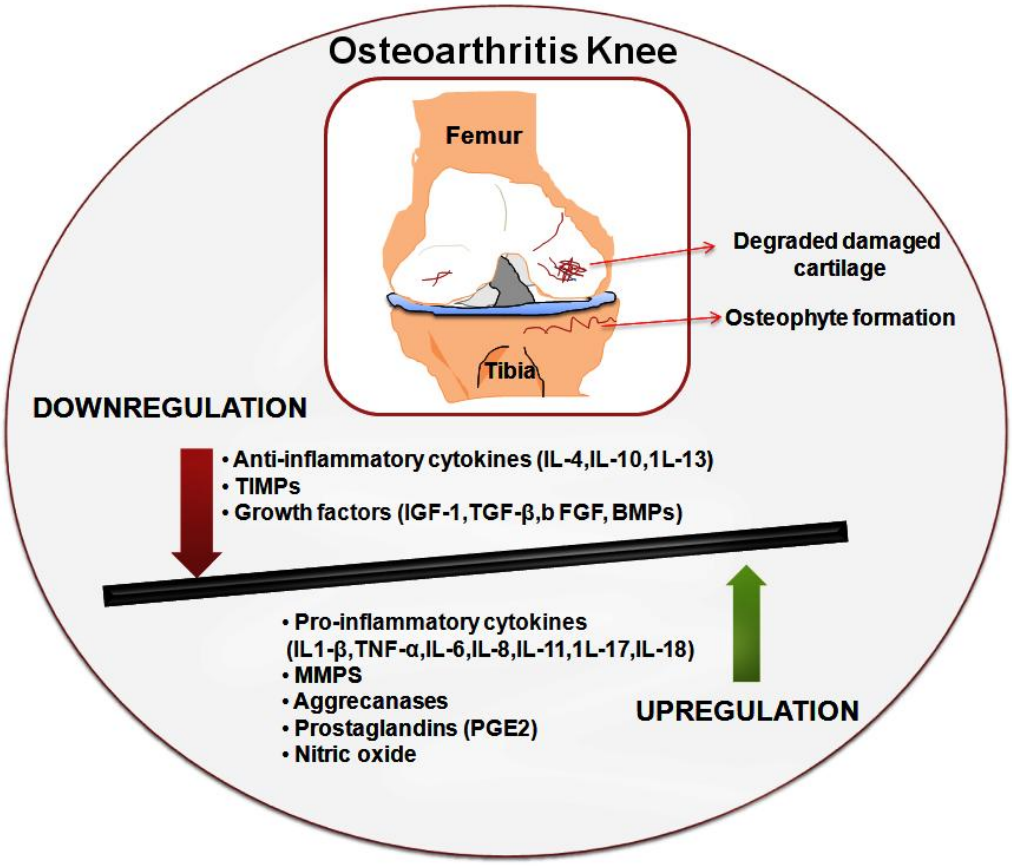



**Figure 1. Pathophysiology of osteoarthritis knee**

The pro-inflammatory cytokines, IL-1β and TNF-α induced mitogen activated protein kinase (MAPK), iNOS and nuclear factor κB (NFκB) pathways that ultimately lead to OA-related pathophysiology (Namdari *et al.,* 2008) including activation of MMPS (particularly, MMP-9, -13 and ADAMTS), COX-2, $PGE_2$, LOX-5, LTB4 and iNOS (Figure 2). All these cascade of events result into inflammation, swelling of the associated joints, apoptosis of chondrocytes; and degradation of cartilage. Inflammation may be a primary event in osteoarthritis progression. Considerable evidence suggest the role of nitric oxide (NO), $PGE_2$, LTB4 and MMPs in OA.

### 4.3.1. Nitric oxide (NO)

NO is a free radical gas and plays a major role as a signaling molecule in a variety of physiological processes (Aktan, 2004). Nitric oxide is synthesised in mammalian cells by the conversion of L-arginine to citrulline and the reaction is catalyzed by one of the three isoforms of enzyme which includes inducible nitric oxide synthase (iNOS), endothelial nitric oxide synthase (eNOS), neuronal nitric oxide synthase (nNOS) (7). In OA, macrophages produce both NO in response to inflammation (Korhonen *et al.,* 2005). NO reacts rapidly with superoxide to form peroxynitrite (a potent oxidant) which causes nitration of tyrosine (a compound inducing DNA injury), which inturn leads to apoptosis of articular chondrocytes. NO has been found to increase the production of COX-2 that leads to increase in the production of $PGE_2$, thereby contributing to the swelling, pain and inflammation associated with OA (Cho *et al.,* 2015; Ying *et al.,* 2013; Toriyabe *et al.,* 2004).

### 4.3.2. Prostaglandin $E_2$ ($PGE_2$)

$PGE_2$ is a prostanoid that is derived from arachidonic acid released from membranes by phospholipase $A_2$ (Ramonda *et al.,* 2015). Under inflammatory conditions, the activated macrophages produce $PGE_2$, which promotes inflammation by increasing vascular permeability, vasodilation in turn giving rise to redness, swelling, stiffness and pain (Ramonda *et al.,* 2015; Krustev *et al.,* 2015). The synthesis of prostaglandins depends mainly on the activity of the cyclooxygenase (COX) enzyme, particularly COX-2 that catalyzes conversion of arachidonic acid to variety of mediator molecules, including prostaglandin (PG) $E_2$, thromboxanes (TXA2), prostacyclins (PGI2), and highly inflammatory leukotrienes (LTB4) (Krustev *et al.,* 2015).

### 4.3.3. Leukotriene B4 (LTB4)

The process of inflammation is often found to be associated with the generation of reactive oxygen species (ROS) and oxidative stress. The latter leads to the synthesis of pro-inflammatory cytokines (IL-1β, TNF-α), nitric oxide (NO), prostaglandins (PGs), leukotrienes, phospholipase A2, nitric oxide synthase (NOS), cyclooxygenases (COXs), and lipoxygenases (LOX) (Krustev *et al.,* 2015). Arachidonic acid (AA) is a key biological intermediate involved in the COX and LOX pathway. 5-Lox metabolizes arachadonic acid to leukotienes (LTB4). Inhibition of one or both of the COX enzymes could shunt AA metabolism down the 5-LOX pathway, which can further exacerbate the severity of the disease (Martel-Pelletier and Pelletier, 2010). In OA, LTB4 acts as a powerful leukocyte chemoattractant. It has also been demonstrated to stimulate TNFα and IL-1β production from human osteoarthritis cartilage explants (Martel-Pelletier and Pelletier, 2010; Oliveira *et al.,* 2008).

### 4.3.4. Matrix Metalloproteinases (MMPs 2, 9 and 13)

In OA, matrix metalloproteinases (MMPs) have been found to play a key role in the progressive destruction of articular cartilage. MMPs are divided into five classes: collagenases (MMP-1.-8,-13), gelatinases (MMP-2,-9), stromelysins (MMP-3,-7,-10,-11,-12), membrane type MMPs (MMP-14,-15,-16,-17), non-classified MMPs (RASI-1, Enamelysin). The collagenases, also known as neutrophil collagenase, have the ability to cleave the triple helical structure of collagen (Yu *et al.,* 2012; Vincenti and Brinckerhoff, 2002). Stromelysin could degrade many components of extracellular matrix, which includes collagen type II, IX, X, XI, proteoglycan and aggrecan. On the other hand, gelatinases could digest collagen types I, III, IV and V (Jannie *et al.,* 2013). Among membrane type MMPs, MMP-14 is involved in cleaving the fibrillar collagen into smaller fragments. The non-classified MMPs have not been found to play any significant role in the pathophysiology of OA.

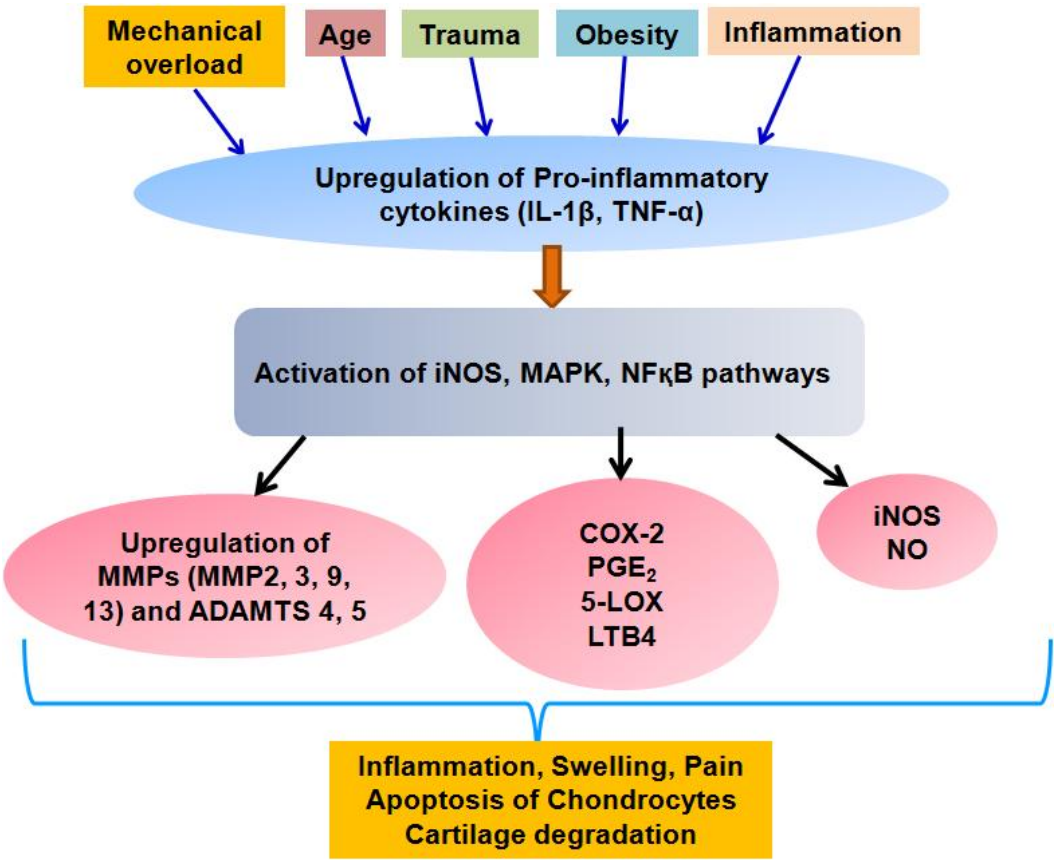



**Figure 2. Molecular mechanisms underlying pathophysiology of OA**

### 4.4. Conservative approaches in the management of Osteoarthritis

The current treatment options for the treatment of OA mainly aim at reducing the symptoms of pain and inflammation, maintenance of joint mobility and prevention of loss of function (Leong *et al.,* 2011). These options include a combination of non-pharmacological and pharmacological therapeutic modalities (Farkas *et al.,* 2010) (Figure 3).

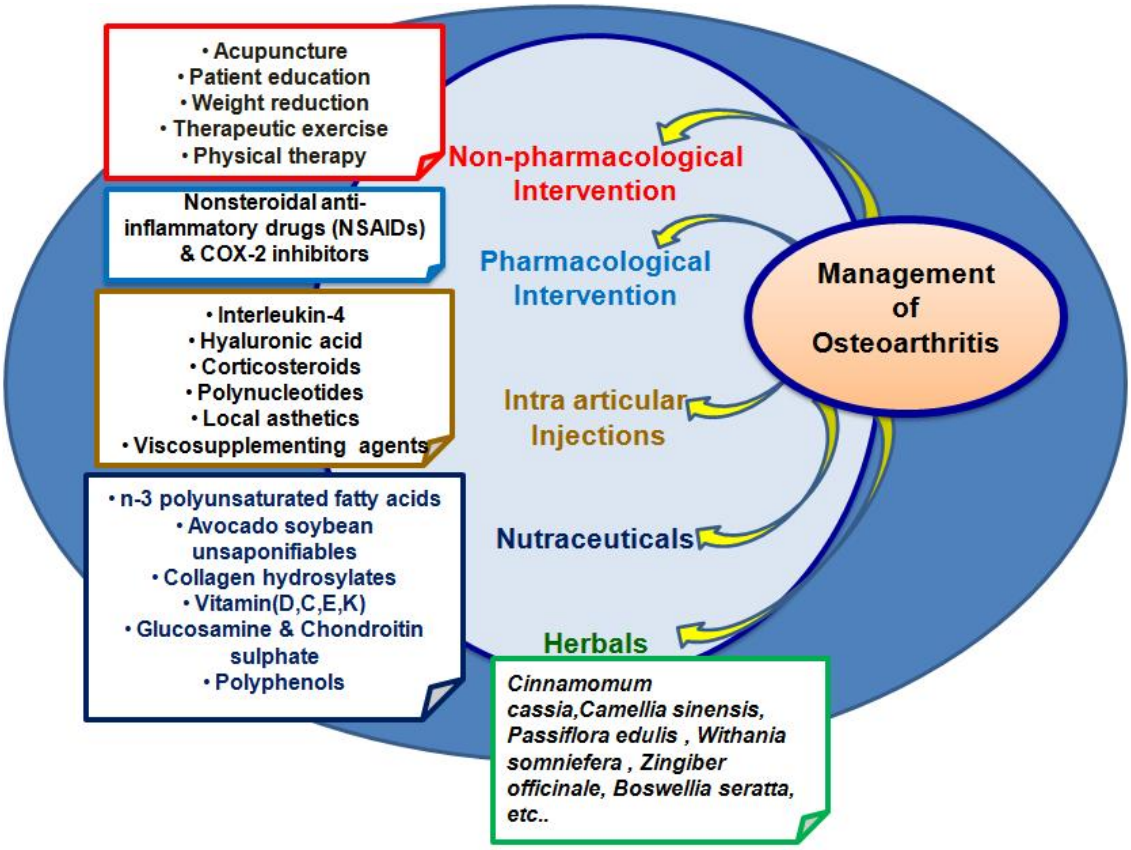



**Figure 3. Treatment options in the management of OA**

### 4.4.1. Non-Pharmacological Intervention

Non-pharmacological approaches have been mostly recommended to combat pain in osteoarthritis. Such interventions do not involve drugs and thus could reduce drug consumption and toxicity or even could help in delaying the need for joint replacement surgery. Non-pharmacological interventions include patient education and self-management (Leong *et al.,* 2011), exercise (Rannou and Poiraudeau, 2010) weight reduction (Norris *et al*, 2005), acupuncture and physical therapy (Vas *et al.,* 2004), the latter involving thermotherapy (Brosseau *et al.,* 2003), transcutaneous electrical nerve stimulation (TENS) (Cetin *et al.,* 2008; Osiri *et al.,* 2000) and short wave diathermy (Cetin *et al.,* 2008). Patient education is an important component of arthritic pain management. It has been proved that through lifestyle modification, particularly inclusion of exercise and weight reduction

programs, it is possible to manage the arthritic pain. Acupuncture is also used as an adjunct therapy for pain relief in osteoarthritic patients (Bennell *et al.,* 2015). Physical therapy is the backbone of OA treatment which includes muscle strengthening programmes, specific for certain joints and general aerobic conditioning (Vas *et al.,* 2004). These regimens have been shown to decrease pain and prevent disability in knee OA. Thermotherapy and sound wave diathermy that are a part of physical therapy have also been used for relieving the symptoms of osteoarthritis. TENS involves non-invasive safe nerve stimulation intended to relax pain by almost by 50-67% in OA (Cetin *et al.,* 2008; Osiri *et al.,* 2007).

### 4.4.2. Pharmacological Intervention

The pharmacological management of OA mainly focuses on the relief of symptoms associated with OA. It has been mainly dominated by the use of NSAIDs and analgesics (Sangdee *et al.,* 2002). It also includes topical treatment and intra-articular therapy.

#### 4.4.2.1. NSAIDs and analgesics

NSAIDs are effective analgesic and anti-inflammatory drugs that are mainly used in the treatment of OA related symptoms, the major one being the OA-related pain. Oral analgesic medications, commonly used to reduce arthritic pain include acetaminophen, ibuprofen, diclofenac and intra-articular corticosteroids, which are cyclooxygenase type 2 (COX-2) inhibitors (McKenna *et al.,* 2001). Though NSAIDs provide short-term pain relief in OA, but there are several side effects associated with their long term use (Mangoni *et al.,* 2010) that include upper and lower gastrointestinal damage, acute renal failure and congestive heart failure.

#### 4.4.2.2. Topical treatment

Topical treatment is an additional treatment option and is available in the form of creams so that less drug is absorbed systemically into the body (Mangoni *et al.,* 2010). Topical treatment modalities include the use of capsaicin, topical lidocaine and topical NSAIDs (Kim *et al.,* 2014). The topical application of NSAIDs reduces adverse effects of oral drugs by maximizing local delivery while simultaneously minimizing systemic toxicity.

#### 4.4.2.3. Intra-articular therapy

##### 4.4.2.3.1. Corticosteroids

Intrarticular injections of corticosteroids are being used frequently for the management of OA. These have been reported to show significant reduction in the pain and stiffness associated with hip osteoarthritis (Nair and Taylor-Gjevr, 2010; Robinson *et al.,* 2007). The major preparations include methylprednisolone acetate (MPA), triamcinolone hexacetonide (TAH), triamcinolone acetonide (TA), betamethasone acetate/betamethasone sodium phosphate (Celestone Chronodose) and betamethasone dipropionate/betamethasone sodium phosphate (Diprospan).

##### 4.4.2.3.2. Interleukin-4

IL-4, is an anti-inflammatory cytokine, has been reported to inhibit the expression of inducible nitric oxide synthase (iNOS) mRNA as well as the production of nitric oxide (NO) by indirectly inhibiting the production and activity of pro-inflammatory cytokines, IL-1β and TNF-α, in synoviocytes (Yorimitsu *et al.,* 2008). IL-4 has also been shown to be chondroprotective in vitro (Assirelli *et al.,* 2014).

#### 4.4.2.3.3. Hyaluronic acid

Hyaluronic acid (HA) or hyaluronan is a linear polysaccharide found in the extracellular matrix and is an important component of synovial fluid. It is essential for maintaining the viscoelastic properties of synovial fluid and acts both as a lubricant and shock absorber (Gigante and Callegari, 2011). Intra-articular injections of HA are being approved worldwide for the treatment and viscosupplementation of the osteoarthritic joints (Masuko *et al.,* 2009). Currently, only five FDA-approved injectable preparations of HA are available for clinical use that include Synvisc®, Hyalgan®, Supartz®, Orthovisc® and Euflexxa® (Fakhari and Berkland, 2013). HA viscosupplementation has been found to decrease the concentration of inflammatory mediators such as prostaglandins, fibronectin and cyclic AMP37 (Moreland, 2003). HA has been reported to increase the synthesis of chondroitin sulfate and proteoglycans and decrease the expression of MMPs and ADAMTS in human chondrocytes (Julovi *et al.,* 2011; Chen *et al.,* 2014).

#### 4.4.2.3.4. Polynucleotides

Polynucleotides, isolated from natural sources (fish sperm) are effectively being used for the management of OA. These are composed of polymeric molecules, which have the ability to bind to a large amount of water. These modulate the organization of water molecules to form a 3D gel-like network that can retain the moisturizing and viscoelastic properties of articular cartilage (Vanelli *et al.,* 2010). A clinical trial has reported intra articular polynucleotides to be more effective in relieving the pain in knee OA compared to the hyluronan supplementation and thus could be a good alternative (Giarratana *et al.,* 2014).

### 4.4.3. Complementary and Alternative Medicines for the Management of OA

Osteoarthritis therapeutic modalities mainly focus towards reduction of either pain or inflammation. However, the focus should be more towards cartilage regeneration and prevention of its degeneration. Recently, people are resorting towards the use of CAM that mostly include chondroprotective drugs, either in the form of nutraceuticals or in the form of herbals.

#### 4.4.3.1. Nutraceuticals

The term 'nutraceutical' is coined from the combination of 'nutrition' and 'pharmaceutical'. It refers to food or food products that provide health and medical benefits in terms of prevention and/or therapy. Nutraceuticals can protect the cartilage from oxidative damage caused by the generation of reactive oxygen species (ROS) (Leong *et al.,* 2013). A large number of nutraceuticals have been reported to be effective in the management of osteoarthritis that have been detailed out in this section (Table 1).

**Table 1. Nutraceuticals used in the management of OA**

| **Nutraceuticals** | **References** |
|---|---|
| n-3 polyunsaturated fatty acids (n-3PUFAs) | Leong *et al.,* 2013 |
| Glucosamine and chondroitin sulphate (CS) | Zeng *et al.,* 2015 |
| Avocado soybean unsaponifiables (ASUs) | Dinubile, 2010 |
| Collagen hydrosylates (CHs) | Walrand *et al.,* 2008 |
| Vitamin D<br>Vitamin C<br>Vitamin E<br>Vitamin K | Bergink *et al.,* 2009<br>Oikonomidis *et al.,* 2014<br>Rhouma *et al.,* 2013<br>Shea *et al.,* 2015 |
| Polyphenols | Shen, 2010 |
| Capsaicin | Laslett and Jones, 2014 |
| Jelly fish mucin | Ohta *et al.,* 2009 |

#### 4.4.3.1.1. n-3 or omega 3 polyunsaturated fatty acids (n-3 PUFAs)

Omega 3 fatty acids are the essential fatty acids that our body cannot synthesize and are available in soybean and canola oils, flaxseeds, walnuts, and fish oils. Omega-3 fatty acids (FAs) are known to modulate cellular signaling events, membrane protein function as well as gene expression (Knott *et al.,* 2011). Various studies have shown the anti-inflammatory effects of the polyunsaturated fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) and their role in cartilage metabolism (Curtis[a] *et al.,* 2000). They have been shown to reduce inflammatory mediators such as IL-1β, COX-2, 5-lipoxygenase (LOX) as well as the catabolic factors such as MMPs or ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs) that contribute to the inflammatory cascades in osteoarthritis (Curtis[b] *et al.,* 2002; Curtis[c] *et al.,* 2002). Recently, an *in vivo* study has reported the role of omega 3 in the regulation of osteoarthritis. Dietary intake of omega 3 FAs increased the cartilage GAG content, reduced denatured type II collagen and reduced pro- and activated MMP-2 production, all indicative of reduced disease severity (Knott *et al.,* 2011). Clinical administration of omega-3 FAs has also been found to reduce the stiffness and pain associated with osteoarthritis (Gruenwald *et al.,* 2009).

#### 4.4.3.1.2. Glucosamine and chondroitin sulphate (CS)

Glucosamine, chondroitin sulphate (CS) and hyaluronic acid (HA) form the backbone of cartilage and synovial fluid. They are not only formed naturally by the body but can also be provided through dietary supplementation. Glucosamine is an aminosaccharide which acts as a preferred substrate for the biosynthesis of glycosaminoglycan and for the production of aggrecan and other proteoglycans (Jerosch, 2011). Chondroitin sulphate is a major component of the extracellular matrix of many connective tissues, including cartilage, bone, skin, ligaments and tendons. CS, glucosamine sulfate (GS) or

both together affect the major bone biomarkers, osteoprotegerin (OPG), receptor activator of nuclear factor-kappa B ligand (RANKL) and the proresorptive activity of OA osteoblasts (El-Arman *et al.,* 2010; Lin *et al.,* 2008; d'Abusco *et al.,* 2008). Glucosamine and CS have been shown to stimulate collagen synthesis and reduce the expression of iNOS, COX-2 and phopholipase-2, thereby inhibiting the inflammatory cascades at the molecular level (El-Arman *et al.,* 2010; Lin *et al.,* 2008; d'Abusco *et al.,* 2008; Schiraldi *et al.,* 2010; Scarpellini *et al.,* 2008). Studies on humans have shown that CS supplements may have an effect in relieving pain and stiffness caused by arthritis (Zeng *et al.,* 2015). Moreover, combination of glucosamine and chondroitin was shown to be effective in relieving pain in OA patients (Clegg *et al.,* 2006) in a Glucosamine/Chondroitin Arthritis Intervention Clinical Trial. Oral administration of glucosamine and intra-articular administration of n-acetyl glucosamine in knees of animals with experimentally induced osteoarthritis have also been demonstrated to reduce the progression of osteoarthritis lesions (Pavelka *et al.,* 2010; Khan *et al.,* 2009).

#### 4.4.3.1.3. Avocado soybean unsaponifiables (ASUs)

These are derived from unsaponifiable residues of avocado and soybean oils. ASUs have been shown to promote the synthesis of anabolic factors that would normally feedback on the cell and will shut down the catabolic pathways (DiNubile, 2010). These have been shown to reduce the expression of inflammatory mediators such as IL1-β, TNF-α, COX-2 and $PGE_2$ (Au *et al.,* 2007). Moreover, clinical studies have revealed the disease modifying effects of ASUs in OA (Boileau *et al.,* 2009; Appelboom *et al.,* 2001).

#### 4.4.3.1.4. Collagen hydrosylates (CHs)

These are obtained by the process of enzymatic hydrolysis of collagen tissue present in the mammalian bone, hide or hide split. CHs are the main source of glycine and proline, the two essential amino acids that can regenerate and stabilize the damaged osteoarthritic cartilage (Benito-Ruiz *et al.,* 2009; Walrand *et al.,* 2008). *In vitro* studies have shown the role of CHs in stimulating the synthesis of extracellular matrix macromolecules by chondrocytes (Bello and Oesser, 2006)

#### 4.4.3.1.5. Vitamin D

It is known to play an essential role in calcium absorption by the body and helps to build cartilage and strong bones. Low serum levels of vitamin D have been shown to increase the progression of knee OA (Bergink *et al,* 2009; Breijawi *et al.,* 2009). Deficiency of Vitamin D has been found to have adverse effects on calcium metabolism, osteoblast activity, matrix ossification and bone density (Paola de *et al.,* 2008).

#### 4.4.3.1.6. Vitamin C

It is also known as ascorbic acid and has been shown to play an essential role in biosynthesis of cartilage molecules. Vitamin C participates in the synthesis of glycosaminoglycan as well as collagen. Its deficiency can impair the production as well as the biomechanical quality of cartilage (Paola de *et al.,* 2008). Ascorbic acid serves as a cofactor for enzymes that are crucial in collagen synthesis. It has been shown that ascorbate and ascorbic acid increased the protein and proteoglycan synthesis by articular chondrocytes as well as the mRNA levels of type I and II collagen, aggrecan and α-prolyl 4-hydroxylase (Clark *et al,* 2002).

#### 4.4.3.1.7. Vitamin E

Alpha-tocopherol or vitamin E is the only significant lipid-soluble, chain-breaking antioxidant present in plasma and red blood cells. The richest food sources of vitamin E are edible plant oils. In OA, vitamin E has been found to decrease the synovial inflammation by blocking the formation of arachidonic acid from phospholipids and inhibit the lipoxygenase activity, without having much effect on cyclooxygenase. Vitamin E has also been reported to promote the synthesis of glycosaminoglycan, involved in synthesis of proteoglycans in cartilage (Sanghi *et al.,* 2009).

#### 4.4.3.1.8. Vitamin K

Vitamin K or phylloquinone plays an essential role in the synthesis of proteins involved in the regulation of bone metabolism (Knapen *et al.,* 2007). Deficiency of vitamin K can result in abnormal cartilage and bone mineralization (Neogi *et al.,* 2008). Vitamin K has been shown to inhibit apoptosis in chondrocytes and its role in chondrocyte development and maturation in OA has been reported (Newman *et al.,* 2001).

#### 4.4.3.1.9. Polyphenols

Polyphenols are important chemical constituents present in food, either vegetables or fruits. These are secondary metabolites of plants and have been found to possess excellent anti-inflammatory activities (Yoon and Baek, 2005). Polyphenols are generally divided into hydrolyzable tannins and phenylpropanoids such as lignins, flavonoids and condensed tannins. Several reports have suggested that due to their rich antioxidant potential, polyphenols could reduce bone loss in men and women (Shen, 2010). Literature study has revealed the anti-inflammatory potential of a wide variety of polyphenols. Pycnogenol®, a standardized polyphenolic extract

from the bark of the French maritime pine *Pinus pinaster* (family Pinaceae), has been studied for its anti-inflammatory, anti-oxidant as well as inhibitory effects on MMPs and iNOS (Farid *et al.,* 2007; Grimm *et al.,* 2004). It has been well-documented to relieve the OA-associated pain and physical stiffness in patients. Quercetin, a plant-derived flavonoid has been found to decrease the expression of TNF-α and monocyte chemoattractant protein-1 (MCP-1) in human synovial cells (Henrotin *et al.,* 2011). Its anti-inflammatory and anti-oxidant properties have been reported.

An in vitro study has investigated the anti-inflammatory effect of prodelphinidins (polymeric tannin found in the pomegranate and green tea leaves) on human chondrocytes (Henrotin *et al.,* 2011). The study showed that prodelphinidins have the potential to increase PG (proteoglycan) and type II collagen and inhibit PGE2 synthesis by acting on COX-2 (Garbacki *et al.,* 2002). Nobiletin, a citrus polymethoxyflavone, has been studied *in vitro* in synovial fibroblasts and articular chondrocytes. It was reported to inhibit the production of $PGE_2$, MMP-3, MMP-9, ADAMTS-4 and 5 in rabbit and human synovial fibroblasts (Imada *et al.,* 2008). Nobiletin was also shown to activate the MMP inhibitor, TIMP-1 in rabbit articular chondrocytes as well as to inhibit cartilage degradation (Ishiwa *et al.,* 2003). Epigallocatechin-3-Gallate (EGCG) is the major polyphenolic component of green tea. It has been shown to inhibit the production of $PGE_2$, NO, COX-2 and iNOS as well as decrease the expression of MAPK and NFκB signaling pathways in osteoarthritic chondrocytes (Leong *et al.,* 2013).

Resveratrol, a natural polyphenol present in grape skin and red wine, has been found to have anti-oxidant as well as anti-inflammatory properties. It has been demonstrated to inhibit MMPs, $PGE_2$ and COX-2 and stimulate the synthesis of matrix components (PG, GAG, type II collagen), thereby preventing cartilage degradation (Zong *et al.,* 2012). Intra-articular injection of resveratrol in anterior cruciate ligament transaction OA model and LPS-induced arthritis model in rabbit, showed its chondroprotective activity (Elmali *et al.,* 2005).

Curcumin, an active component of *Curcuma longa*, commonly known as turmeric, is used as a spice, flavoring agent, food preservative as well as a coloring agent. Curcumin has been extensively studied for its anti-cancer (Gaurisankar and Tanya, 2008; Wani *et al.,* 2011)**,** anti-oxidant (Jackson *et al.,* 2006; Ramadan et al., 2011) and anti-inflammatory (Mathy-Hartert *et al.,* 2009) activities. Its potential in OA has been widely studied *in vitro*. Curcumin inhibits the activation of NF-κB in human articular chondrocytes. Recently, the anti-apoptotic effect of curcumin on osteoarthritic chondrocytes has also been demonstrated. It has been found to inhibit IL-1β, TNF-α, MMP-1, MMP-3, MMP-9, and MMP-13 as well as to restore the type II collagen and GAG synthesis (Shakibaei *et al.,* 2011; Chowdhury *et al.,* 2008)

#### 4.4.3.1.10 Capsaicin

Capsaicin (8-methyI-N-vanillyl-6-nonenamide) is the pungent vanilloid found in red peppers. It exerts its analgesic effect through exhaustion of transmitters and desensitization resulting into silencing of the afferents. The efficacy of capsaicin cream has been reported in osteoarthritis, diabetic neuropathy and psoriasis at clinical level (Laslett and Jones, 2014).

#### 4.4.3.1.11 Jelly fish mucin (Qniumucin)

It is a glycoprotein isolated from jelly fish and clinical studies have demonstrated its potential to reduce the articular cartilage degeneration in OA (Ohta *et al.,* 2009).

### 4.4.4 Herbals used in the management of OA

Medicinal plants have been used from time immemorial for the prevention as well as treatment of various disease conditions. Currently, the research on herbals is at its peak and more attention is being focussed towards elucidation of molecular mechanisms underlying the action of herbal drugs. Some of the herbals that have been proven to be effective in the management of osteoarthritis alongwith their molecular targets have been discussed in Table 2.

***Cissus quadrangularis*** is a perennial plant of the grape family. It is also commonly known as Veldt Grape. It has been shown to inhibit IL-1β induced inflammatory responses (inhibited nitric oxide and glycosaminoglycan release) on chondrocytes and alleviate bone deterioration in osteotomized rats via p38 MAPK signalling (Kanwar *et al.,* 2015).

***Phyllanthus amarus*** belonging to the family Euphorbiaceae, is a small herb that is well known for its medicinal properties. It has been reported to exhibit anti-inflammatory properties (Kassuya *et al.,* 2005). In IL-1β-induced cartilage explant degradation, it has been shown to decrease GAG level and MMP-2 activity (Pradit *et al.,* 2015).

***Harpagophytum procumbens*** (devils' claw) belongs to the family Pedaliacea and is a plant found in Kalahari region of South Africa. Its main active compound is harpagoside that has been shown to inhibit IL-1β induced production of MMP-1, MMP-3 and MMP-9 in human chondrocytes (Schulze-Tanzil *et al.,* 2004).

***Passiflora edulis*** is a vine belonging to the family Passifloraceae and is widely grown in almost all parts of the world. The antioxidant and anti-inflammatory properties in bioflavonoids of *P. edulis* have been reported (Farid *et al.,* 2010; Watson *et al.,* 2008). Plant flavonoids attenuate inflammation through inhibition of regulatory enzymes (lipoxygenase and cyclooxygenase) involved in arachidonic acid metabolism (Hooshmand *et al.,* 2007).

***Lonicera japonica*** is a Chinese herb belonging to the family Caprifoliaceae. Its flowers are of high medicinal value and have been found

to have anti-bacterial (Rhee and Lee, 2011) and anti-inflammatory (Kwak *et al.,* 2003) properties. The anti-nociceptive and anti-inflammatory activity of *L. japonica* in osteoarthritic animal models has been reported (Kang *et al.,* 2010).

***Anemarrhena asphodeloides*** is a Chinese herb belonging to the family Agavaceae. In traditional medicine, its rhizome is used as an anti-inflammatory (Kim *et al.,* 2009), anti-diabetic (Miura *et al.,* 2001) and antidepressant (Ren *et al.,* 2006). The anti-inflammatory as well as protective effect of *A. asphodeloides* in osteoarthritic cartilage has been reported (Kim *et al.,* 2009).

An in vitro study has shown that WIN-34B [a mixture of *Lonicera japonica* flowers and *Anemarrhena asphodeloides* root (2 : 1, w/w)] protected cartilage degradation through the regulation of matrix proteinases (aggrecanases and MMPs/TIMPs), inflammatory mediators (PGE2, NO, IL-1β, and TNF-α), and MAPK pathways in osteoarthritic human cartilage explant cultures and chondrocytes (Kang *et al.,* 2010).

***Rosa canina*** belongs to the family Rosaceae and is widely cultivated in Europe, Northwest Africa and Western Asia. Hyben vital is a phytomedicinal preparation of rose-hip powder, the fruit from a subtype of *R. canina*. Some reports have shown safety and efficacy of Hyben vital for the treatment of OA (Reina *et al.,* 2004). *R. canina* has also been reported to have anti-inflammatory (Orhan *et al.,* 2007) and antioxidant properties (Ghazghazi *et al.,* 2010). It has been reported to inhibit the production of NO and $PGE_2$ and reduce the secretion of cytokines (TNF-α, IL-1β), chemokines (RANTES) and MMPs (MMP-1, MMP-3 and MMP-13) in OA (Schwager *et al.,* 2011). Several clinical studies have reported the use of *R. canina* in the management of OA pain (Christensen *et al.,* 2008; Chrubasik *et al.,* 2006).

***Camellia sinensis*** is commonly known as green tea and belongs to the family Theaceae. It is available in the form of fresh or dried leaves and has been reported to inhibit iNOS, COX-2 as well as NF-kB pathways (Singh *et al.,* 2003). It has also been shown to inhibit cartilage degradation and provide

protection to proteoglycans and collagen II. It has been shown to suppress the aggrecanases ADAMTS-1, -4, and -5 (Vankemmelbeke *et al.,* 2003).

***Boswellia seratta*** belongs to the family Burseraceae and is widely found in Rajasthan and Madhya Pradesh in India. It is a moderate-to-large branching tree found in India, Northern Africa, and the Middle East. Extracts of this gummy exudate have been traditionally used in the Ayurvedic system of medicine in arthritis (Kulkarni *et al.,* 1991). *Boswellia seratta* extract has been shown to inhibit the glycosaminoglycan degradation thereby preventing the destruction of articular cartilage (Sontakkae *et al.,* 2007). It has been shown to relieve the symptoms of OA in a randomized placebo-controlled trial in OA knees (Sontakkae *et al.,* 2007; Kimmatkar *et al.,* 2003).

***Zingiber officinale,*** commonly known as ginger, belongs to Zingiberaceae family, and is a very popular spice in cuisine. Ginger has been used traditionally in Japanese, Indian and Chinese medicine as an anti-inflammatory agent for musculoskeletal disorders (Srivastava and Mustafa, 1992). Ginger extract has been shown to decrease IL1β- and LPS-induced production of NO and $PGE_2$ in osteoarthritic cartilage samples (Shen *et al.,* 2003). In synoviocytes, ginger has been shown to decrease the IL1β- or TNF-α-induced expression of TNF-α mRNA and protein as well as the expression of COX2 and NF-κB by reducing IκB (Frondoza *et al.,* 2004). Clinically, *Z. officinale* has been proven to be effective in reducing the symptoms associated with osteoarthritis (Haghighi *et al.,* 2005).

***Emblica officinalis***, also known as *Phyllanthus emblica*, belongs to Euphobiaceae family. It has been shown to exhibit immunomodulatory (Xiaoli *et al.,* 2012), anti-cancer (Xiaoli *et al.,* 2012), anti ulcer (Xiaoli *et al.,* 2012; Ngamkitidechakul *et al.,* 2010) and antioxidant activities (Shukla *et al.,* 2009). The chondroprotective activity of aqueous extract of *P. emblica* fruit powder has been reported wherein the extract was shown to strongly inhibit the activities of hyaluronidase and collagenase type 2 enzymes in vitro on human cartilage explants (Sumantran *et al.,* 2007). Moreover, *P. emblica* fruit extract caused a statistically significant, long-term decrease in

the levels of glycosaminoglycans released from human cartilage explants in a subset of OA patients (Sumantran *et al.,* 2007).

***Withania somnifera***, commonly known as *Ashwagandha*, belongs to the family Solanaceae. It has been reported to exhibit anti-inflammatory (Gupta and Singh, 2014) antitumor (Choudhary *et al.,* 2010), antistress (Archana and Namasivayan, 1999), antioxidant (Jaleel *et al.,* 2008), immunomodulatory (Rasool and Varalakshmi, 2006) properties. It has been mentioned in the Indian Ayurvedic medicine as a herbal tonic and health food with rejuvenating properties. Among all the parts of this plant, the root has been considered to be most active for therapeutic purposes. The chondroprotective activity of aqueous extract of *W. somnifera* root powder has been reported wherein the extract was shown to strongly inhibit the activities of the gelatinase and collagenase type 2 enzymes *in vitro* on OA cartilage explants (Sumantran *et al.,* 2007). Moreover, *W. somnifera* root extract caused a significant decrease in levels of glycosaminoglycans released from human cartilage explants in a subset of OA patients (Sumantran *et al.,* 2008).

***Triphala guggulu*** is an Ayurvedic formulation prepared through a combination of three powdered fruits, namely *Phyllanthus emblica* (amala), *Terminalia chebula* (haritaki) and *Terminalia belerica* (bibhitaki), *Commiphora wightii (guggulu*). It has been shown to possess anti-inflammatory (Kumawat *et al.,* 2013), anti-oxidant (Hazra *et al.,* 2010), anti-cancer (Shi *et al.,* 2008), radioprotective (Sandhya *et al.,* 2006) as well as anti-microbial (Biradar *et al.,* 2007) properties. ***Triphala guggulu*** has been reported to have chondroprotective activity. It has been found to inhibit the activities of hyaluronidase and collagenase type 2 enzymes *in vitro* on OA cartilage explants (Sumantran *et al.,* 2007).

***Urtica dioica*** (Stinging nettle) belongs to the family Uricaceae and is widely found in European, Asian and African countries. It has been reported to have potent anti-inflammatory properties (Hajhashemi and Klooshani, 2013). Clinical studies have reported the use of *U. dioica* in providing mild to moderate relief in OA symptoms (Randall *et al.,* 1999).

**Willow bark** belongs to the family Salicaceae is widely found in European, Asian and African countries. Willow bark has been reported to have potent anti-inflammatory properties. *In vivo* studies have demonstrated the potential of willow bark in reducing nitric oxide, TNF-α, IL-1β and IL-6 in OA (Drummond *et al.,* 2013). Clinically, it has been proven to alleviate the symptoms associated with OA (Schmid *et al.,* 2001).

***Clerodendrum phlomidis*** belongs to the family Verbinaceae and is widely found in some parts of South India. In the Indian system of medicine, it has been reported to have antinociceptive, anti-inflammatory and antipyretic properties (Narayanan *et al.,* 1999). *In vivo* studies have demonstrated the potential of *C. phlomidis* in reducing the swelling associated with OA (Kilimozhi *et al.,* 2009).

***Perna canaliculus*** (Green-lipped Mussel) belongs to the family Mytilidae and is widely found in New Zealand. It has been reported to have anti-inflammatory properties (Halpern, 2000). *In vivo* studies have demonstrated the potential of *P. canaliculus* in alleviating the pain associated with osteoarthritis (Hielm-Bjorkman *et al.,* 2009).

***Punica granatum*** belongs to the family Punicaceae and is widely found in Persia. It has been reported to have anti-oxidant (Gil *et al.,* 2000) and anti-inflammatory properties (Longtin, 2003). *In vitro* studies have demonstrated the potential of *P. granatum* in reducing the expression of MAPK and NFκB in OA chondrocytes (Ahmed *et al.,* 2005).

***Humulus lupulus*** (Common hop) belongs to the family Cannabaceae and is widely found in Northern hemisphere. It has been reported to have anti-inflammatory (Bohr *et al.,* 2005), anti-cancer (Miranda *et al.,* 1999), antioxidant and antibacterial (Yamaguchi et al., 2009) properties. *In vivo* studies have demonstrated the potential of *H. lupulus* in inhibiting $PEG_2$ and COX-2 production in OA (Hougee *et al.,* 2006).

***Arnica Montana*** belongs to the family Asteraceae and is widely found in Europe. It has been reported to have anti-inflammatory properties (Macedo *et al.,* 2004). *In vitro* studies have demonstrated the potential of *A.*

*montana* in reducing the symptoms associated with OA (Knuesel *et al.,* 2002).

***Tripterygium wilfordii*** belongs to the family Celastraceae and has been reported to have anti-inflammatory properties (Chen, 2001). *In vitro* studies have demonstrated the potential of *T. wilfordii* in reducing the expression of COX-2, $PGE_2$, MMP-3, MMP-13, AP-1 and NFκB in OA chondrocytes (Sylvester *et al.,* 2001).

***U. guianensis*** belongs to the Rubiaceae family and has been traditionally used in South America for their anti-inflammatory properties (Sandoval *et al.,* 2002). In IL-1β-stimulated chondrocytes, *U. guianensis* has been reported to upregulate the gene expression of the anabolic insulin-like growth factor 1 (Miller *et al.,* 2006).

**Turmeric** is a widely used spice and coloring/ flavoring agent that is obtained from the root of *C. longa*. Curcumin the active ingredient of Turmeric, has been extensively investigated for its antitumor (Khar *et al.,* 1999), antioxidant (Suryanarayana *et al.,* 2007) and anti-inflammatory (Chainani-Wu, 2003) properties. The anti-arthritic potential of curcumin has been widely studied *in vitro*. In human chondrocytes, curcumin has been reported to inhibit the production of MMPs-3, 9 and 13 by inhibiting JNK, AP-1 and NF-κB pathways (Henrotin *et al.,* 2009; Csaki *et al.,* 2009). In IL-1β stimulated chondrocytes curcumin has ben found to restore type II collagen and GAG synthesis (Clutterbuck *et al.,* 2009). Curcumin has also been reported to inhibit the incorporation of arachidonic acid into membrane lipids, $PGE_2$ production, leukotriene B4 and leukotriene C4 synthesis, as well as the secretion of collagenase, elastase, and hyaluronidase by macrophages (Chainani-Wu, 2003).

***A. comosus*** Merr belongs to Bromeliaceae family. Bromelain, a crude, aqueous extract obtained from the stems and immature fruits of the pineapple plant has been reported to reduce leukocyte activation by decreasing th levels of $PGE_2$, thromboxane A2 and through modulation of certain immune cell

surface adhesion molecules, which play an important role in the pathogenesis of arthritis (Lim, 2012).

**Table 2. List of herbals used in the management of osteoarthritis along with their molecular targets**

| Medicinal Plants | Molecular Targets | References |
|---|---|---|
| *Cissus quadrangularis* | Nitric oxide, Glucosaminoglycan (GAG), p38 MAPK | Kanwar *et al.,* 2015 |
| *Phyllanthus amarus* | GAG, MMP-2 | Pradit *et al.,* 2015 |
| *Harpagophytum procumbens* | MMPs (1,3,9) | Schulze-Tanzil *et al.,* 2004 |
| *Passiflora edulis* | COX, LOX | Hooshmand *et al.,* 2007 |
| *Rosa canina* | NO, $PGE_2$, IL-1β, TNF-α, MMPS (MMP 1,-3,-13) | Schwager *et al.,* 2011 |
| *Lonicera japonica* *A. asphodeloides* | MMPs, TIMPs, PGE2, NO, IL-1β, TNF-α and MAPK pathways | Kang *et al.,* 2010 |
| *Uncaria guianensis (cat's claw)* | Insulin-like growth factor 1 (IGF-1) | Miller *et al.,* 2006 |
| *Camellia sinensis* | iNOS, NFκB, COX-2 | Vankemmelbeke *et al.* 2003; Singh *et al.,* 2003 |
| *Boswellia seratta* | glycosaminoglycan | Sontakkae *et al.,* 2007; Kimmatkar *et al.,* 2003 |
| *Zingiber officinale* | NFκB,COX-2 | Frondoza *et al.,* 2004; Shen *et al.,* 2003 |
| *Emblica officinalis* | GAG, Hyaluronidase and Collagenase type II inhibition activity | Sontakkae *et al.,* 2007 |
| *Withania somniefera* | MMPs | Sontakkae *et al.,* 2007; Sumantran *et al.,* 2008 |
| *Triphala guggulu* | MMPs (1, 3 and 8), hyaluronidase and collagenase type-II inhibition activity | Sontakkae *et al.,* 2007 |

| | | |
|---|---|---|
| Willow bark | NO, TNF-α, IL-16 | Drummond *et al.,* 2013 |
| *Punica granatum* | MAPK, NFκB | Ahmed *et al.,* 2005 |
| *Humulus lupulus* | $PEG_2$, COX-2 | Hougee *et al.,* 2006 |
| *Tripterygium wilfordii* | COX-2, PGE2, MMP-3, MMP-13, AP-1, NFκB | Sylvester *et al.,* 2001 |
| *Curcuma longa (Turmeric)* | $PGE_2$,MMP-3, MMP-9, MMP-13, JNK, AP-1 and NF-κB | Henrotin *et al.,* 2009; Csaki *et al.,* 2009; Chainani-Wu, 2003 |
| *Ananas comosus* | $PGE_2$, thromboxane A2 | Lim, 2012 |

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### 4.4.5. Herbals with anti-inflammatory potential proposed for their use in osteoarthritis

Recent research focusses towards identification of natural products that would not only target the inflammatory mediators in OA but would also prevent the further deterioration of the affected regions. In the previous section, we have enlisted herbals that have been specifically tested for OA, but a large number of plants have been investigated for their anti-inflammatory potential in vitro using macrophage and chondrocytic cell lines. These plants have been shown to modulate the inflammatory mechanisms via inhibition of key enzymes (COX, LOX), as well as pathways (MAPK, NFκB). In Table 3, based on the available literature, we have attempted to enlist majority of medicinal plants that have been shown to exhibit anti-inflammatory potential by targeting various molecular mechanisms involved in the process of inflammation. Such plants could be explored further for their potential in the management of OA at clinical level.

**Table 3. List of herbals shown to possess anti-inflammatory potential along with their molecular targets**

| Medicinal plants | Molecular targets | References |
|---|---|---|
| *Dendropanax morbifera* | iNOS, COX-2, $PGE_2$ | Hyun *et al.,* 2015 |
| *Orostachys japonicus* | iNOS, COX-2, MMP-2, MMP-9, NF-κB, MAPK | Kim[a] *et al.,* 2015 |
| *Sargassum horneri* | iNOS, COX-2, NF-κB | Kim[b] *et al.,* 2015 |
| *Portulaca oleracea* | NO, iNOS, $PGE_2$, IL-6 | Kim[c] *et al.,* 2015 |
| *Taxillus tsaii* | iNOS, COX-2, $PGE_2$ | Liu *et al.,* 2015 |
| *Juncus effusus* | iNOS, COX-2, $PGE_2$ | Park *et al.,* 2015 |
| *Gouania leptostachya* | NO, iNOS, COX-2, NF-κB, MAPK | Dung *et al.,* 2015 |
| *Rumex crispus* | iNOS, COX-2 | Im *et al.,* 2014 |
| *Tinospora cordifolia* | COX-2, iNOS, ICAM-1 | Tiwari *et al.,* 2014 |
| *Scutellaria baicalensis* | MAP kinase | Kim *et al.,* 2009 |
| Seabuckthorn | NO, iNOS | Padwad *et al.,* 2006 |
| *Rhizoma coptidis* | MCP-1/CCL2, AP-1, NFκB | Remppis *et al.,* 2010 |
| *Cudrania tricuspidata* | NO, iNOS COX-2, PGE2, pro-inflammatory cytokines (IL-1β, TNF-α) | Jeong *et al.,* 2009 |
| *Lilium lancifolium* | NO, iNOS COX-2, NFκB, pro-inflammatory cytokine (IL-6, TNF-α) | Kwon *et al.,* 2010 |

| | | |
|---|---|---|
| Jeju endemic seaweeds: *Acer pictum*, *Viburnum dilatatum*, *Melia azedarach*, *Lonicera japonica*, *Osmun japonica*, *Alnus firma*, *Lindera erythrocarpa*, *Platycarya strobilacea*, *Rhododendron werrichii*, *Weigela subsessilis*, *Salix koreensis*, *Magnolia kobus*, *Corylus sieboldiana*, *Cornus walteri*, *Ulmus parvifolia*, *Morus bombycis*, *Aria alnifolia*, *Neoshirakia japonica*, *Actinodaphne lancifolia*, *Triadica sebifera*, *Elaeagnus umbellata*, *Oenothera glazioviana*, *Ficus erecta* var. *sieboldii*, *Rubus buergeri*, *Orixa japonica*, *Cnidium japonicum* | NO, iNOS | Yang *et al.,* 2010 |
| *Laurencia okamurae*, *Grateloupia elliptica*, *Sargassum thunbergii*, *Gloiopeltis furcata* and *Hizikia fusiformis* | NO, iNOS COX-2, NFκB, PGE2, pro-inflammatory cytokines (IL-1β, IL-6, TNF-α) | Yang *et al.,* 2010 |
| *Dioscorea batatas* | NO, iNOS, NFκB, ERK1/2 | Jin *et al.,* 2010 |
| *Acanthopanax senticosus* | NO, iNOS, NFκB | Lin *et al.,* 2008 |
| *Glycyrrhiza glabra* | NO, iNOS, NFκB, COX-2, pro-inflammatory cytokines (IL-1β, IL-6) | Franceschelli *et al.,* 2011 |
| *Cinnamomum cassia*, *Cinnamomum zeylanicum* | TNF-α, NO, iNOS COX-2, PGE2 | Gunawardena *et al.,* 2015; Joshi *et al.,* 2010 |
| *Pleurospermum kamtschatidum* | TNF-α, NO, iNOS COX-2, $PGE_2$, NFκB | Won *et al.,* 2006 |

| | | |
|---|---|---|
| *Dictyota dichotoma* | NO, iNOS COX-2, pro-inflammatory cytokines (IL-1β, IL-6, TNF-α) | Yoon *et al.,* 2009 |
| *Alpinia officinarium* | pro-inflammatory cytokines (IL-1β, IL-8, TNF-α), TLR2 | Subramanian *et al.,* 2009 |
| *Daphne genkwa* | NO, COX-2, $PGE_2$, NFκB | Yesilada *et al.,* 2001 |
| *Chrysopogon aciculatis* | NO, iNOS, COX-2, $PGE_2$, NFκB, JNK/p38 MAPK | Hsieh *et al.,* 2011 |
| *Sargassum micracanthum* | NO, iNOS, COX-2, pro-inflammatory cytokines (IL-1β, IL-6, TNF-α) | Yoon *et al.,* 2009 |
| *Citrus reticulata* | NO, iNOS, NFκB | Jung *et al.,* 2007 |
| *Phellinus linteus* | NO, iNOS, MAPK(JNK) | Kim *et al.,* 2006 |
| Vietnamese oriental medicine: *Crinum latifolium*, *Evodia rutaecarpa*, *Polygonum cuspidatum*, *Perilla ocymoides*, *Rubia cordifolia*, *Scutellaria barbata, Sparganium stenophyllum* | NF-κB | Nam *et al.,* 2009 |
| *Mume fructus* | NO, $PGE_2$, IL-6, iNOS, COX-2, p38 MAPK | Choi *et al.,* 2007 |
| *Moutan cortex* | NO, $PGE_2$, iNOS, COX-2, p-IkB, NFkB, TNF-α, IL-1β, IL-6 | Chun *et al.,* 2007 |
| *Pistacia terebinthus* | LTB4, 5-LOX | Giner-Larza *et al.,* 2002 |
| *Artemisia copa* | $PGE_2$, COX-2 | Moscatelli *et al.,* 2005 |
| *Capparis spinosa* | $PGE_2$ | Panico *et al.,* 2005 |
| *Juniperus communis* | PG release | Tunon *et al.,* 1995 |
| *Hypericum perforatum* | 5-LOX | Herold *et al.,* 2003 |
| *Humulus lupulus* | $PGE_2$ | Tripp *et al.,* 2005 |
| *Salvia aethiopis* | 5-LO | Hernández-Pérez *et al.,* 1995 |
| *Rosmarinus officinalis* | COX, LOX | Benincá *et al.,* 2011 |

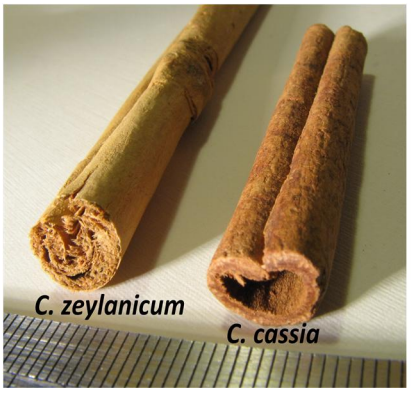
| | | |
|---|---|---|
| *Plantago lanceolata* | NO, COX-2, $PGE_2$ | Vigo *et al.,* 2005 |
| *Scrophularia auriculata* | iNOS, COX-2, $PGE_2$ | Bas *et al.,* 2007 |
| *Smilax china, Smilax glabra* | IL-1β, TNF-α, NO, COX-2 | Shu *et al.,* 2006 |
| *Physalis peruviana* | NO, iNOS, COX-2, $PGE_2$ | Wu *et al.,* 2006 |
| *Pinus sylvestris* | NO, iNOS, COX-2 | Laavola *et al.,* 2015 |
| *Plantago lanceolata* | NO, iNOS, COX-2 | Vigo *et al.,* 2005 |
| *Daphne oleoides* | IL-1β, IL-1α, TNF-α | Yesilada *et al.,* 2001 |
| *Medicago sativa* (alfalfa sprouts) | TNF-α, IL-6, IL-1β, NFkB | Hong *et al.,* 2009 |
| *Aloe vera* | NO | Sarkar *et al.,* 2005 |
| *Chrysanthemum indicum* | IL-1β, TNF-α | Lee *et al.,* 2009 |
| *Sophora flavescens* | iNOS, COX-2, PGE-2 | Jin *et al.,* 2010 |
| *Eucalyptus globules* | iNOS | Sugimoto *et al.,* 2011 |
| *Tanacetum parthenium* (Feverfew) | $PGE_2$, TNF-*α*, IL-2, IFN-*γ*, IL-4 | Sur *et al.,* 2009 |
| *Commiphora myrrha* | $PGE_2$ | Su *et al.,* 2011 |
| *Silybum marianum* | iNOS, COX-2 | Vaid and Katiyar, 2010 |
| *Pimpinella corymbosa* | NFkB | Tabanca *et al.,* 2007 |
| *Physalis alkekengi* | NFκB (IKKβ) | Ji *et al.,* 2012 |
| *Silybum marianum* | TNF-α, TGF-β, MAPK (JNK) | Aghazadeh *et al., 2011* |
| *Ocimum sanctum* | NO, iNOS, IL-1β, TNF-α | Eshraghian, 2013; Basak *et al., 2014* |
| *Cymbopogon giganteus* | Prostaglandin H synthase (PGHS), 5-LOX | Sahouo *et al.,* 2003 |
| *Epimedium brevicornum* | NO, IL-3, IL-10, IL-12p40, interferon- inducible protein-10, keratinocyte-derived chemokine, vascular endothelial growth factor (VEGF), monocyte chemotactic protein (MCP)-1 and granulocyte macrophage-colony stimulating factor (GMCSF) | Yuk *et al.,* 2010 |

| | | |
|---|---|---|
| *Prunella vulgaris* | $PGE_2$ | Huang *et al*., 2009; Psotova *et al*., 2003 |
| *Angelica gigas Nakai* | AKT, ERK, p38, NF-κB, iNOS, COX-2 , ROS, PARP and caspase-3 | Choi *et al*., 2014 |
| *Panax ginseng* | NF-κB, IL-1β, IL-6, IL-12, IL-18, IFN-γ | Choi *et al*., 2007; Lee and Lau, 2011 |

### 4.4.6. Review of Literature on the medicinal plants selected for the study

#### 4.4.6.1. *Cinnamomum cassia* and *Cinnamomum zeylanicum*

| Scientific Classification | | |
|---|---|---|
| | ***Cinnamon cassia*** | ***Cinnamon zeylanicum*** |
| Kingdom | Plantae | Plantae |
| Order | Laurales | Laurales |
| Family | Lauraceae | Lauraceae |
| Genus | *Cinnamomum* | *Cinnamomum* |
| Species | *C. cassia* | *C. verum* |




Cinnamon is commonly used as a culinary spice and flavoring agent (Balasubramanian *et al.,* 2015). It is obtained from the inner bark of trees from the genus Cinnamomum, a tropical evergreen plant that has two main varieties *Cinnamomum zeylanicum* and *Cinnamomum cassia*. *C. cassia* is an evergreen tree originating in southern China and eastern Asia (India, Indonesia, Laos, Malaysia, Taiwan, Thailand, and Vietnam). On the other hand, Sri Lanka produces 80–90% of the world's supply of *C. zeylanicum*. Cinnamon is cultivated by growing the tree for two years, then cutting the stems at ground level (Ito, 2008; Zhengyi *et al*., 2008; Ranatunga *et al*., 2004). After harvesting the cut stems are processed by scraping off the outer bark followed by beating of the branch evenly with a hammer to loosen the inner bark, which is then pried off in long rolls (Ito, 2008; Ranatunga *et al*., 2004). The processed bark is completely dried, cut into 5- to 10-cm (2- to 4-in) lengths for sale. *C. cassia* and *C. zeylanicum* differ from each other in a number of ways which are included in the table below (Zhengyi *et al*., 2008; Ranatunga *et al*., 2004):

**Table 4. Differences between *C. cassia* and *C. zeylanicum***

| | ***C. cassia*** | ***C. zeylanicum*** |
|---|---|---|
| **1.** | The taste is strong to peppery | It is sweet and delicate |
| **2.** | It is a reddish brown to dark brown | It is light brown/ tan color |
| **3.** | Its appearance is like a hollow tube | Its appearance is like a rolled up newspaper |
| **4.** | The surface is rough and uneven | The surface is smooth |
| **5.** | It is cultivated in China, Vietnam, Indonesia | It is cultivated in India, Srilanka |
| **6.** | Coumarin content is high | Coumarin content is low |

Almost every part of the cinnamon tree including the bark, leaves, flowers, fruits and roots, has some medicinal or culinary use. The different parts of the plant possesses the same array of hydrocarbons in varying proportions, with primary constituents such as cinnamaldehyde (bark), eugenol (leaf) and camphor (root) (Gruenwald *et al.,* 2010). Cinnamaldehyde, the active component of cinnamon, has been reported to downregulate the production of major inflammatory mediators (iNOS, COX-2, NF-$\kappa$B) in RAW264.7 cells (Zhang *et al.,* 2012; Liao *et al.,* 2012).

*C. cassia* has been widely used in Indian traditional medicine for the management of various disease conditions (Rao and Gan, 2014). Various studies have shown that *C. cassia* has anti-inflammatory properties and decreased the expression of IL-1β, IL6, and TNF-α (Hong *et al.,* 2012). It has been shown to exhibit anti-inflammatory (Gunawardena *et al.,* 2015), antipyretic (Sini *et al.,* 2011), antimicrobial (Ooi *et al.,* 2006), antidiabetic (Wickenberg *et al.,* 2014) and antitumor activities (Kwon *et al.,* 2010).

*C. zeylanicum*, also known as Ceylon cinnamon (the source of its Latin name, zeylanicum) or 'true cinnamon' is indigenous to Sri Lanka and southern parts of India (Paranagama *et al.,* 2010). Three of the main components of the essential oils obtained from the bark of *C. zeylanicum* are trans-cinnamaldehyde, eugenol and linalool, which represent 82.5% of the total composition (Chericoni *et al.,* 2005). *C. zeylanicum*, has been used

traditionally for its antidiabetic (Ranasinghe *et al.,* 2012), anti-nociceptive (Zhang *et al.,* 2014), astringent (Joshi *et al.,* 2010) and diuretic activities (Joshi *et al.,* 2012). Procyanidine polyphenols, a compound extracted from *C. zeylanicum*, has been reported to regulate inflammation and arthritis (Vetala *et al.,* 2013). **Although several studies have reported the anti-inflammatory activity of cinnamon bark from either *C. cassia* or *C. zeylanicum,* however, no one has compared their anti-inflammatory activities in human chondrocytic cell line (SW1353) and human primary chondrocytes, this would help in selection of the most potent variety that could be used in the management of OA related pathophysiology.**

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#### 4.4.6.2. *Ocimum basilicum*

| Scientific Classification | |
|---|---|
| **Kingdom** | Plantae |
| **Phylum** | Magnoliophyta |
| **Class** | Magnoliopsida |
| **Order** | Lamiales |
| **Family** | Lamiacaea |
| **Genus** | Ocimum |
| **Species** | *O. basilicum* |

Basil belongs to the genus Ocimum, derived from the Greek word "ozo" which means to smell, with reference to the strong odor of the species within the genus. It grows as a perennial plant in tropical climates and is planted as an annual in temperate regions, where it may be sown directly from the seeds or transplanted. Basil grows between 30–130 cm tall, with opposite, light green, silky leaves, 3–11 cm long and 1–6 cm broad (Boning, 2010). Although basil grows best outdoors, it can be grown indoors in a pot and, like most herbs. It grows best in strong sunlight (Tilebeni, 2011). The flavor and smell of basil varieties is largely determined by their chemical components. Basil varieties contain different oils in varying quantities that include cinnamate, citronellol, geraniol, linalool, methyl chavicol, myrcene, pinene, ocimene and terpineol (Husain, 1994).

*Ocimum basilicum* (Sweet basil) is native to tropical Asia. It is cultivated commercially in southern Europe, Egypt, Morocco, Indonesia, and California. It is a popular culinary herb used in many cuisines including Italian and Thai [Gernot Katzer, Spice Pages: Basil (gernot-katzers-spice-pages.com)]. The leaves can be eaten as a salad. Basil is also used in perfumery, soap-making, and to flavour liqueours (Gernot Katzer, Spice Pages). The seeds are edible and when soaked in water becomes mucilaginous (Gernot Katzer, Spice Pages).

*O. basilicum,* an important medicinal herb, has been used in Ayurveda as an antiseptic, preservative, sedative, digestive regulator and diuretic (Shirazi et al., 2014; Dashputre and Naikwade, 2010). It has also been reported to offer protection from radiation induced toxic effects (Monga et al., 2011). Various *in vivo* studies have reported anti-inflammatory activity of *O.basilicum* (Benedec et al., 2007; Yadav et al., 2009; Rakha et al., 2010). Its methanolic extract has been shown to exhibit anti-inflammatory activity in PBMCs and RAW264.7 (Thyagaraj et al., 2013). **However, to our knowledge, the anti-inflammatory activity of aqueous and methanolic extracts of aerial parts of *O. basilicum* has not been compared in RAW264.7, SW1353 and human primary chondrocytes in terms of their efficacy to manage OA related pathophysiology.**

#### 4.4.6.3. *O. sanctum*

| Scientific Classification | |
|---|---|
| **Kingdom** | Plantae |
| **Order** | Lamiales |
| **Family** | Lamiacaea |
| **Genus** | Ocimum |
| **Species** | *O.sanctum or O. tenuiflorum* |

*Ocimum sanctum* L. (also known as *Ocimum tenuiflorum*, Tulsi) is widely distributed, covering the entire Indian sub-continent, ascending upto 1800 m in the Himalayas and in Andaman and Nicobar Islands (Ajjan *et al*., 2009). *O. sanctum* is an erect, much branched sub-shrub 30-60 cm tall, with simple opposite green or purple leaves that are strongly scented and has hairy stems (Ajjan *et al*., 2009). Tulsi is native throughout the world tropics and widespread as a cultivated plant. Ocimum belongs to an important group of aromatic and medicinal plants which yield many essential oils and aroma chemicals and finds diverse use in perfumery, cosmetic industries and in indigenous systems of medicine (Verma and Singh, 2009).

*O. sanctum* has been used in Ayurveda for treating common cold, headache, heart disease, stomach and skin disorders and against various forms of poisons as well as in the management of neurological, inflammatory and allergic disorders (Prakash and Neelu, 2005). The plant has also been investigated extensively for its immunotherapeutic (Mukherjee *et al.,* 2005), antioxidant (Basu *et al.,* 2013), anti-inflammatory (Kumar *et al.,* 2015; Basak *et al.,* 2014; Kalabharathi *et al.,* 2011), antibacterial (Singh *et al.,* 2005), antidiabetic (Hannan *et al.,* 2015), analgesic (Kumar *et al.,* 2015), antipyretic (Kumar *et al.,* 2015), hepatoprotective (Lahon and Das, 2011), radio protective (Joseph *et al.,* 2011) and chemopreventive (Singh *et al.,* 2012)

properties. Although several studies have shown the medicinal value of *O. sanctum*, there is still an ample scope for further research on this plant.

The chemical composition of Tulsi is highly complex, containing many nutrients and other biologically active compounds, the proportions of which may vary considerably between strains and even among plants within the same field. The leaf volatile oil contains eugenol, euginal, urosolic acid, oleonolic acid, carvacrol, linalool, limatrol, caryophyllene, methyl carvicol (Shishodia *et al.,* 2000; Kelm *et al.,* 2000). In addition, other phenolic bioactives that have been identified include rosmarinic acid, apigenin, cirsimaritin, isothymusin and isothymonin, which also exhibit antioxidant and anti-inflammatory activities. Two water-soluble flavonoids of *O. sanctum*, orientin and vicenin, have shown to provide protection against radiation-induced chromosomal damage in human blood lymphocytes (Joksic *et al.,* 2008).

**Even though *O. sanctum* has been effectively studied, however, no has compared the anti-inflammatory properties of aqueous and methanolic extracts of *O. sanctum* as well as mixture of different ratios of aqueous and methanolic extracts in human chondrosarcoma cells (SW1353) and human primary chondrocytes.**

# SECTION-5

# STUDY DESIGN AND OBJECTIVES

## 5. Study Design

The present study aimed at comparing the anti-inflammatory properties of selected medicinal plants and identifying the most effective variety or a blend of the potent varieties for their use in the management of OA related pathophysiology. We tested the effect **of aqueous and alcoholic extracts of 2 species of cinnamon** [*Cinnamomum zeylanicum* (True cinnamon/Ceylon cinnamon) and *Cinnamomum cassia* (Cassia, Chinese cinnamon)] **and 2 species of Ocimum** [*Ocimum basilicum and Ocimum sanctum*] **(total 8 extracts) on anti-inflammatory markers (**NO release and inhibition of $PGE_2$, LTB4 and MMP) in RAW264.7, SW1353 and primary human chondrocytes. The best **extract/ blend of active varieties would be selected** based on NO scavenging and inhibition of $PGE_2$, LTB4 and MMP activities. **The selected extract/blend would be studied for its efficacy to regulate the expression of** proinflammatory cytokines (IL-1β and TNF-α), matrix metalloproteinases (MMPs) and COX enzymes. **This would be followed by safety studies of the selected active extract/ blend by performing s**ub acute toxicity assays in Wistar rats.

**The study aimed at following objectives:**

1. Comparing the anti-inflammatory activity of aqueous and methanolic extracts of *C. cassia* and *C. zeylanicum* in RAW264.7, SW1353 and human primary chondrocytes.

2. Comparing the anti-inflammatory activity of aqueous and methanolic extracts of *O. basilicum* in RAW264.7, SW1353 and human primary chondrocytes.

3. Comparing the effect of aqueous and methanolic extracts of *O. sanctum* on IL-1β induced $PGE_2$ and LTB4 levels in human chondrosarcoma (SW1353) cell line and human primary chondrocytes.

4. Evaluation of anti-inflammatory potential of combination of aqueous and methanolic extracts of *O. sanctum* (LOT001, LOT002 and LOT02) in SW1353 and human primary chondrocytes.

5. Evaluating the safety of the selected active extract/ blend by performing sub acute toxicity study in Wistar rats.

# 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, Hams 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$^{o}$C for 1h 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$^{o}$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$^{o}$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$^{o}$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$^{o}$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 $<75^0$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 ($1 \times 10^6$ cells/ml) in 35 mm primaria coated culture dishes. The cells were cultured in DMEM: Hams F12 containing 2 mM L-glutamine, 10% FBS, 100Units/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. $PGE_2$ 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 $4^0$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 $4^0$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 comparision 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±20ºC 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), prothombin time (PT), prothombin 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 (3-4$\mu$m 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.

# SECTION-7

# OBSERVATIONS AND RESULTS

## 7.1 Comparing the anti-inflammatory activity of aqueous and methanolic extracts of *C. cassia* and *C. zeylanicum* in RAW264.7, SW1353 and primary chondrocytes

**The manuscript has been accepted in International Journal of Pharmacy and Pharmaceutical Science (2015)**

We have compared the anti-inflammatory activity of aqueous and methanolic extracts of *C.cassia* (CC) and *C. zeylanicum* (CZ) in mouse macrophage (RAW264.7) and human chondrosarcoma (SW1353) cells as well as in human primary chondrocytes to correlate their efficacy in modulation of NO release and expression of $PGE_2$, LTB4 and MMPs.

### 7.1.1. Cinnamon extracts were non-toxic to the cells

RAW264.7, SW1353 and human chondrocytes were treated with different concentrations of extracts (0-100 µg/ml) to test their effect on cell viability. The methanolic and aqueous extracts of *C. cassia* (CC) (Table 5a) and that of *C. zeylanicum* (CZ) (Table 5b) were found to be non toxic to the cells, thereby suggesting their safety for further studies.

### 7.1.2. Cinnamon extracts reduced NO levels in RAW264.7

RAW264.7 cells were treated with different concentrations (0-100 µg/ml) of methanolic ($CC_M$, $CZ_M$) and aqueous extracts ($CC_W$, $CZ_W$) of *C. cassia* and *C. zeylanicum*. A significant dose dependent decrease in nitrite production was observed with both the extracts as compared to LPS stimulated control cells. We found that at 100 µg/ml dose, $CC_M$ exhibited 45.40 % ($p<0.001$) decrease in NO levels compared to $CC_W$ (24.64 %; $p<0.001$) (Table 6). Interestingly, at the same dose $CZ_M$ effectively reduced the NO levels by 65.98 % ($p<0.001$) compared to $CZ_W$ (28.67 %; $p<0.001$) (Table 6). Thus, methanolic extracts of CC and CZ effectively reduced NO levels compared to their respective aqueous extracts.

**Table 5a. Effect of $CC_M$ and $CC_W$ on cell viability in RAW264.7, SW1353 and human primary chondrocytes**

| | $CC_M$ | | | $CC_W$ | | |
|---|---|---|---|---|---|---|
| **Conc. (µg/ml)** | **RAW264.7** | **SW1353** | **human primary chondrocytes** | **RAW264.7** | **SW1353** | **human primary chondrocytes** |
| **0.1** | 101.1 ± 1.4 | 100.2 ± 0.06 | 100.4 ± 0.32 | 101.4 ± 2.3 | 102.7 ± 3.4 | 101.8 ± 2.4 |
| **1** | 100.1 ± 0.76 | 100.1 ± 0.04 | 100.1 ± 0.05 | 101.8 ± 2.5 | 103.6 ± 3.6 | 104.3 ±0.99 |
| **10** | 100.2 ± 0.78 | 100.0 ±0.17 | 100.8 ± 0.37 | 100.7 ± 0.70 | 105.8 ± 3.1 | 109.7 ± 3.3 |
| **100** | 100.5 ± 0.55 | 102.8 ± 2.5 | 104.0 ± 1.07 | 100.8 ± 0.88 | 104.3 ± 1.1 | 112.0 ± 1.7 |

Values have been represented as mean±SD of three independent experiments.

**Table 5b. Effect of $CZ_M$ and $CZ_W$ on cell viability in RAW264.7, SW1353 and human primary chondrocytes**

| | $CZ_M$ | | | $CZ_W$ | | |
|---|---|---|---|---|---|---|
| **Conc. (µg/ml)** | **RAW264.7** | **SW1353** | **human primary chondrocytes** | **RAW264.7** | **SW1353** | **human primary chondrocytes** |
| **0.1** | 100.1 ± 0.06 | 100.0 ± 0.01 | 100.4 ± 0.42 | 102.1 ± 1.4 | 101.06 ± 1.4 | 100.05 ± 0.04 |
| **1** | 100.7 ± 0.93 | 102.1 ± 1.3 | 102.0 ± 0.50 | 104.6 ± 0.92 | 101.04 ± 0.11 | 101.6 ± 0.63 |
| **10** | 101.8 ± 1.9 | 101.8 ± 1.9 | 110.2 ± 2.2 | 106.8 ± 2.1 | 105.06 ± 2.01 | 108.02 ± 0.67 |
| **100** | 105.4 ± 4.3 | 104.3 ± 0.55 | 118.6 ± 0.76 | 109.9 ± 0.69 | 105.6 ±0.72 | 115.5 ± 1.05 |

Values have been represented as mean±SD of three independent experiments.

**Table 6. Effect of $CC_M$, $CC_W$, $CZ_M$ and $CZ_W$ on NO levels in LPS stimulated RAW264.7 cells**

| Conc. (µg/ml) | $CC_W$ | $CC_M$ | $CZ_W$ | $CZ_M$ |
|---|---|---|---|---|
| | | **% decrease in NO levels** | | |
| **0.1** | 5.16 ± 4.5 | 5.71 ± 4.93 | 7.42 ± 3.8 | 14.79 ± 8.4 |
| **1** | 11.53 ± 5.4 | 9.50 ± 3.83 | 14.97 ± 9.2 | 19.74 ± 4.5 |
| **10** | 12.69 ± 9.06 | 23.12 ± 6.94 | 18.38 ± 7.07 | 48.36 ± 7.6 |
| **100** | 24.64 ± 6.11 | 45.40 ± 8.56[a] | 28.67 ± 6.7[c] | 65.63 ± 5.7[b] |

Values have been represented as mean±SD of three independent experiments. Tukey's multiple comparisons test: [a]$p<0.05$ compared to $CC_W$, [b]$p<0.01$ compared to $CZ_W$, [b]$p<0.05$ compared to "a", [c]$p>0.05$ compared to $CC_W$

### 7.1.3. Cinnamon extracts reduced $PGE_2$ levels in RAW264.7, SW1353 and primary human chondrocytes

We compared the effect of $CC_M$, $CC_W$, $CZ_M$ and $CZ_W$ on $PGE_2$ levels. Since the extracts induced maximum inhibition in the nitrite levels in RAW264.7 cells at 100µg/ml dose, this dose was selected for our further experiments. It was observed that at 100 µg/ml dose, $CC_M$ and $CC_W$ reduced the $PGE_2$ production by 79.88 % ($p<0.001$) and 80.07 % ($p<0.001$), respectively in RAW264.7 cells. At the same dose, $CZ_M$ reduced $PGE_2$ levels by 95.91 % ($p<0.001$) compared to $CZ_w$ (11.18 %) (Table 7). Both the extracts of *C. cassia* seemed to be equally effective in reducing $PGE_2$ levels in RAW264.7 cells.

In IL-1β stimulated SW1353 cells, at 100µg/ml dose, $CC_M$ significantly reduced $PGE_2$ production by 68.86 % ($p<0.001$) compared to $CC_w$ (22.36 %; $p<0.001$) whereas $CZ_M$ was found to decrease $PGE_2$ production by 70.18 % ($p<0.001$) compared to $CZ_w$ (59.93 %; $p<0.001$) (Table 7). Interestingly, in human primary chondrocytes, the methanolic extracts of cinnamon reduced $PGE_2$ levels more effectively compared to the aqueous extracts. At 100µg/ml dose, $CC_M$ reduced $PGE_2$ production by

36.07 % ($p<0.01$), compared to $CC_W$ (6.74%) whereas $CZ_M$ decreased the $PGE_2$ production by 52.25 % ($p<0.001$), compared to $CZ_W$ (16.23%) (Table 7). The data shows that methanolic extracts of CC and CZ reduced $PGE_2$ levels significantly in chondrocytic cell line and primary chondrocytes.

**Table 7. Effect of $CC_W$, $CC_M$, $CZ_W$ and $CZ_M$ on $PGE_2$ levels in RAW264.7, SW1353 and primary human chondrocytes**

| Values | Conc. (100µg/ml) | % decrease in $PGE_2$ levels | | |
|---|---|---|---|---|
| | | **RAW264.7** | **SW1353** | **primary human chondrocytes** |
| | **$CC_W$** | 80.07 ± 3.8 | 22.36 ± 20.7 | 6.74 ± 4.2 |
| | **$CC_M$** | 79.88 ± 1.2 [a] | 68.86 ± 6.4[d] | 36.07 ± 9.5[g] |
| | **$CZ_W$** | 11.18 ±11.57[c] | 59.93 ± 4.8[f] | 16.23 ± 3.7[i] |
| | **$CZ_M$** | 95.91 ±0.32[b] | 70.18 ± 2.3[e] | 52.25 ± 5.4[h] |

Values have been represented as mean±SD of three independent experiments. Tukey's multiple comparisons test: [a]$p>0.05$ compared to $CC_W$, [b]$p<0.001$ compared to $CZ_W$, [b]$p<0.001$ compared to "a", [c]$p<0.001$ compared to $CC_W$, [d]$p<0.05$ compared to $CC_W$, [e]$p>0.05$ compared to $CZ_W$, [e]$p>0.05$ compared to "d", [f]$p<0.05$ compared to $CC_W$, [g]$p<0.05$ compared to $CC_W$, [h]$p<0.05$ compared to $CZ_W$, [h]$p>0.05$ compared to "g", [i]$p>0.05$ compared to $CC_W$

### 7.1.4. Cinnamon extracts reduced LTB4 levels in SW1353 and primary human chondrocytes

$CC_M$, $CC_W$, $CZ_M$ and $CZ_W$ were further compared for their potential to modulate IL-1β induced LTB4 production in SW1353 and human chondrocytes. In SW1353, at 100µg/ml dose, $CC_M$ reduced LTB4 levels by 85.47 % ($p<0.001$) compared to $CC_W$ (61.59 %; $p<0.001$) (Table 8). At the same dose $CZ_M$ reduced LTB4 by 67.5 % ($p<0.001$) as compared to $CZ_W$ (26.83 %; $p<0.001$). In human primary chondrocytes, at 100µg/ml dose, both $CC_M$ and $CC_W$ significantly reduced the LTB4 levels by 99.56 % ($p<0.001$)

and 90.27 % ($p<0.001$), respectively. On the other hand, $CZ_M$ reduced LTB4 levels by 75.57 % ($p<0.001$) compared to $CZ_W$ (48.78 %; $p<0.001$) (Table 8). The methanolic extracts of CC and CZ showed more reduction in LTB4 activity compared to aqueous extracts.

**Table 8. Effect of $CC_W$, $CC_M$, $CZ_W$ and $CZ_M$ on LTB4 levels in SW1353 and primary human chondrocytes**

| | % decrease in LTB4 levels | |
|---|---|---|
| **Conc. (100µg/ml)** | **SW1353** | **primary human chondrocytes** |
| **$CC_W$** | 61.59 ± 4.6 | 90.27 ± 0.09 |
| **$CC_M$** | 85.47 ± 3.03[a] | 99.56 ± 0.2[d] |
| **$CZ_W$** | 26.83 ± 6.1[c] | 48.78 ± 0.89[f] |
| **$CZ_M$** | 67.50 ± 5.6[b] | 75.57 ± 1.2[e] |

Values have been represented as mean±SD of three independent experiments. Tukey's multiple comparisons test: [a]$p<0.05$ compared to $CC_W$, [b]$p<0.01$ compared to $CZ_W$, [b]$p>0.05$ compared to "a", [c]$p<0.01$ compared to $CC_W$, [d]$p<0.01$ compared to $CC_W$, [e]$p<0.001$ compared to $CZ_W$, [e]$p<0.001$ compared to "d", [f]$p<0.001$ compared to $CC_W$

### 7.1.5. Cinnamon extracts reduced MMP levels in chondrocytes

We compared the effect of $CC_M$, $CC_W$ and $CZ_M$, $CZ_W$ on IL-1β induced MMP levels in primary chondrocytes. Compared to control stimulated cells, at 100µg/ml dose, $CC_M$ reduced MMP 2, 9 and 13 production by 55.68 % ($p<0.001$), 57.52 % ($p<0.001$) and 90.11% ($p<0.001$), respectively. At the same dose, $CC_W$ reduced MMP 2, 9 and 13 production by 16.06 %, 59.51 % ($p<0.001$) and 41.52 % ($p<0.001$), respectively (Table 9). Similarly, at 100µg/ml dose, $CZ_M$ significantly decreased MMP 2, 9 and 13 production by 73.06 % ($p<0.001$), 39 % ($p<0.001$) and 71.17% ($p<0.001$), respectively, whereas $CZ_W$ reduced MMP 2, 9 and 13 production by 15.62 %, 6.43 % and 40.05% ($p<0.01$), respectively, compared to the control cells (Table 9). These

data showed that overall methanolic extracts significantly reduced MMP levels compared to aqueous extracts, however, with few exceptions.

**Table 9. Effect of $CC_W$, $CC_M$, $CZ_W$ and $CZ_M$ on MMP levels in primary human chondrocytes**

| | Primary human chondrocytes | | |
|---|---|---|---|
| **Conc. (100µg/ml)** | **% decrease in MMP levels** | | |
| | **MMP-2** | **MMP-9** | **MMP-13** |
| **$CC_W$** | 16.06 ± 17.02 | 59.51 ± 4.2 | 41.53 ± 7.8 |
| **$CC_M$** | 55.68 ± 5.2[a] | 57.52 ± 4.7[d] | 90.12 ± 2.6[g] |
| **$CZ_W$** | 15.62 ± 22.09[c] | 6.43 ± 3.2[f] | 40.06 ± 5.74[i] |
| **$CZ_M$** | 73.06 ± 7.1[b] | 74.5 ± 5.2[e] | 71.18 ± 12.5[h] |

Values have been represented as mean±SD of three independent experiments. Tukey's multiple comparisons test: [a]$p>0.05$ compared to $CC_W$, [b]$p>0.05$ compared to $CZ_W$, [b]$p>0.05$ compared to "a", [c]$p>0.05$ compared to $CC_W$, [d]$p>0.05$ compared to $CC_W$, [e]$p<0.001$ compared to $CZ_W$, [e]$p>0.05$ compared to "d", [f]$p<0.001$ compared to $CC_W$, [g]$p<0.01$ compared to $CC_W$, [h]$p>0.05$ compared to $CZ_W$, [h]$p>0.05$ compared to "g", [i]$p>0.05$ compared to $CC_W$

## 7.2. Comparing the anti-inflammatory activity of aqueous and methanolic extracts of *Ocimum basilicum* in RAW264.7, SW1353 and human primary chondrocytes

**The manuscript is under revision**

The present study has compared the anti-inflammatory activity of aqueous ($OB_W$) and methanolic ($OB_M$) extracts of aerial parts of *O. basilicum* in mouse macrophage (RAW264.7) and human chondrosarcoma (SW1353) cell lines, and human primary chondrocytes to correlate their efficacy in modulation of NO release and expression of $PGE_2$, LTB4 and MMPs.

### 7.2.1. Phytochemical finger printing of *O. bascilicum*

$OB_M$ and $OB_W$ were compared for their efficacy in modulating the expression of proinflammatory molecules in the management of OA. The two extracts were subjected to HPLC profiling (Figure 7.2.1 A and B) to test different polarities of constituents present in the plant material. Some of the constituents of Ocimum species reported in the literature viz., oleanolic acid, ursolic acid, rosmarinic acid and eugenol have been quantified and the data has been presented in Table 10. These constituents were present in methanolic extract ($OB_M$) while the water extract ($OB_W$) had negligible quantities except for the presence of rosmarinic acid and traces of eugenol.

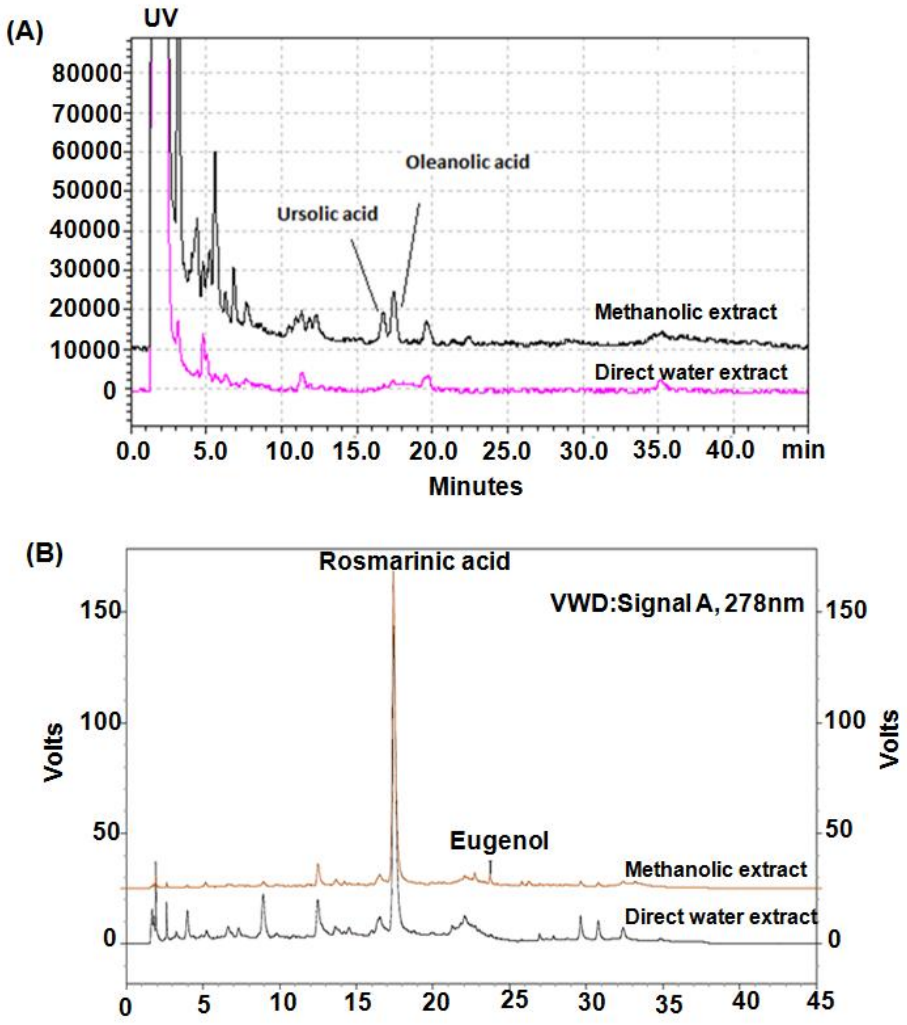


***Figure 7.2.1. Phytochemical finger printing of O. bascilicum.*** *The constituents of Ocimum species viz., oleanolic acid, ursolic acid, rosmarinic acid and eugenol were quantified in aqueous and methanolic extracts using HPLC.*

**Table 10. Phytochemical analysis of $OB_M$ and $OB_W$**

| SI. No. | Phytocompounds | Methanolic extract ($OB_M$) (%) | Aqueous extract ($OB_W$) (%) |
|---|---|---|---|
| 1. | Oleanolic acid | 0.27 | - |
| 2. | Ursolic acid | 0.48 | - |
| 3. | Rosmarinic acid | 6.25 | 1.47 |
| 4. | Eugenol | 0.11 | 0.01 |

### 7.2.2. *O.basilicum* extracts were non-toxic to the cells

$OB_M$ and $OB_W$ were evaluated for their cytotoxic effect in RAW264.7, SW1353 and human chondrocytes. The cells were treated with different concentrations of $OB_M$ and $OB_W$ (0-100 µg/ml) and both the extracts were found to be non toxic (Figure 7.2.2 A and B), thereby suggesting their safety for further applications.

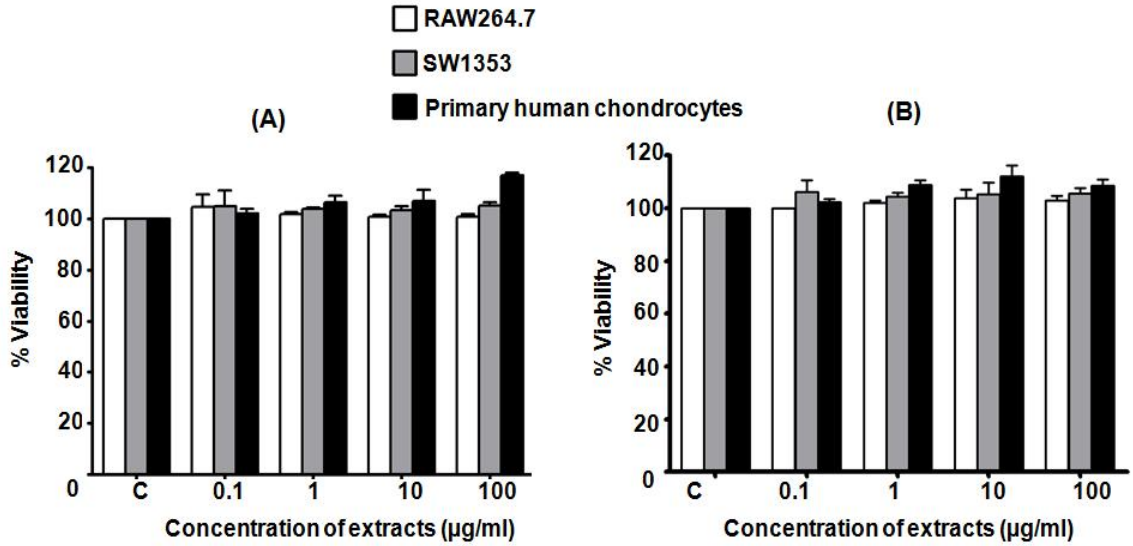


***Figure 7.2.2. Effect of $OB_M$ and $OB_W$ on cell viability in RAW264.7, SW1353 and primary human chondrocytes.*** *The cells were treated with different concentrations (0-100 µg/ml) of $OB_M$* ***(A)*** *and $OB_W$* ***(B)*** *and cell viability was determined by MTT dye uptake method. The data represents mean±SD of three independent experiments, each performed in triplicates.*

### 7.2.3. *O. basilicum* extracts reduced NO levels with simultaneous decrease in iNOS expression in RAW264.7

$OB_M$ and $OB_W$ were found to decrease nitric oxide (NO) by 20.4% and 35.01% ($p<0.01$), respectively, at 100 µg/ml dose (Figure 7.2.3 A). 1400W dihydrochloride, used as a positive control, was found to decrease the NO levels by 67.02% ($p<0.001$). Interestingly, at 100 µg/mL concentration, $OB_M$ and $OB_W$ significantly decreased LPS induced iNOS expression by 29.68 % ($p<0.05$) (Figure 7.2.3 B) and

71.4% ($p<0.001$) (Figure 7.2.3 C), respectively. 1400W reduced iNOS expression by 47.5% ($p<0.001$). Thus, $OB_W$ decreased NO and iNOS levels more significantly than $OB_M$ suggesting that it could be exploited for alleviation of NO to reduce inflammation in osteoarthritis.

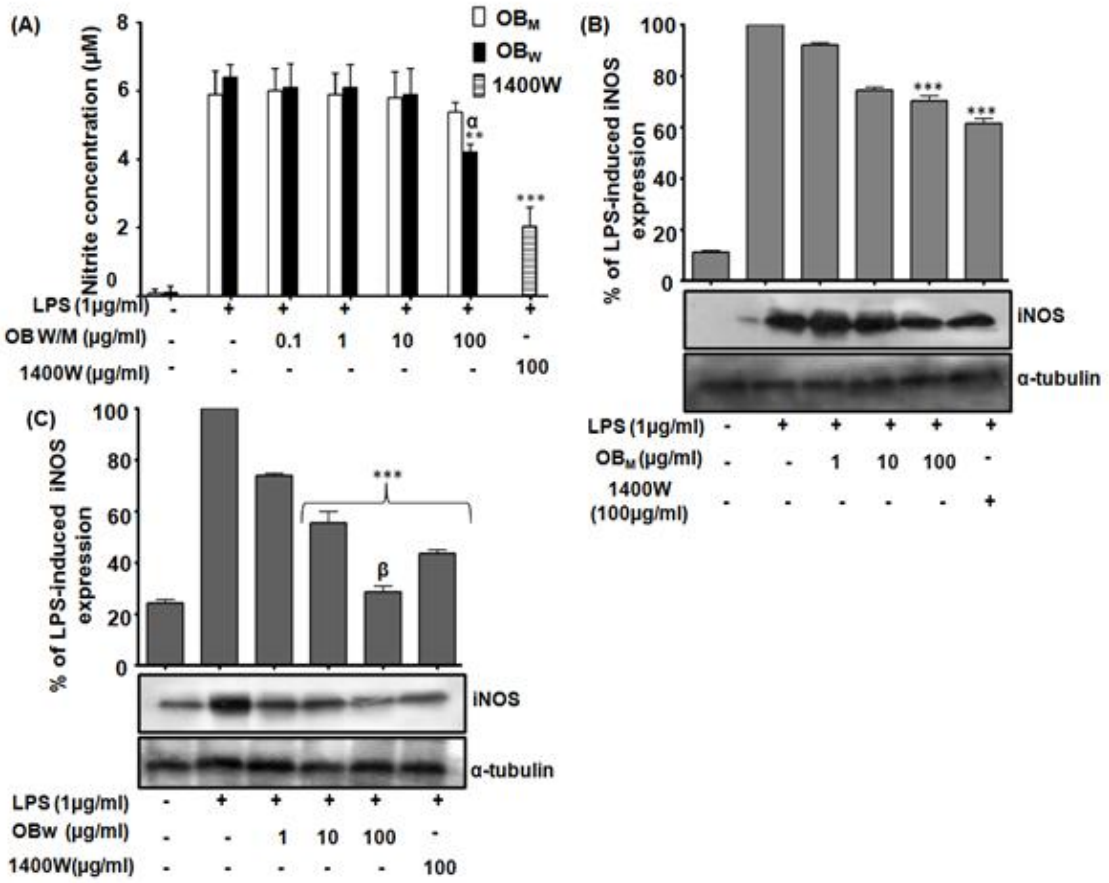


***Figure 7.2.3. Effect of $OB_M$ and $OB_W$ on NO and iNOS expression in LPS stimulated RAW264.7 cells.*** *The cells were pre-treated with $OB_M$ and $OB_W$ and stimulated with LPS followed by analysis of secreted NO in the cell-free culture media measured by Griess reaction* ***(A)****; iNOS protein expression by western blotting in cells treated with $OB_M$* ***(B)*** *and $OB_W$* ***(C).*** *α-tubulin was used as a loading control. *p<0.05, **p<0.01, ***p<0.001 indicated statistically significant differences. "α" compared with $OB_M$ p<0.01; "β" compared with $OB_M$ p<0.001 (Tukeys multiple comparison test).*

### 7.2.4. *O. basilicum* decreased COX-2 and NFκB expression in RAW264.7

In RAW264.7, $OB_M$ was found to decrease COX-2 and NFkB expression by 87.4% ($p<0.001$) and 35.4% ($p<0.01$), respectively (Figure 7.2.4 A) at 100 µg/ml concentration. At this dose, $OB_w$ reduced COX-2 and NFkB expression by 83.87% ($p<0.001$) and 79.28% ($p<0.001$), respectively (Figure 7.2.4 B). Since $OB_W$ reduced NFκB more significantly than $OB_M$, showed good potential in the management of OA related inflammation.

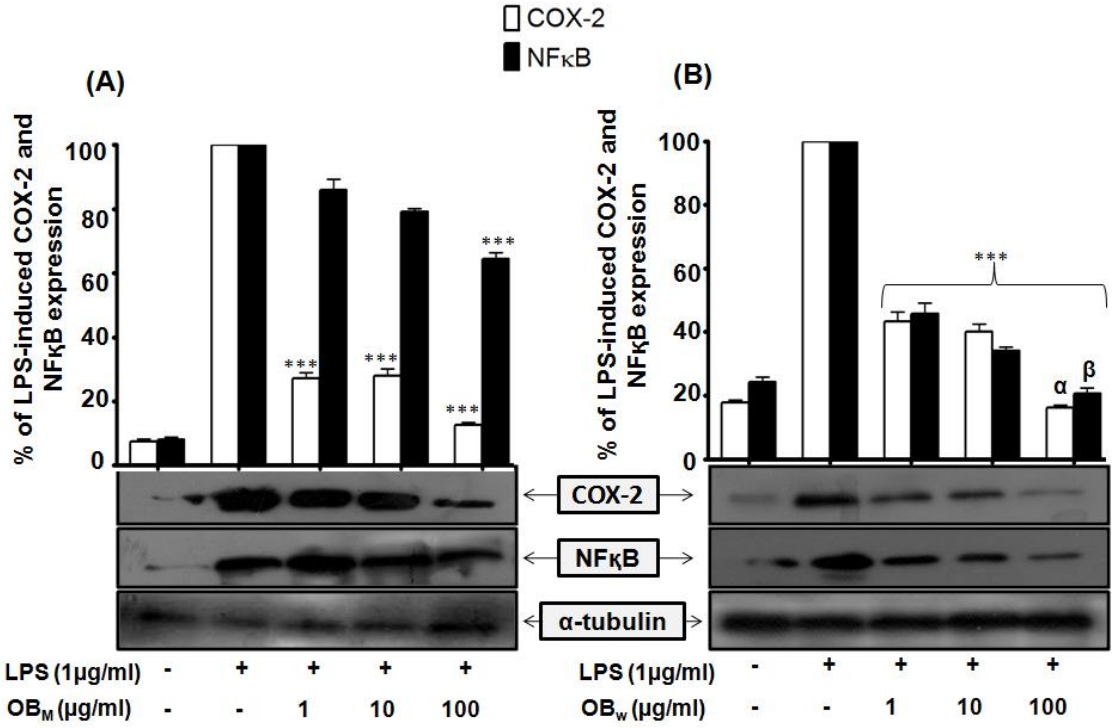


***Figure 7.2.4. Effect of $OB_M$ and $OB_W$ on COX-2 and NFκB protein expression in LPS stimulated RAW264.7 cells.*** *The cells were pre-treated with $OB_M$* ***(A)*** *and $OB_W$* ***(B)*** *and stimulated with LPS, followed by analysis of COX-2 and NFκB expression by western blotting.* *α-tubulin was used as a loading control. *p<0.05, **p<0.01, *** p<0.001 indicated statistically significant differences. "α" compared with $OB_M$ p>0.05NS; "β" compared with $OB_M$ p<0.001 (Tukeys multiple comparison test). NS: Not significant.*

### 7.2.5. *O. basilicum* reduced $PGE_2$ levels in RAW264.7, SW1353 and primary human chondrocytes

In RAW264.7, at 100 μg/ml dose, $OB_M$ and $OB_W$ significantly reduced $PGE_2$ production by 12.97 and 70.88% ($p<0.001$), respectively, compared to LPS stimulated control cells (Figure 7.2.5 A). In SW1353 (Figure 7.2.5 B) and chondrocytes (Figure 7.2.5 C), at this dose, $OB_W$ significantly reduced $PGE_2$ production by 76.11 and 41.96% ($p<0.001$), respectively, compared to IL-1β stimulated control cells. However, $OB_M$ did not reduce $PGE_2$ in either SW1353 (Figure 7.2.5 B) or chondrocytes (Figure 7.2.5 C). Dexamethasone, used as a positive control, significantly reduced $PGE_2$ by 63.01% ($p<0.001$). Since $OB_W$ was more effective than $OB_M$ in reducing $PGE_2$ levels, it holds a promise in preventing cartilage degradation.

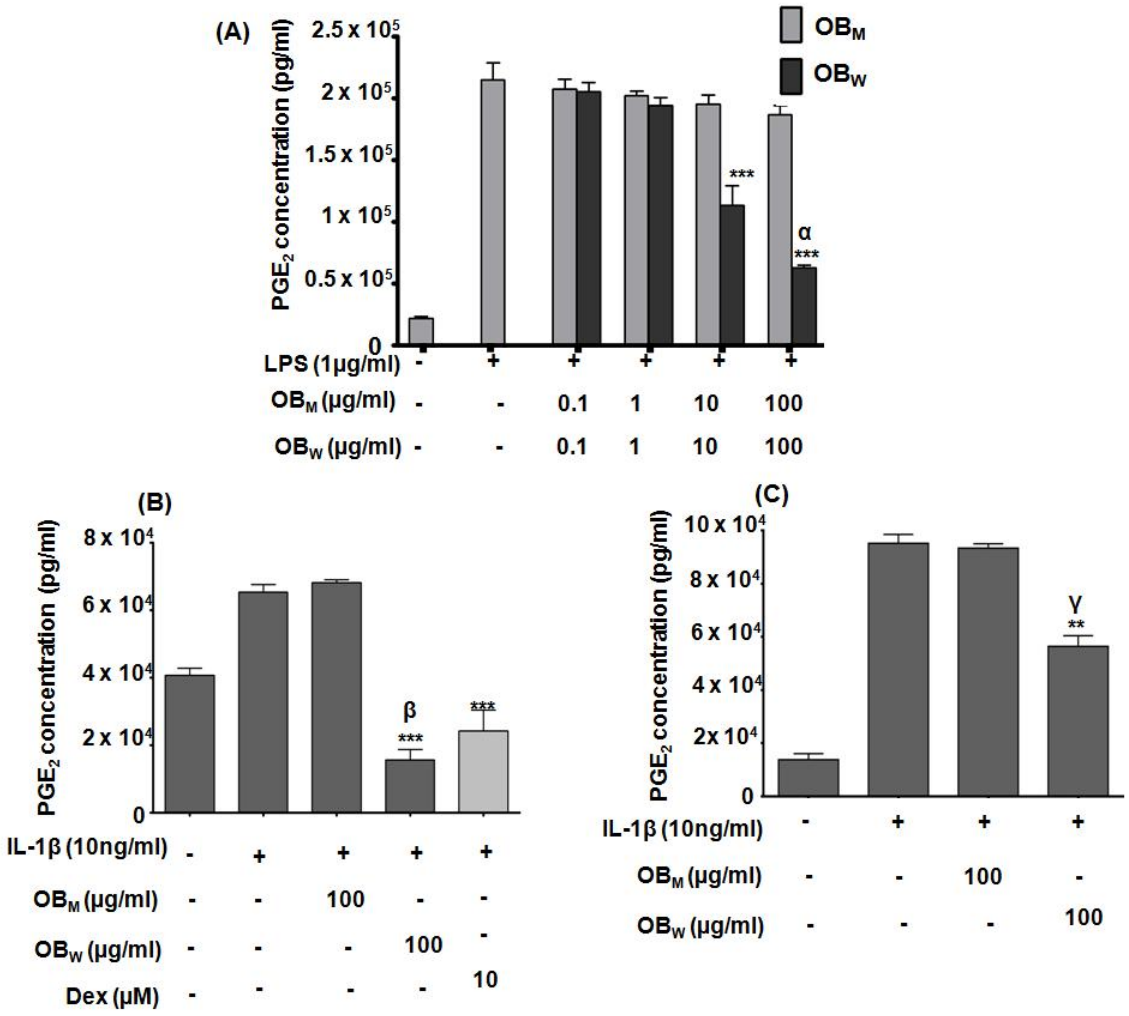


***Figure 7.2.5. Effect of $OB_M$ and $OB_W$ on $PGE_2$ levels in Raw264.7, SW1353 cell line and human chondrocytes.*** *Raw264.7 cells were pre-treated with $OB_M$ and $OB_W$, stimulated with LPS and evaluated for their effect on PGE2 levels* ***(A)****. SW1353* ***(B)*** *and human chondrocytes* ***(C)*** *were pre-treated with $OB_M$ and $OB_W$, stimulated with IL-1β and evaluated for their effect on $PGE_2$ that was measured by EIA-Monoclonal based kits. The data represents mean±SD of three independent experiments, each performed in triplicates. *p<0.05, **p<0.01, ***p<0.001 indicated statistically significant differences. "α" compared with $OB_M$ p<0.001; "β" compared with $OB_M$ p<0.001; "γ" compared with $OB_M$ p<0.001 (Tukeys multiple comparison test).*

### 7.2.6. *O. basilicum* decreased LTB4 levels in SW1353 and primary human chondrocytes

In SW1353, at 100 µg/ml concentration, $OB_M$ and $OB_W$ significantly reduced LTB4 levels by 91.1% ($p<0.001$) and 84.1%, ($p<0.001$), respectively (Figure 7.2.6 A) compared to IL-1β treated cells. In chondrocytes, at this dose, $OB_M$ and $OB_W$ decreased LTB4 levels by 99.3 ($p<0.001$) and 59.6% ($p<0.001$), respectively (Figure 7.2.6 B). Dexamethasone reduced LTB4 levels by 29.36% ($p<0.001$). It is important to note that in chondrocytes, $OB_M$ decreased LTB4 levels below the basal values that may otherwise lead to severe complications and hence needs careful evaluation.Thus, $OB_W$ could be considered for future applications.

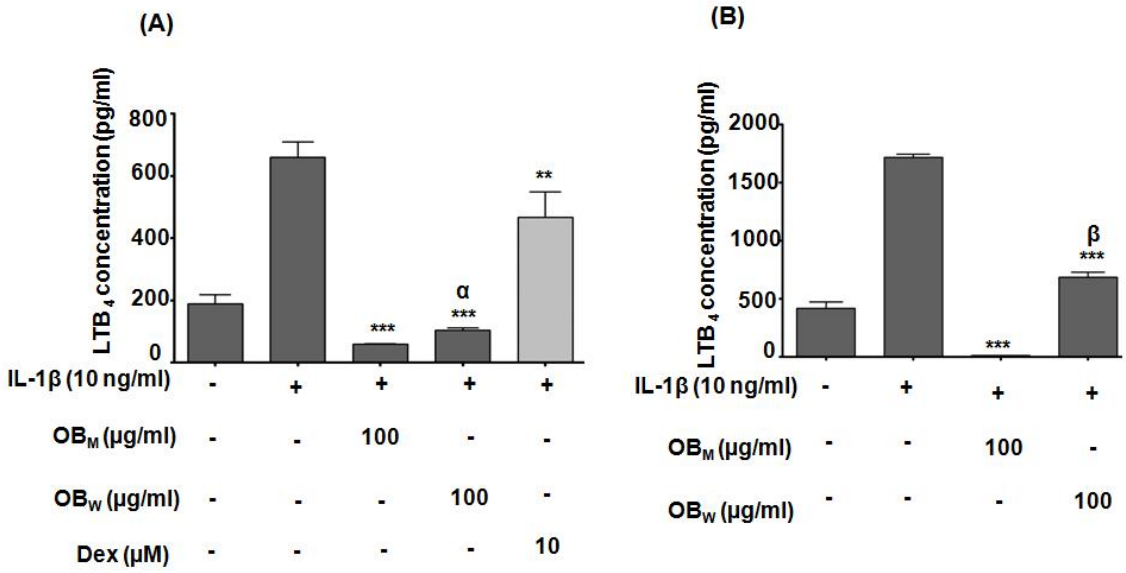


***Figure 7.2.6. Effect of $OB_M$ and $OB_W$ on LTB4 levels in IL-1β stimulated SW1353 cells and human chondrocytes.*** *SW1353* ***(A)*** *and human chondrocytes* ***(B)*** *were pre-treated with $OB_M$ and $OB_W$ and analyzed for their effect on production of LTB4 in the cell-free culture media that was measured by EIA-Monoclonal based kits. The data represents mean±SD of three independent experiments, each performed in triplicates. *p<0.05, **p<0.01, ***p<0.001 indicated statistically significant differences. "α" compared with $OB_M$ p>0.05NS; "β" compared with $OB_M$ p<0.001(Tukeys multiple comparison test). NS: Not significant.*

### 7.2.7. *O. basilicum* reduced MMP production in human primary chondrocytes

$OB_W$ and $OB_M$ reduced the expression of MMP 2, 9 and 13 in chondrocytes compared to IL-1β treated control cells. At 100µg/ml dose, $OB_M$ reduced MMP 2,-9, -13 by 26.41, 27.45 ($p<0.05$) and 41.81% ($p<0.01$), respectively (Figure 7.2.7 A, B, C, respectively). Contrarily, $OB_W$ reduced MMP 2,-9,-13 production by 58.49 ($p<0.01$), 43.13 ($p<0.05$) and 54.54% ($p<0.01$), respectively (Figure 7.2.7 A, B, C, respectively), compared to IL-1β treated cells. The gelatinases are known to degrade collagen types IV, V, and XI and MMP-13 plays an important role in cell enlargement and/or cartilage calcification. These results showed that $OB_W$ was more effective than $OB_M$ in reducing MMP levels in human chondrocytes. Thus, by modulating the expression of these MMPs by $OB_W$, the continued degradation of articular cartilage could be prevented. All these observations suggest that the aqueous extract of *O. basilicum* could be explored as an anti-inflammatory and chondroprotective agent.

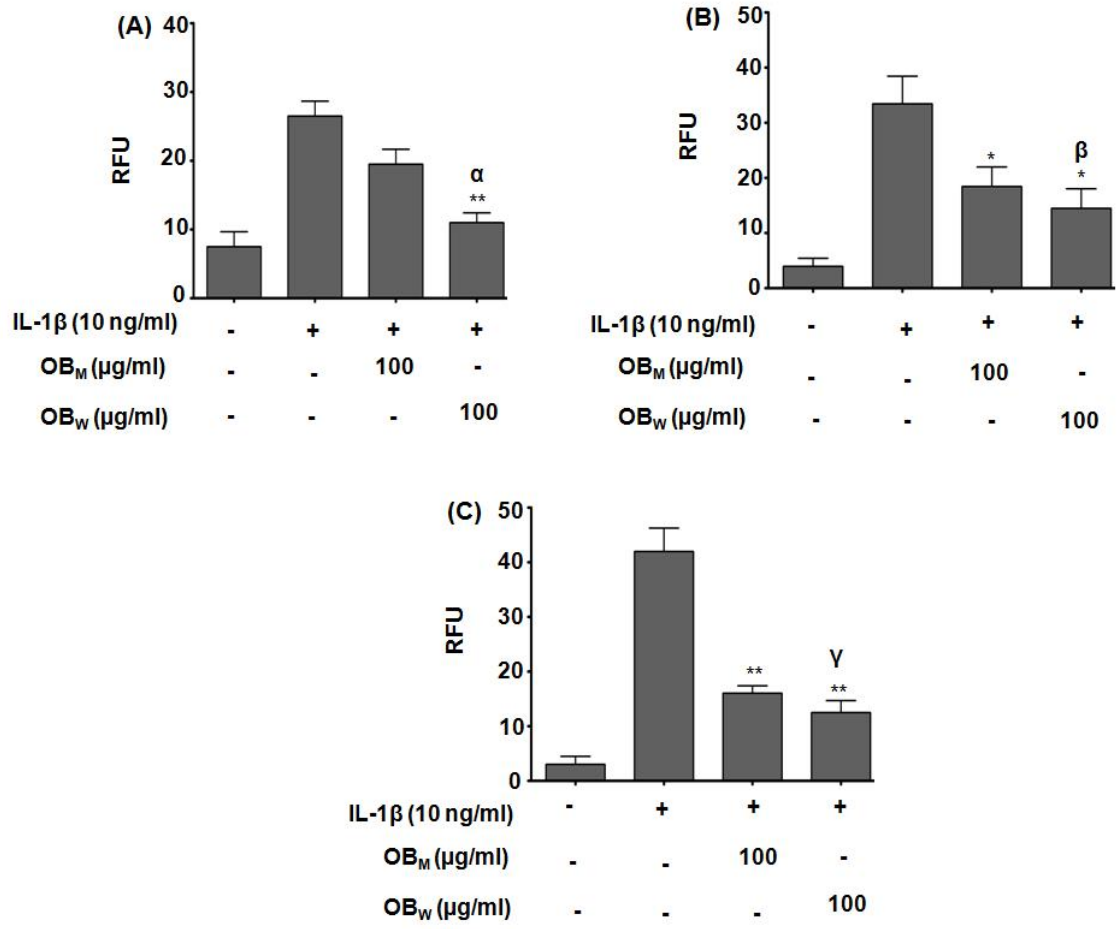


***Figure 7.2.7. Effect of $OB_M$ and $OB_W$ on MMP levels in IL-1β stimulated human chondrocytes.*** *Human chondrocytes were pre-treated with $OB_M$ and $OB_W$ and analyzed for their effect on production of MMP-2* ***(A),*** *MMP-9* ***(B)*** *and MMP-13* ***(C)*** *in the cell-free culture media by using SensoLyte® 520 Generic MMP Activity Kit.. The data represents mean±SD of three independent experiments, each performed in triplicates. *p<0.05, **p<0.01, ***p<0.001 indicated statistically significant differences. "α" compared with $OB_M$ p<0.01; "β" compared with $OB_M$ p>0.05NS; "γ" compared with $OB_M$ p>0.05NS (Tukeys multiple comparison test).NS: Not significant.*

## 7.3 Comparing the effect of aqueous and methanolic extracts of *O. sanctum* on IL-1β induced $PGE_2$ and LTB4 levels in human chondrosarcoma (SW1353) cell line

**The manuscript is under preparation**

The anti-inflammatory potential of the aerial parts of aqueous ($OS_W$) and methanolic ($OS_M$) extracts of *O. sanctum* was evaluated in SW1353 cell line. *B. serrata*, a well known anti-inflammatory herbal, was used as a positive control in the study.

### 7.3.1. Effect of $OS_W$, $OS_M$, $BS_W$ and $OS_{WM}$ on cell viability

Initially, different concentrations (0.1-100 µg/ml) of $OS_M$ and $OS_W$ extracts of *O. sanctum* as well as aqueous extract of *B. serrata* ($BS_W$) were evaluated for their effect on viability of SW1353 cells. Interestingly, the extracts did not exhibit any apparent cytotoxicity upto 10 µg/ml (Table 11). At 100 µg/ml dose, 91.2, 86.8, 78.3% viability was observed in $OS_M$, $OS_W$ and $BS_W$ treated cells, respectively. Thus, the doses till 10 µg/ml were taken for further assays.

**Table 11. Effect of $OS_M$, $OS_W$ and $BS_W$ on cell viability in SW1353**

| | % Viability | | |
|---|---|---|---|
| **Conc. (µg/ml)** | **$OS_M$** | **$OS_W$** | **$BS_W$** |
| **0.1** | 100±0.27 | 100±3.6 | 100±3.7 |
| **1** | 100±3.6 | 100±1.8 | 100±1.8 |
| **10** | 100±7.1 | 100±4.3 | 100±3.6 |
| **100** | 91.2±8.8 | 86.8±3.1 | 78.3±4.7 |

### 7.3.2. Effect of $OS_M$, $OS_W$ and $BS_W$ on NO levels

The effect of $OS_M$, $OS_W$ and $BS_W$ on NO levels was evaluated in SW1353. The cells were pre-treated with different concentrations (0.1-10µg/ml) of $OS_W$, $OS_M$ and $BS_W$. It was observed that compared to IL-1β stimulated control cells, at the concentrations of 0.1, 1 and 10 µg/ml, $OS_M$ reduced the NO levels by 44.5 ($p<0.05$), 46.4 ($p<0.01$) and 78.2 ($p<0.001$) %, respectively, whereas $OS_W$ reduced the NO levels by 54.5 ($p<0.01$), 63.2 ($p<0.01$) and 73.6 ($p<0.001$) %, respectively (Fig. 7.3.2). On the other hand, at the concentrations of 0.1, 1 and 10 µg/ml, $BS_W$ decreased NO levels by

21.9, 45.4 ($p<0.05$) and 71.5 ($p<0.001$)%, respectively (Fig. 7.3.2). The results of $OS_W$ and $OS_M$ were comparable to the positive control, *B. serrata*.

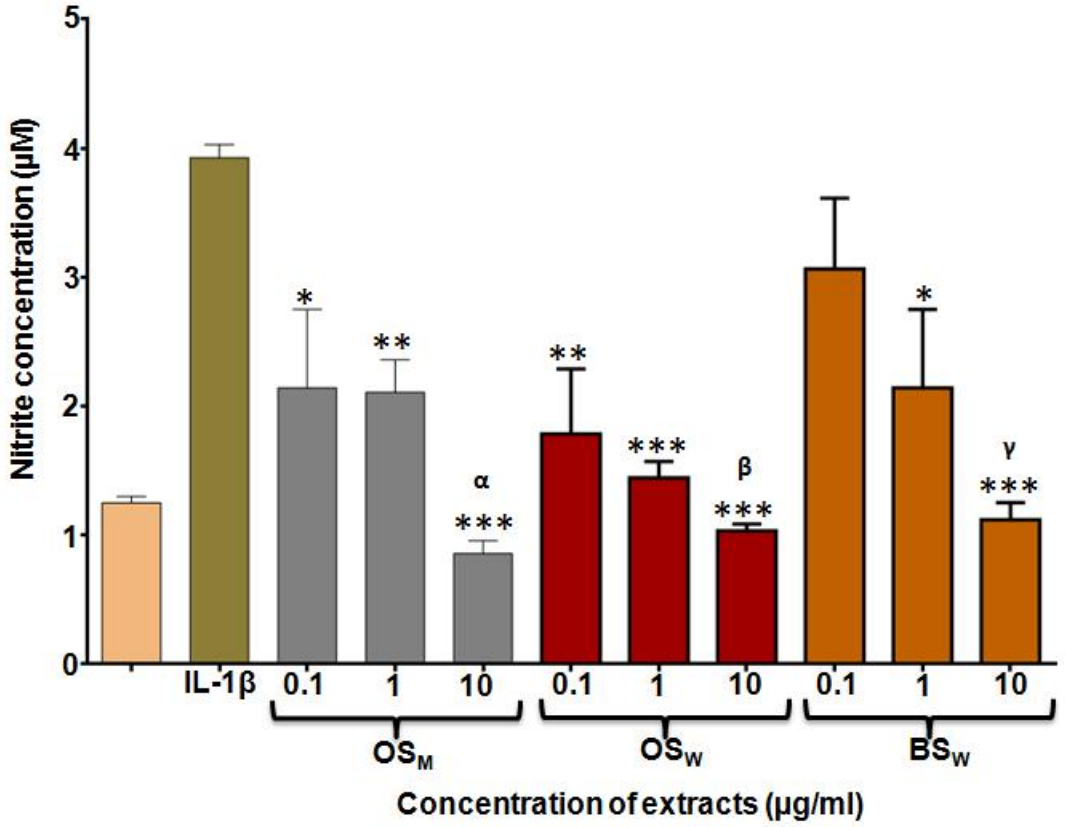


***Fig 7.3.2. Effect of $OS_M$, $OS_W$ and $BS_W$ on NO levels in SW1353****. The cells were pre-treated with $OS_W$, $OS_M$ and $BS_W$ and stimulated with IL-1β and evaluated for their effect on NO that was measured by Griess reagent. The data represents mean±SD of three independent experiments, each performed in triplicates. *p<0.05, **p<0.01, ***p<0.001 indicated statistically significant differences. "α" compared with "β" p>0.05NS; "α" compared with "γ" p>0.05NS; "β" compared with "γ" p>0.05NS (Tukeys multiple comparison test).NS: Not significant.*

### 7.3.3. Effect of $OS_M$, $OS_W$ and $BS_W$ on $PGE_2$ levels

The effect of $OS_M$, $OS_W$ and $BS_W$ on $PGE_2$ levels was evaluated in SW1353. The cells were pre-treated with different concentrations (0.1-10µg/ml) of $OS_W$, $OS_M$ and $BS_W$. It was observed that compared to IL-1β stimulated control cells, at the

concentration of 1 and 10µg/ml, $OS_M$ and $OS_W$ were found to be almost equally effective in reducing $PGE_2$ levels. At 1 µg/ml dose, $OS_M$ and $OS_W$ decreased $PGE_2$ levels by 48.36 ($p<0.001$) and 48.52 ($p<0.001$), respectively whereas at 10µg/ml dose, $OS_M$ and $OS_W$ decreased $PGE_2$ levels by 52.5 ($p<0.001$), 54.6 ($p<0.001$), respectively (Fig. 7.3.3). Interestingly, it was found that $OS_W$ at a lower dose of 0.1 µg/ml, significantly reduced PGE2 levels by 46.2 ($p<0.001$) %. $BS_W$ at 1 and 10µg/ml dose was found to reduce $PGE_2$ levels by 19.75% ($p<0.05$) and 19.95% ($p<0.05$), respectively. Thus, from the above results we found that at lower doses both $OS_W$ and $OS_M$ decreased $PGE_2$ levels more significantly compared to the positive control *B. serrata*.

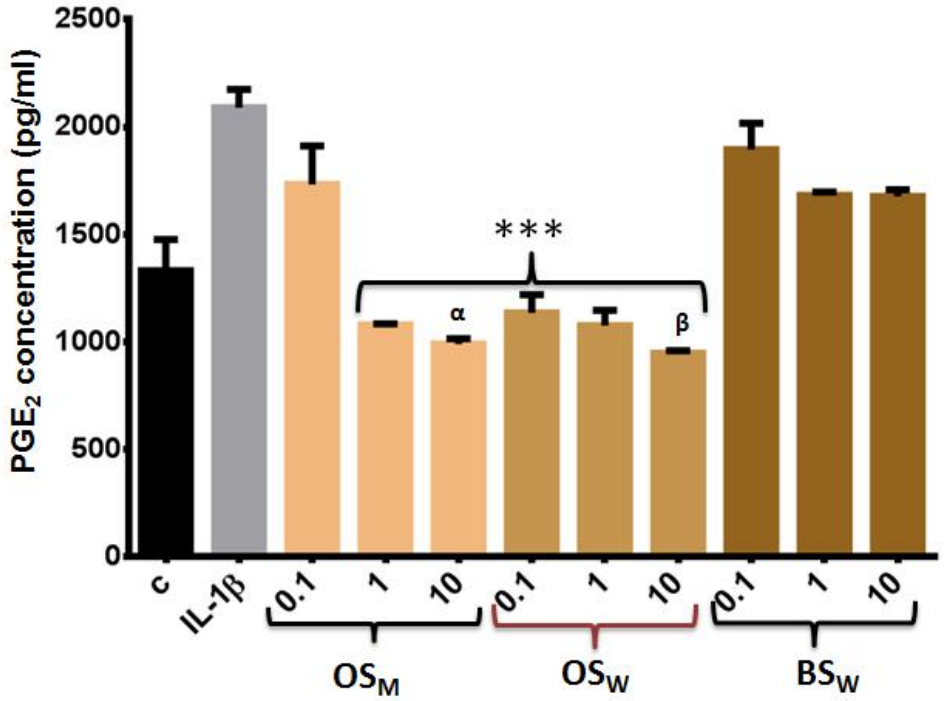


***Fig 7.3.3. Effect of $OS_M$, $OS_W$ and $BS_W$ on $PGE_2$ levels in SW1353****. The cells were pre-treated with $OS_W$, $OS_M$ and $BS_W$ and stimulated with IL-1β and evaluated for their effect on $PGE_2$ that was measured by EIA-Monoclonal based kit. The data represents mean±SD of three independent experiments, each performed in triplicates. *p<0.05, **p<0.01, ***p<0.001 indicated statistically significant differences. "α" compared with "β" p>0.05NS; "α" compared with "γ" p<0.001; "β" compared with "γ" p<0.001 (Tukeys multiple comparison test).NS: Not significant.*

### 7.3.4. Effect of $OS_M$, $OS_W$ and $BS_W$ on LTB4 levels

The effect of $OS_M$, $OS_W$ and $BS_W$ was evaluated on LTB4levels in SW1353. The cells were pre-treated with different concentrations (0.1-10µg/ml) of $OS_W$, $OS_M$ and $BS_W$. It was observed that compared to IL-1β stimulated control cells, at the concentration of 10µg/ml, $OS_M$, $OS_W$ and $BS_W$ decreased the LTB4 levels by, 70.3($p<0.001$), 72.7($p<0.001$) and 81.5($p<0.001$) %, respectively (Fig. 7.3.4).

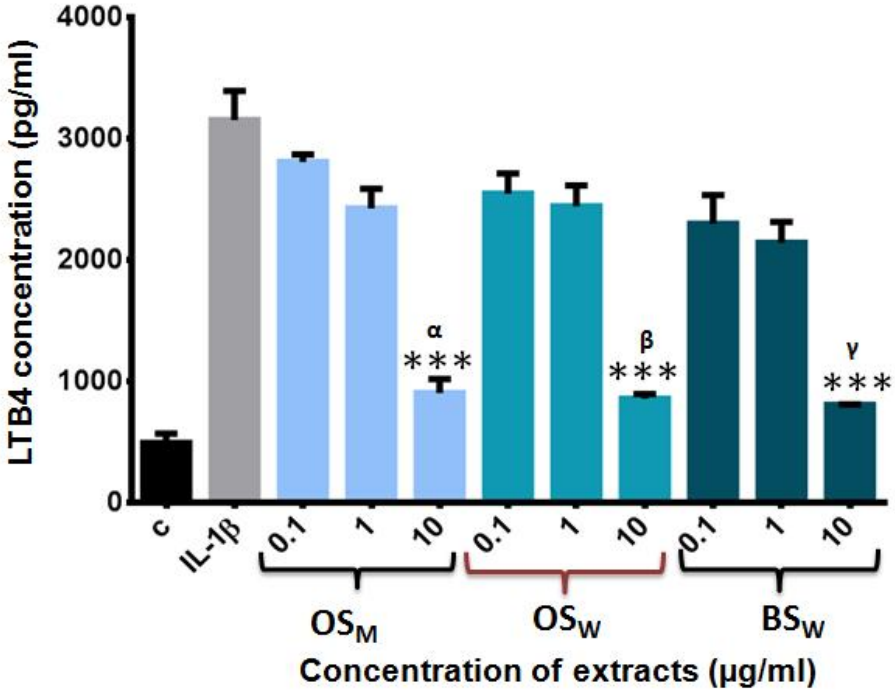


***Fig. 7.3.4. Effect of $OS_W$, $OS_M$ and $BS_W$ on LTB4 levels in SW1353****. The cells were pre-treated with* $OS_{WM}$ combinations *stimulated with IL-1β and evaluated for their effect on LTB4 that was measured by EIA-Monoclonal based kits. The data represents mean±SD of three independent experiments, each performed in triplicates. *p<0.05, **p<0.01, ***p<0.001 indicated statistically significant differences. "α" compared with "β" p>0.05NS; "α" compared with "γ p>0.05NS; "β" compared with "γ" p>0.05NS (Tukeys multiple comparison test).NS: Not significant.*

## 7.4. Evaluation of anti-inflammatory potential of combination of aqueous and methanolic extracts of *O. sanctum* (LOT001, LOT002 and LOT02) in SW1353 and human primary chondrocytes

**Why different combinations of aqueous and methanolic extracts of *O. sanctum* were done**?

Extensive literature survey revealed that *O. sanctum* showed the presence of many chemical constituents, which are responsible for varied pharmacological and medicinal properties (Ravi *et al*., 2012). The aqueous extract of *O. sanctum* has been shown to contain primary metabolites [amino acid, nucleotides, carbohydrates (sugar, starch) and lipids (fats, essential oils, waxes terpenoids and oleoresin)] where as the alcoholic extract has been shown to contain secondary metabolites (total phenols, tannins, steroids and alkaloids) in large proportions (Jamal., 2011). Thus, on the basis of the available literature on the phytochemical constitution of *O. sanctum*, we wanted to find out whether the mixing of aqueous and alcoholic extracts in different proportions could induce more decrease in expression of pro-inflammatory molecules compared to either of the two extracts at lower doses.

The leaves of *O.* sanctum have been shown to contain many volatile oils like eugenol, euginal, urosolic acid, carvacrol, linalool, limatrol, caryophyllene, and methyl carvicol (Temimi *et al*., 2015; Chandrasekaran *et al.,* 2013; Rahman *et al*., 2011) The seed volatile oils have been reported to contain fatty acids and sitosterol; the seed mucilage has been shown to contain some levels of sugars and anthocyanins (Rahman *et al*., 2011; Klem et al., 2000). In addition, the stem and leaves of *O. sanctum* contains variety of other constituents which includes saponins, flavonoids, triterpenoids (ursolic acid, oleonolic acid etc.) and tannins that have been reported to have biological activity (Jaggi *et al*., 2003). The phenolic compounds such as apigenin, cirsimaritin, isothymusin, rosmarinic acid, isothymonin have been shown to exhibit antioxidant and antiinflammatory activities (Rahman *et al*., 2011). The flavonoids such as orientin and vicenin have been shown to provide protection against radiation induced chromosomal damage in human blood lymphocytes (Pattanayak *et al*., 2010).

### 7.4.1. Evaluation of anti-inflammatory potential of LOT001, LOT002, LOT02 in SW1353 cells

Different ratios of aqueous and methanolic extracts of *O. sanctum* were prepared as 1:1 (LOT001), 1:2 (LOT002) and 1:4 (LOT02). The level of total triterpene acids (ursolic acid, oleanolic acid) was normalized in different lots of *O. sanctum* to ≥2.5%. [For drug development the level of triterpene acids in a herbal product should be ≥2.5% (Mathuna and Larimore, 2006)].

We further evaluated the anti-inflammatory potential of different combinations (LOT001, LOT002 and LOT02) of aqueous and methanolic extracts of *O. sanctum* on IL-1β stimulated human chondrosarcoma cells (Table 12).

**Table 12. Different combinations of aqueous and methanolic extracts of *O. sanctum* (LOT001, LOT002 and LOT02)**

| OS LOTS | $OS_W$ | $OS_M$ | Approximate Ratio |
|---|---|---|---|
| **LOT001** | 38% | 62% | 1:1 |
| **LOT002** | 31% | 69% | 1:2 |
| **LOT02** | 19% | 81% | 1:4 |

#### 7.4.1.1. Effect of different OS LOTs on cell viability of SW1353

The different combinations (LOT001, LOT002 and LOT02) of aqueous and methanolic extracts of *O. sanctum* were evaluated for their effect on viability of SW1353. The cells were treated with different concentrations (1-100 µg/ml) of the LOTs. Interestingly, the extracts did not exhibit any apparent cytotoxicity upto 100µg/ml (Table 13).

**Table 13. Effect of OS LOTs on cell viability in SW1353**

| Conc.(µg/ml) | LOT001 | LOT002 | LOT02 |
|---|---|---|---|
| | | % Viability | |
| **0.1** | 100±3.35 | 100±1.7 | 100±5.6 |
| **1** | 100±10.9 | 100±3.4 | 100±1.7 |
| **10** | 100±8.1 | 100±7.6 | 100±6.2 |
| **100** | 100±11.7 | 100±4.5 | 100±7.3 |

### 7.4.1.2. Effect of OS LOTs (LOT001, LOT002 and LOT02) on the intracellular nitric oxide (NO) levels

The nitric oxide assay was performed in SW1353 by pretreating the cells with different concentrations (1-100 µg/ml) of LOT001, LOT002 and LOT02. It was observed that compared to IL-1β stimulated control cells, LOT001, LOT002 and LOT02, at all the concentrations, showed a significant reduction in intracellular NO levels (Fig. 7.4.1.2). At the lower concentration of 1µg/ml, LOT001, LOT002 and LOT02, significantly reduced the NO levels by 94.1 ($p<0.001$), 93.1 ($p<0.001$) and 92.31 ($p<0.001$) %, respectively (Fig. 7.4.1.2). At all the concentrations (0.1-100 µg/ml), the effect on NO was found to be almost similar in all the three LOTs. The reason could be probably a saturation effect that may be observed at higher concentrations of the extract.

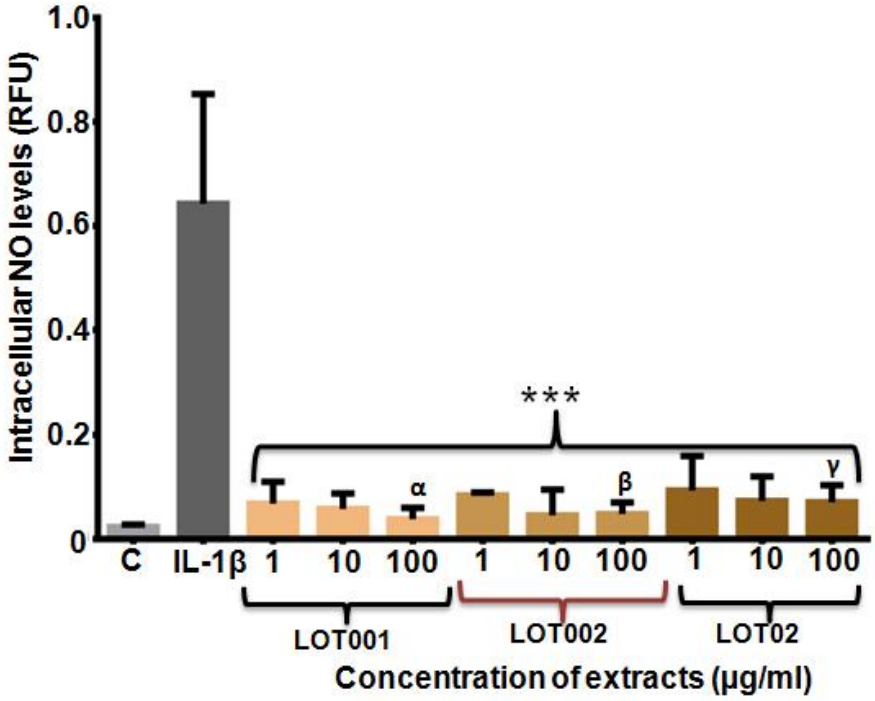


***Fig. 7.4.1.2 Effect of OS LOTs on the intracellular nitric oxide (NO) levels.*** *The cells were pre-treated with* $OS_{WM}$ combinations *stimulated with IL-1β and evaluated for their effect on intracellular NO levels that was measured by DAF-FM dye. The data represents mean±SD of three independent experiments, each performed in triplicates. *p<0.05, **p<0.01, ***p<0.001 indicated statistically significant differences. "α" compared with "β" p>0.05NS; "α" compared with "γ p>0.05NS; "β" compared with "γ" p>0.05NS (Tukeys multiple comparison test).NS: Not significant.*

### 7.4.1.3. Effect of OS LOTs on $PGE_2$ levels

The effect of OS LOTs on $PGE_2$ levels was evaluated in SW1353. The cells were pre-treated with different concentrations (1-10 µg/ml) of the three LOTs. It was observed that compared to IL-1β stimulated control cells, at the concentration of 1, 10 and 100 µg/ml, LOT001 decreased $PGE_2$ levels by 6.6, 25.7 and 51.2 ($p<0.05$) %, respectively. On the other hand, LOT002 decreased $PGE_2$ levels by 0, 2.6 and 44.3 ($p<0.05$) %, respectively, at the concentrations of 1, 10 and100

µg/ml. Interestingly, LOT02 significantly reduced $PGE_2$ levels by 86.1 ($p<0.001$), 97.9 ($p<0.001$) and 92.1 ($p<0.001$) % at the concentrations of 1, 10 and100 µg/ml, respectively (Fig. 7.4.1.3)**.** As compared to other ratios, LOT02 showed a significant decrease in the $PGE_2$ levels at lower concentrations. It was also observed that LOT02 lowered $PGE_2$ levels below the basal levels. However, SW1353, being a human chondrosarcoma cell line, the basal $PGE_2$ levels are high in these cells. Thus, lowering $PGE_2$ below the basal levels in SW1353 by LOT02, could not be considered to be detrimental.

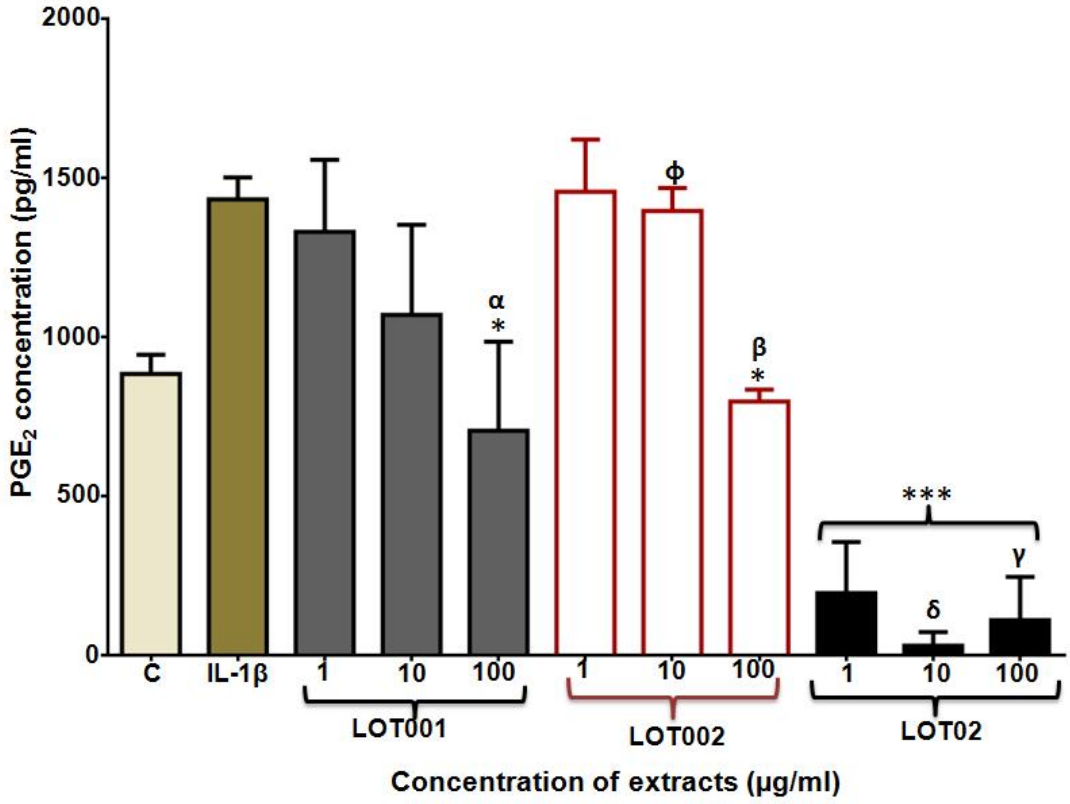


***Fig. 7.4.1.3. Effect of OS LOTs (LOT001, LOT002 and LOT02) on PGE₂ levels in SW1353****. The cells were pre-treated with* $OS_{WM}$ combinations, *stimulated with IL-1β and evaluated for their effect on PGE₂ that was measured by EIA-Monoclonal based kits. The data represents mean±SD of three independent experiments, each performed in triplicates. *p<0.05, **p<0.01, ***p<0.001 indicated statistically significant differences. "δ" compared with "λ" p<0.01; "δ" compared with "φ" p<0.001; "λ" compared with "φ" p>0.05NS; "γ" compared with "α" p>0.05NS;*

*"γ" compared with "β" p<0.05; "α" compared with "β" p>0.05NS; "γ" compared with "δ" p>0.05NS; (Tukeys multiple comparison test). NS: Not significant.*

#### 7.4.1.4. Effect of OS LOTs on LTB4 levels

We compared the effect of the LOTs on LTB4 levels in SW1353. It was observed that compared to IL-1β stimulated control cells, at the concentration of 1, 10 and 100 µg/ml doses, LOT001 decreased LTB4 levels by 31.3, 29.9 and 44.5 ($p<0.05$) %, respectively (Fig. 7.4.1.4). On the other hand, LOT002 decreased LTB4 levels by 44.6, 59.5 ($p<0.001$) and 53.1 ($p<0.05$) %, respectively, at the concentrations of 1, 10 and100 µg/ml. Interestingly, LOT02 reduced LTB4 levels by 59.1 ($p<0.01$), 84.9 ($p<0.001$) and 73.7 ($p<0.001$) % respectively, at the concentrations of 1, 10 and100 µg/ml, (Fig. 7.4.1.4). From the above results it is clear that, compared to other LOTs, LOT02 showed a significant decrease in LTB4 levels at lower doses.

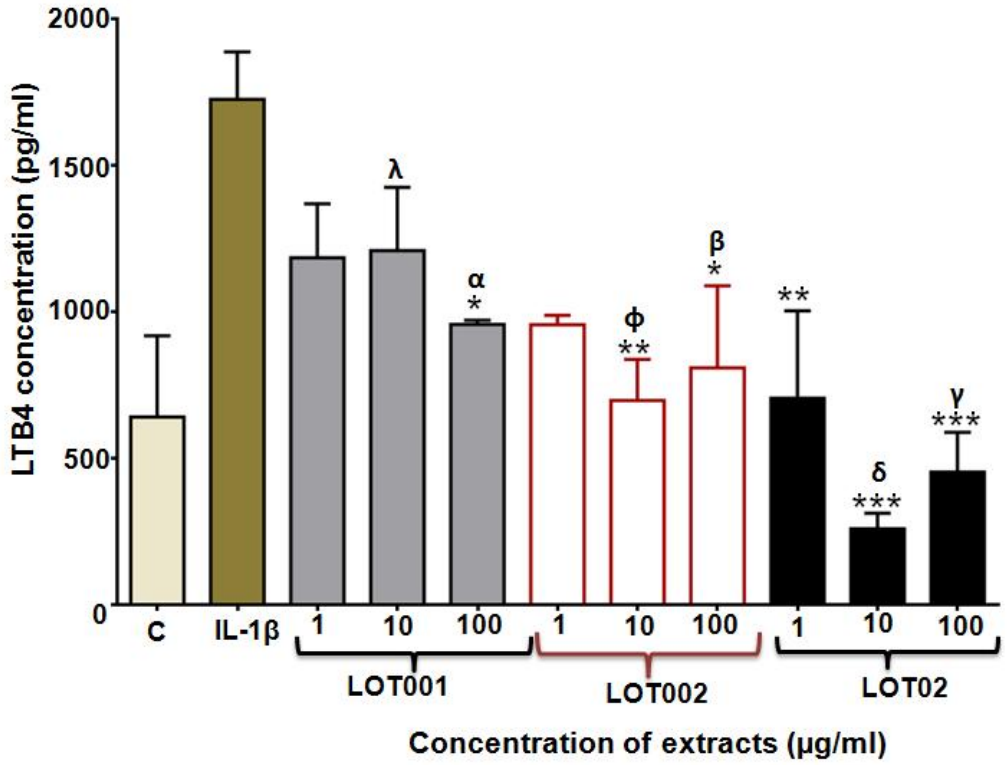


***Fig. 7.4.1.4. Effect of OS LOTs (LOT001, LOT002 and LOT02) on LTB4 levels in SW1353***. *The cells were pre-treated with* $OS_{WM}$ combinations *stimulated with IL-1β and evaluated for their effect on LTB4 that was measured by EIA-Monoclonal based kit. The data represents mean±SD of three independent experiments, each performed in triplicates. *p<0.05, **p<0.01, ***p<0.001 indicated statistically significant differences. "δ" compared with "λ" p<0.05; "δ" compared with "φ" p>0.05NS; "λ" compared with "φ" p>0.05NS; "γ" compared with "α" p>0.05NS; "γ" compared with "β" p>0.05NS; "α" compared with "β" p>0.05NS; "γ" compared with "δ" p>0.05NS (Tukeys multiple comparison test).NS: Not significant.*

**On the basis of the results of $PGE_2$ and LTB4, LOT02 further analyze for its effect on IL-1β, TNF-α and COX-2 expression in SW1353 cells**

### 7.4.1.5. Effect of LOT02 on IL-1β, TNF-α and COX expression

LOT02 was analyzed for its effect on the expressions of IL-1β, TNF-α and COX-2 proteins in SW1353. Our previous results showed that at 10 and 100 µg/ml concentrations, LOT02 showed almost equal inhibitory potential in terms of $PGE_2$ and LTB4 inhibition, so only 1 and 10 µg/ml concentrations were taken for further studies. It was observed that at 1 µg/ml dose, LOT02 significantly reduced the expression of IL-1β, TNF-α and COX proteins by 58.9, 68.5 and 49.8% respectively, compared to IL-1β stimulated control (Fig.7.4.1.5 A, B and C, respectively). On the other hand, at 10 µg/ml concentration, LOT02 significantly reduced the expression of IL-1β, TNF-α and COX proteins by 57, 72 and 52% respectively (Fig.7.4.1.5 A, B and C, respectively). Thus, at protein level, both the concentrations (1 and 10 µg/ml) were found to be almost equally effective suggesting that both concentrations could be useful for future studies.

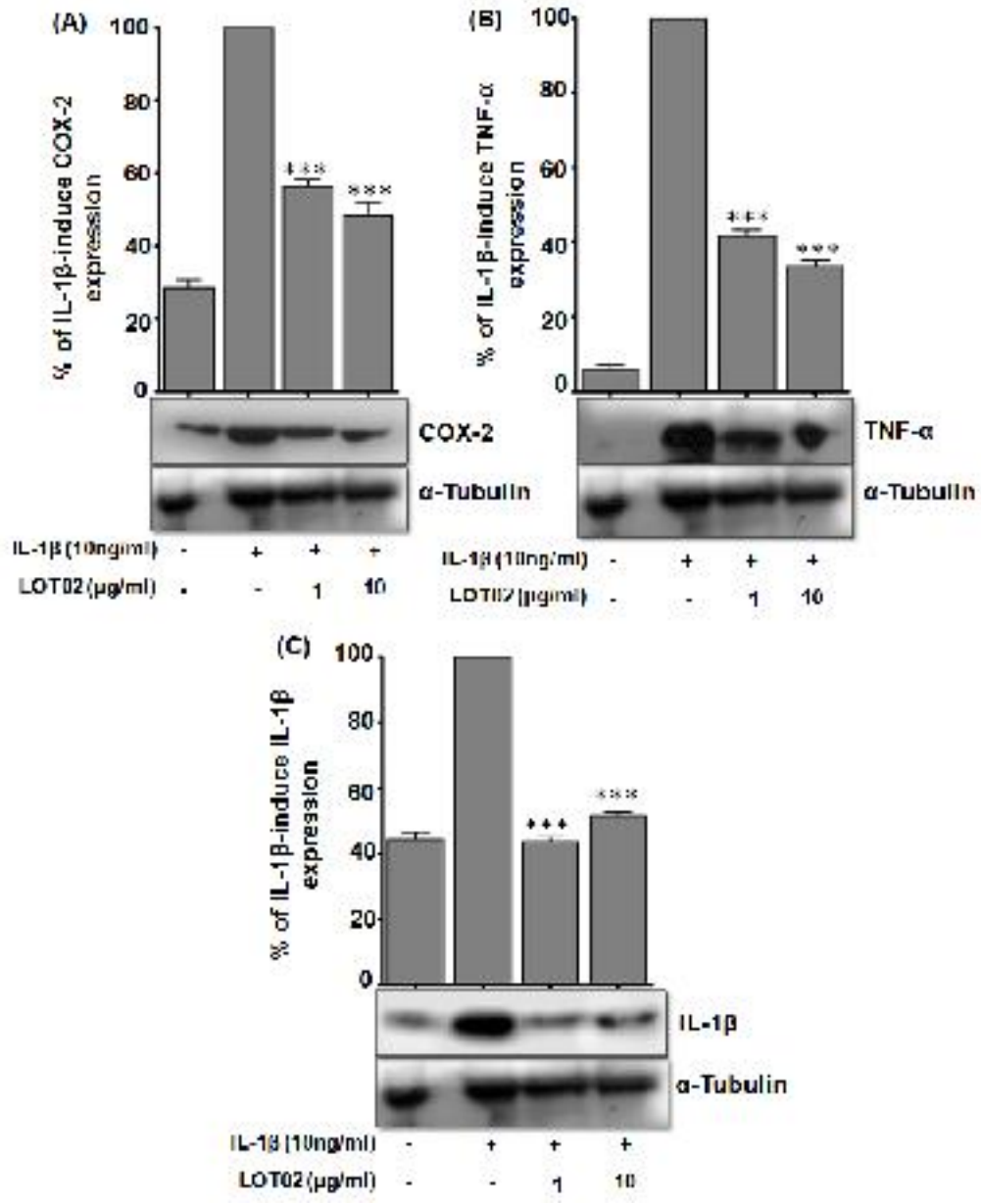


***Fig. 7.4.1.5. Effect of LOT02 on the expression of IL-1β (A), TNF-α (B) and COX (C) proteins.*** *The cells were pre-treated with LOT02 and stimulated with IL-1β, followed by analysis of IL-1β, TNF-α and COX expression by western blotting. α-tubulin was used as a loading control. *p<0.05, **p<0.01, ***p<0.001 indicated statistically significant differences.*

## 7.4.2. Evaluation of anti-inflammatory potential of LOT02 in human primary chondrocytes

**On the basis of the results of $PGE_2$ and LTB4, LOT02 was taken further for mechanistic studies in human primary chondrocytes**

### 7.4.2.1. Effect of LOT02 on IL-1β induced $PGE_2$ levels

We evaluated the effect of LOT02 on $PGE_2$ levels in human primary chondrocytes. At all the concentrations (0.1-100 µg/ml) tested, a significant decrease in $PGE_2$ levels was observed compared to IL-1β stimulated control cells. LOT02 reduced the $PGE_2$ levels by 93.2 ($p<0.001$), 94.5 ($p<0.001$), 97.3 ($p<0.001$) and 95.8 ($p<0.001$) % at the concentrations of 0.1, 1, 10 and 100 µg/ml, respectively (Fig. 7.4.2.1).

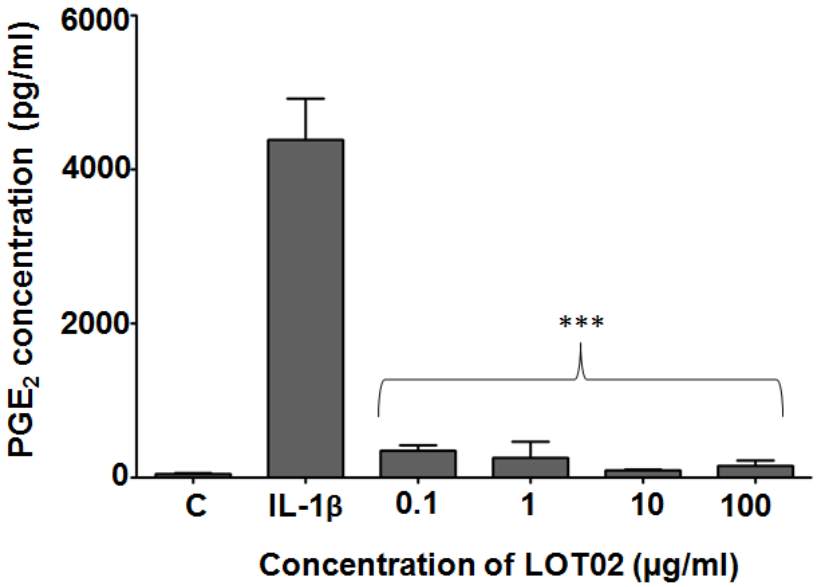


***Fig. 7.4.2.1. Effect of LOT02 on $PGE_2$ levels in human primary chondrocytes****. The cells were pre-treated with LOT02, stimulated with IL-1β and evaluated for their effect on $PGE_2$ that was measured by EIA-Monoclonal based kit. The data represents mean±SD of three independent*

*experiments, each performed in triplicates. *p<0.05, **p<0.01, ***p<0.001 indicated statistically significant differences.*

### 7.4.2.2. Effect of LOT02 on IL-1β induced LTB4 levels

We evaluated the effect of LOT02 on LTB4 levels in human primary chondrocytes. At all the concentrations (1-100 µg/ml) tested, a significant decrease in LTB4 levels was observed, compared to IL-1β stimulated control cells. LOT02 reduced LTB4 levels by 23.2, 55.2 ($p<0.05$), 62.8 ($p<0.01$) and 57.3 ($p<0.001$) % at the concentrations of 0.1, 1, 10 and 100 µg/ml, respectively (Fig.7.4.2.2).

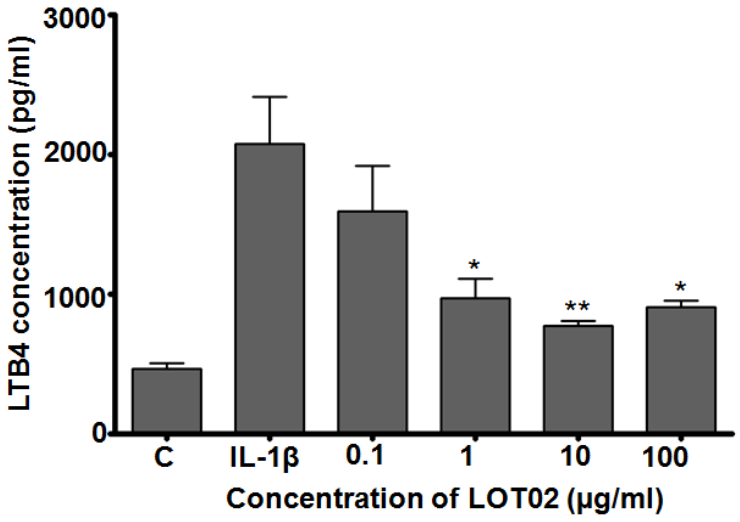


***Fig.7.4.2.2. Effect of LOT02 on LTB4 levels in human primary chondrocytes****. The cells were pre-treated with LOT02 stimulated with IL-1β and evaluated for their effect on LTB4 that was measured by EIA-Monoclonal based kit. The data represents mean±SD of three independent experiments, each performed in triplicates. *p<0.05, **p<0.01, ***p<0.001 indicated statistically significant differences.*

### 7.4.2.3 Chondroprotective potential of LOT02

In human primary chondrocytes, compared to IL-1β stimulated control cells, LOT02 significantly stimulated the proliferation of chondrocytes. At the concentrations of 0.1, 1, 10 and 100 µg/ml about 32.4 ($p<0.001$), 33.7 ($p<0.001$), 32.8 ($p<0.001$) and 32.8 ($p<0.001$) % increase in cell viability was observed, thereby indicating that it exhibited chondroprotective activity (Fig. 7.4.2.3).

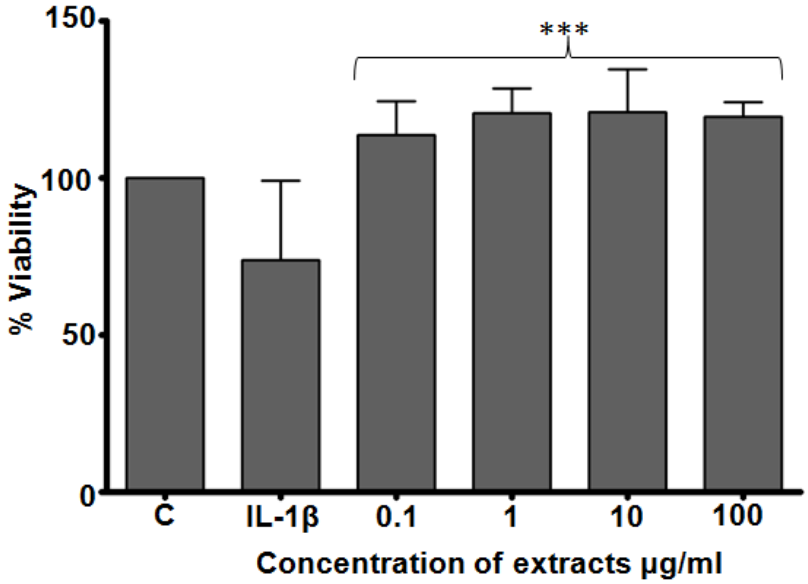


***Fig. 7.4.2.3. Effect of LOT02 on cell viability in human primary chondrocytes.*** *The cells were treated with different concentrations (0.1-100 µg/ml) of LOT02 and cell viability was determined by MTT dye uptake method. The data represents mean±SD of three independent experiments, each performed in triplicates.*

### 7.4.2.4 Effect of LOT02 on proteoglycan release and hyaluronidase activity in IL-1β stimulated chondrocytes

In human primary chondrocytes, compared to IL-1β stimulated control cells, at 10 µg/ml dose LOT02 significantly decreased the proteoglycan content by ~42 ($p<0.05$) % (Fig. 7.4.2.4).

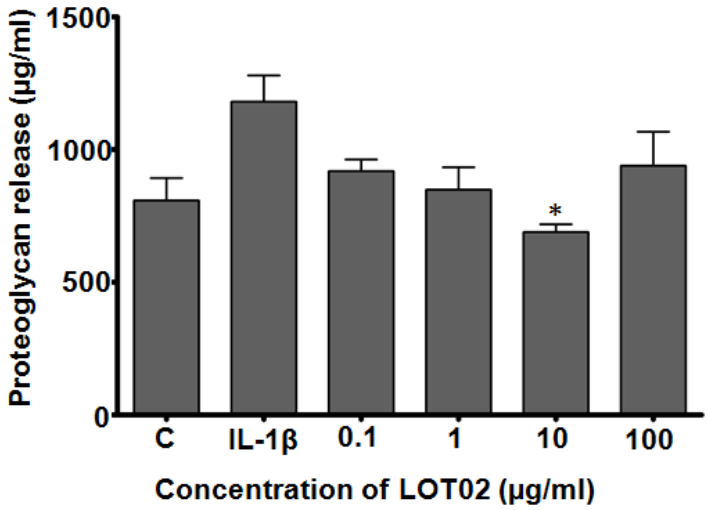


***Fig. 7.4.2.4. Effect of LOT02 on proteoglycan release in IL-1β stimulated human chondrocytes.*** *Total GAG content in supernatants of IL-1β stimulated chondrocytes was measured by the dimethylmethylene blue dye binding assay using chondroitin sulphate (CS) as a standard. The data represents mean±SD of three independent experiments, each performed in triplicates. *p<0.05, **p<0.01, ***p<0.001 indicated statistically significant differences.*

LOT02 inhibited hyaluronidase activity in a dose-dependent manner. It was observed that at the concentrations of 0.1, 1, 10 and 100 µg/ml, LOT02 decreased the hyaluronidase activity by 0, 0, 23.9 and 30.01 ($p<0.05$) % (Table 14).

**Table 14. Effect of LOT02 on hyaluronidase activity**

| Concentration of LOT02 in enzyme reaction (µg/ml) | (%) Enzyme inhibition by LOT02 |
|---|---|
| 0.1 | No Inhibition |
| 1 | No Inhibition |
| 10 | 23.9±4.33 |
| 100 | 30.01±6.69 |

### 7.4.2.5 Effect of LOT02 on MMP levels in chondrocytes

The expression of various MMPs (MMP-2, -9, -13) in response to treatment with LOT02 was analyzed in the chondrocytes. Since, 0.1 and 1 µg/ml concentrations did not show any significant effect on proteoglycan release and hyaluronidase activity in IL-1β stimulated chondrocytes, so these concentrations were not taken for MMP assay. It was observed that LOT02, at the concentration of 10 µg/ml, decreased MMP-2, 9 and 13 levels by 35.93 ($p<0.05$), 71.2 ($p<0.001$) and 85.3 ($p<0.001$) %, respectively (Fig. 7.4.2.5 A, B, C, respectively). On the other hand, at 100 µg/ml concentration, LOT02 reduced MMP-2, 9 and 13 levels by 33.3, 70.8 ($p<0.001$) and 84.9 ($p<0.001$) %, respectively. These results suggested that 10 µg/ml could be an effective dose for LOT02.

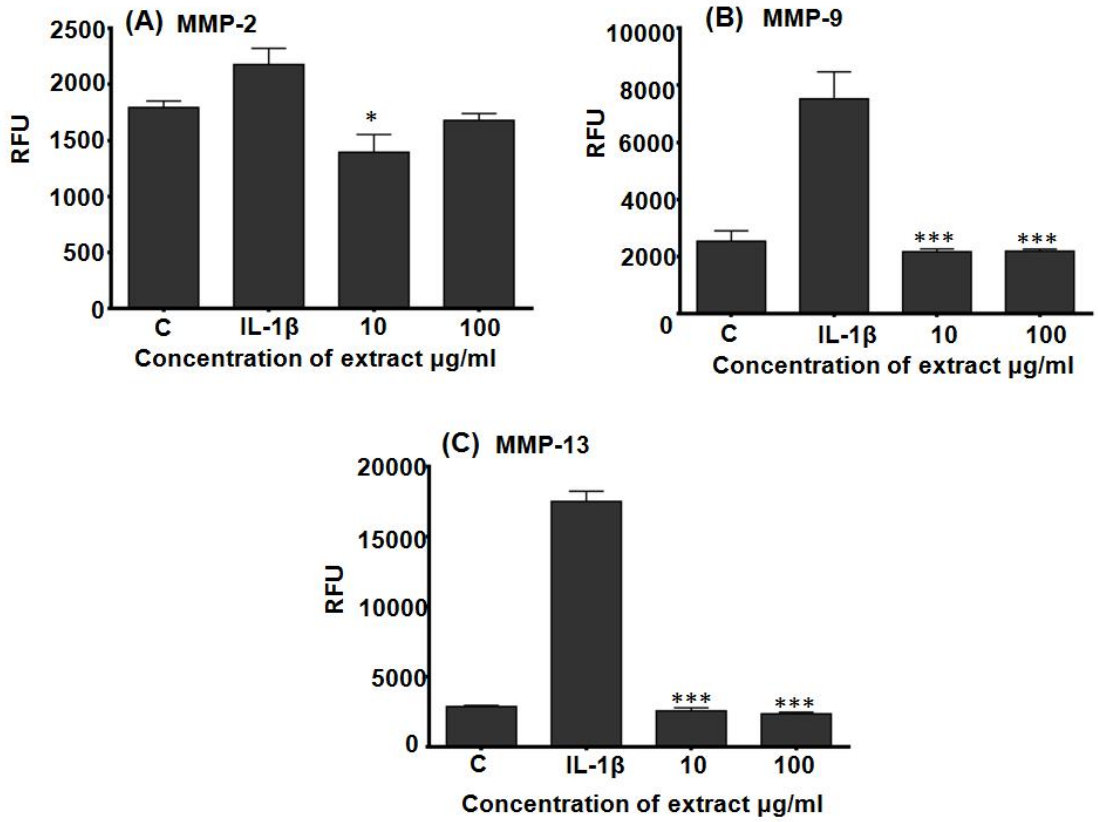


***Fig. 7.4.2.5 Effect of LOT02 on MMP levels in IL-1β stimulated human chondrocytes.*** *Human chondrocytes were pre-treated with LOT02 and analyzed for their effect on production of MMP-2 (A)**,** MMP-9 (B) and MMP-13 (C). The data represents mean±SD of three independent experiments, each performed in triplicates. *p<0.05, **p<0.01, ***p<0.001 indicated statistically significant differences*

# SECTION-8

## Evaluation of subacute toxicity of LOT02 (OciBest™ ) in Wistar Rats (The study was outsourced to Natural Remedies, Bangalore)

**The manuscript has been accepted in Journal of Ethanopharmacology (2015)**

Since LOT02 showed significant decrease in the inflammatory molecules at in vitro level, we evaluated the subacute toxicity of combination of methanolic and aqueous preparation of aerial parts of *O.sanctum* (LOT02), now named as OciBest™, in Wistar rats. The rats were orally gavaged with different doses (250, 500 and 1000 mg/kg/day) of OciBest™extract for 28 consecutive days. The clinical, pathological, hematological, biochemical and histopathological parameters of the rats were studied. The study was done according to the Organization for Economic Cooperation and Development (OECD) guidelines for testing of chemicals (No. 407, Section 4: Health Effects).

### 8.1. General observations

Daily oral administration of OciBest™ at doses of 250, 500 and 1000 mg/kg for 28 days did not produce any abnormality and toxicity symptoms in rats of either sex. The doses were selected based on the results of acute toxicity study wherein, the test item was found to be safe upto a dose of 5000 mg/kg body weight. A limit dose of 1000 mg/kg body weight was selected as a high dose.

### 8.2. Physical parameters

An increase in body weight was observed in OciBest™ (250, 500 and 1000 mg/kg) treated groups compared with control group after 28 days of study period (Table 14a and b). The rats of either sex showed little or no change in food consumption as compared to the control groups (Table 15a and b)

**Table 14a. Effect on body weight of male rats after 28 days oral administration of OciBest™**

| Oral treatment (mg/kg/day) | Body weight (g) | | | | |
|---|---|---|---|---|---|
| | 1 day | 8 day | 15 day | 22 day | 28 day |
| **Control 0** | 145.4±15.9 | 180.2±22.8 | 212.4±36.2 | 229.4±40.4 | 248.0±44.2 |
| **250** | 147.2±14.3 | 182.6±19.2 | 206.4±19.0 | 221.8±24.5 | 249.8±40.7 |
| **500** | 149.0±13.2 | 183.2±17.0 | 217±15.6 | 226±16.6 | 254.6±21.3 |
| **1000** | 149.4±13.5 | 185.0±13.9 | 211.8±15.6 | 226.4±18.1 | 249.4±28.3 |
| **Control Recovery Group 0** | 151.6±10.4 | 186.6±20.1 | 219.6±31.0 | 233.0±34.0 | 257.8±45.9 |
| **High Dose Reversible Group 1000** | 155.0±11.6 | 182.6±12.3 | 211.2±16.0 | 224.4±17.5 | 247.8±31.5 |

Data represented as Mean±SD of five animals.

**Table 14b. Effect on body weight of female rats after 28 days oral administration of OciBest™**

| | Body weight (g) | | | | |
|---|---|---|---|---|---|
| **Oral treatment (mg/kg/day)** | **1 day** | **8 day** | **15 day** | **22 day** | **28 days** |
| **Control 0** | 129.2±9.2 | 148.6±11.2 | 161.6±10.1 | 179.4±12.4 | 194.6±17.9 |
| **250** | 132.2±9.7 | 144.8±13.3 | 153.0±13.5 | 166.8±15.9 | 184.8±18.9 |
| **500** | 132.8±11.8 | 150.8±12.9 | 160.8±11.4 | 176.2±12.1 | 195.6±12.1 |
| **1000** | 135.6±11.4 | 152.2±14.7 | 116.4±16.7 | 178.4±17.9 | 208.4±13.5 |
| **Control Recovery Group 0** | 137.0±9.5 | 151.6±10.2 | 164.8±11.4 | 176.8±13.2 | 188.2±21.7 |
| **High Dose Reversible Group 1000** | 135.8±9.5 | 151.8±13.5 | 164.0±11.6 | 184.6±18.3 | 197.8±21.4 |

Data represented as Mean±SD of five animals.

**Table 15a. Effect on food intake of male rats after 28 days of the oral administration of OciBest™**

| | Food intake (g/day) | | | | | |
|---|---|---|---|---|---|---|
| | **Control 0 mg/kg** | **250 mg/kg** | **500 mg/kg** | **1000 mg/kg** | **Control Recovery Group 0 mg/kg** | **High Dose Reversible Group 1000 mg/kg** |
| **Day 8** | 223.5±62.9 | 233.0±26.2 | 223.0±58.0 | 222.0±62.2 | 225.5±48.8 | 184.6±41.0 |
| **Day 15** | 247.5±67.2 | 246.0±27.6 | 247.5±58.7 | 244.5±58.7 | 237.0±62.2 | 200.9±50.2 |
| **Day 22** | 265.0±77.8 | 262.5±33.6 | 270.5±70.0 | 275.7±77.1 | 272.5±74.2 | 223.7±77.8 |
| **Day 28** | 313.0±79.2 | 302.0±35.4 | 297.0±52.3 | 312.0±69.3 | 339.5±72.8 | 258.6±72.8 |

Data represented as Mean±SD of five animals.

**Table 15b. Effect on food intake of female rats after 28 days of the oral administration of OciBest™**

| | Food intake (g/day) | | | | | |
|---|---|---|---|---|---|---|
| | **Control 0 mg/kg** | **250 mg/kg** | **500 mg/kg** | **1000 mg/kg** | **Control Recovery group 0 mg/kg** | **High Dose Reversible Group 1000 mg/kg** |
| **Day 8** | 211.5±71.4 | 204.5±28.6 | 221.5±98.3 | 222±69.3 | 220.5±55.9 | 181.7±66.5 |
| **Day 15** | 244.0±72.1 | 238.5±32.9 | 236.5±84.1 | 237.5±57.3 | 233.0±53.7 | 197.3±68.6 |
| **Day 22** | 266.0±91.9 | 253.0±28.3 | 257.0±69.3 | 250.0±66.5 | 244.0±76.4 | 210.1±69.3 |
| **Day 28** | 241.5±68.6 | 242.5±30.1 | 246.5±68.6 | 243.0±60.8 | 264.5±74.2 | 205.5±58.0 |

Data represented as Mean±SD of five animals.

### 8.3. Motor activity and behavioural observations

No changes were observed in motor activity measurements of males and females compared to the recovery groups of both the sexes (Table 16a and b). Sensory reactivity measurements, grip strength and foot splay measurements were comparable to their respective control groups (Table 17a and b).

**Table 16a. Effect on motor activity of male rats after 28 days of the oral administration of OciBest™**

| Parameters | Control 0 mg/kg | 250 mg/kg | 500 mg/kg | 1000 mg/kg | Control Recovery Group 0 mg/kg | High Dose Reversible Group 1000 mg/kg |
|---|---|---|---|---|---|---|
| **DT(cm)** | 2580.2±1277.2 | 1876.0±821.9 | 1708.6±803.4 | 2430.8±1078.2 | 1344.8±519.08 | 1573.6±278.5 |
| **Rt (sec)** | 169.6±72.1 | 205.2±36.1 | 250.4±559 | 175.4±47.9 | 245.0±82.89 | 218.0±41.63 |
| **ST (sec)** | 114.4±20.18 | 133.4±42.3 | 126.2±29.4 | 109.4±20.2 | 134.6±25.42 | 128.6±25.5 |
| **AT(sec)** | 316.0±89.6 | 261.4±43.2 | 223.4±58.76 | 315.2±62.4 | 220.4±67.75 | 253.4±24.96 |
| **BSM** | 80.2±12.0 | 91.6±20.4 | 78.6±10.3 | 81.4±8.9 | 87.0±14.68 | 81.6±19.85 |
| **HC** | 1843.4±835.3 | 1435.2±292.4 | 1184.8±361.9 | 1804.8±592.4 | 1220.8±438.13 | 1335.4±153.2 |
| **AC** | 1306.2±762.7 | 924.8±251.03 | 698.8±264.3 | 1277.6±554.6 | 760.6±337.6 | 882.80±103.3 |
| **HB** | 0.8±0.4 | 1.4±0.8 9 | 2.6±4.1 | 3.0±5.05 | 0.8±0.447 | 1.8±1.09 |
| **CR** | 29.2±10.9 | 28.4±7.43 | 19.4±6.1 | 33.0±15.8 | 22.80±8.43 | 23.4±3.7 |
| **CCR** | 31.6±13.7 | 22.8±4.08 | 17.0±5.05 | 23.6±6.3 | 18.40±6.87 | 25.0±5.7 |

Data represented as Mean±SD of five animals. DT=Distance travelled (cm), RT=Resting time (sec), ST=Stereotypic time (sec), AT=Ambulatory time (sec), BSM= Burst of stereotypic movements, HC= Horizontal counts, AC=Ambulatory count, HB=Horizontal break, CR=Clockwise rotation and CCR=Counter clockwise rotation

**Table 16b. Effect on motor activity of female rats after 28 days of the oral administration of OciBest™**

| Parameters | Control 0 mg/kg | 250 mg/kg | 500 mg/kg | 1000 mg/kg | Control Recovery Group 0 mg/kg | High Dose Reversible Group 1000 mg/kg |
|---|---|---|---|---|---|---|
| **DT(cm)** | 1359.0±474.9 | 2509.2±1452.3 | 1570.2±1067.2 | 1978.6±453.9 | 899.0±450.2 | 1064.8±362.5 |
| **Rt (sec)** | 243.8±48.67 | 219.8±56.5 | 257.0±88.9 | 215.0±38.3 | 322.0±108.6 | 233.8±51.5 |
| **ST (sec)** | 154.8±41.00 | 121.6±63.6 | 128.0±25.1 | 122.0±48.9 | 130.4±50.35 | 184.2±30.9 |
| **AT(sec)** | 201.4±60.89 | 258.6±40.9 | 215.0±95.2 | 263.0±27.79 | 147.6±70.8 | 182.0±44.9 |
| **BSM** | 84.2±14.25 | 76.40±33.1 | 80.6±14.8 | 77.6±25.17 | 76.6±21.7 | 103.0±18.8 |
| **HC** | 1152.2±234.8 | 1476.8±416.7 | 1175.4±376.6 | 1474.4±231.7 | 866.8±414.8 | 1152.8±317.6 |
| **AC** | 652.0±184.4 | 968.2±373.7 | 709.8±257.6 | 976.0±179.09 | 500.6±266.6 | 631.0±225.3 |
| **HB** | 2.6±3.050 | 1.6±1.9 | 2.0±2.8 | 0.6±0.54 | 1.8±1.3 | 1.0±1.2 |
| **CR** | 15.8±8.899 | 24.4±6.5 | 18.6±8.0 | 25.4±5.9 | 12.2±3.49 | 16.0±4.2 |
| **CCR** | 20.2±3.421 | 25.6±9.07 | 19.8±9.03 | 22.2±1.9 | 15.2±9.09 | 13.2±6.05 |

Data represented as Mean±SD of five animals. DT=Distance travelled (cm), RT=Resting time (sec), ST=Stereotypic time (sec), AT=Ambulatory time (sec), BSM= Burst of stereotypic movements, HC= Horizontal counts, AC=Ambulatory count, HB=Horizontal break, CR=Clockwise rotation and CCR=Counter clockwise rotation

**Table 17a. Effect on Sensory reactivity of male rats after 28 days of the oral administration of OciBest™**

| | | Control 0 mg/kg | 250 mg/kg | 500 mg/kg | 1000 mg/kg | Control Recovery group 0 mg/kg | High Dose Reversible Group 1000 mg/kg |
|---|---|---|---|---|---|---|---|
| **Parameters** | Observations | No. of Animals Showing Observation | | | | | |
| **Approach response** | Fast | 1 | 2 | 3 | 2 | 2 | 3 |
| | Moderate | 4 | 3 | 2 | 3 | 3 | 2 |
| **Touch response** | Normal | 5 | 5 | 5 | 5 | 5 | 5 |
| **Click response** | Normal | 5 | 5 | 5 | 5 | 5 | 5 |
| **Pupil response** | Normal | 5 | 5 | 5 | 5 | 5 | 5 |
| **Tail pinch response** | Flinch | 5 | 5 | 5 | 5 | 5 | 5 |
| **Air righting reflex** | Normal | 5 | 5 | 5 | 5 | 5 | 5 |

**Table 17b. Effect on Sensory reactivity of female rats after 28 days of the oral administration of OciBest™**

| | | Control 0 mg/kg | 250 mg/kg | 500 mg/kg | 1000 mg/kg | Control Recovery group 0 mg/kg | High Dose Reversible Group 1000 mg/kg |
|---|---|---|---|---|---|---|---|
| **Parameters** | Observations | No. of Animals Showing Observation | | | | | |
| **Approach response** | Fast | 3 | 3 | 3 | 2 | 3 | 3 |
| | Moderate | 2 | 2 | 2 | 3 | 2 | 2 |
| **Touch response** | Normal | 5 | 5 | 5 | 5 | 5 | 5 |
| **Click response** | Normal | 5 | 5 | 5 | 5 | 5 | 5 |
| **Pupil response** | Normal | 5 | 5 | 5 | 5 | 5 | 5 |
| **Tail pinch response** | Flinch | 5 | 5 | 5 | 5 | 5 | 5 |
| **Air righting reflex** | Normal | 5 | 5 | 5 | 5 | 5 | 5 |

### 8.4. Clinical signs

No apparent treatment related clinical signs were observed in any of the animals throughout the treatment and recovery period (Table 18). Ophthalmological examination did not reveal any test item related changes in both the eyes of any of the experimental animals, at the end of treatment and recovery periods (Table 19).

**Table 18. Effect on Clinical Signs/ Symptoms of male and female rats after 28 days of the oral administration of OciBest™**

| | | | Sex: Male/Female | | | |
|---|---|---|---|---|---|---|
| **Group No.** | **Group** | **Dose (mg/kg bodywt)** | **No. of Animals/Group** | **Day of Observations** | **Clinical signs** | **Incidences** |
| **G1** | Control | 0 | 5 | 1-28 | Normal | 5/5 |
| **G2** | Low Dose | 250 | 5 | 1-28 | Normal | 5/5 |
| **G3** | Mid Dose | 500 | 5 | 1-28 | Normal | 5/5 |
| **G4** | High Dose | 1000 | 5 | 1-28 | Normal | 5/5 |
| **G5** | Control Recovery | 0 | 5 | 1-42 | Normal | 5/5 |
| **G6** | High Dose Recovery | 1000 | 5 | 1-42 | Normal | 5/5 |

**Table 19. Ophthalmological observations in male and female rats after 28 days of the oral administration of OciBest™**

| | | Control 0 mg/kg | 250 mg/kg | 500 mg/kg | 1000 mg/kg | Control Recovery group 0 mg/kg | High Dose Reversible Group 1000 mg/kg |
|---|---|---|---|---|---|---|---|
| **Pre-treatment** | | | | | | | |
| **Eye** | **Observations** | **M/F** | **M/F** | **M/F** | **M/F** | **M/F** | **M/F** |
| **Right** | *NAD | 5 | 5 | 5 | 5 | 5 | 5 |
| **Left** | NAD | 5 | 5 | 5 | 5 | 5 | 5 |
| **Post-Treatment** | | | | | | | |
| **Eye** | **Observations** | **M/F** | **M/F** | **M/F** | **M/F** | **M/F** | **M/F** |
| **Right** | NAD | 5 | 5 | 5 | 5 | 5 | 5 |
| **Left** | NAD | 5 | 5 | 5 | 5 | 5 | 5 |
| **Post-Recovery** | | | | | | | |
| **Eye** | **Observations** | **M/F** | **M/F** | **M/F** | **M/F** | **M/F** | **M/F** |
| **Right** | NAD | 5 | 5 | 5 | 5 | 5 | 5 |
| **Left** | NAD | 5 | 5 | 5 | 5 | 5 | 5 |

*NAD: No abnormality detected

### 8.5. Hematological studies

Hematological parameters like mean Hgb, WBC, RBC and differential cell counts were not significantly different in OciBest™ treated rats compared to control group (Table 20a and b). A significant increase in haemoglobin (Hb) was observed in males dosed with 1000 mg/kg/body weight of OciBest™ (Table 20a). A significant increase in mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) were observed in males and females dosed with 1000 and 500 mg/kg/body weight of OciBest™. No alterations were observed in recovery groups of animals in both the sexes. The minimal changes observed in the males dosed with 1000 mg/kg/body weight (Hb, MCH and MCHC) and in the females with 1000 and 500 mg/kg/body weight (MCH and MCHC) of OciBest™ were well compared with baseline data. (Males, Hb = 16.883 ± 0.605, MCH = 19.283 ± 0.608 and MCHC = 40.933 ± 0.344 and in females MCH = 20.967 ± 0.948 and MCHC = 45.667 ± 2.362).

**Table 20a. Effect on Hematological parameters of male rats after 28 days of the oral administration of OciBest™**

| Parameters | Control Group | OSE 250 mg/kg | OSE 500 mg/kg | OSE 1000 mg/kg | Control Recovery Group 0 mg/kg | High Dose Reversible Group 1000 mg/kg |
|---|---|---|---|---|---|---|
| **RBC x 106(µl)** | 7.9±0 .2 | 8.4±0.3 | 8.3±0.8 | 8 .3±0.6 | 8.4±0.4 | 8.4±0.9 |
| **HCT( %)** | 37.4±1.2 | 39.2±0.7 | 39.8±1.7 | 39.4±3.0 | 38.9±0.8 | 39.3±2.8 |
| **MCV (µm3)** | 46.8±1.1 | 46.3±0.8 | 48.1±3.2 | 47.4±1.6 | 46.2±1.9 | 46.5±1.8 |
| **Hgb (g/dl)** | 14.1±0.4 | 15.3±0.4 | 15.4±0.6 | 16.5*±1.5 | 15.2±0.2 | 15.9±1.7 |
| **MCH (pg)** | 17.7±0.3 | 18.1±0.4 | 18.6±1.3 | 19.8*±0.8 | 18.1±0.8 | 18.7±0.7 |
| **MCHC (g/dl)** | 37.8±0.5 | 39.1±0.4 | 38.7±1.1 | 41.8*±1.1 | 39.1±0.8 | 40.4±1.5 |
| **Platelet x$10^3$ (µl)** | 346.6±33.6 | 372.4±70.3 | 374.2±36.0 | 399.2±72.1 | 391.4±31.2 | 411. 2±51.8 |
| **WBC x $10^3$ (µl)** | 8.7±1.2 | 9.4±1.3 | 9.6±2.04 | 8.88±1.5 | 8.8±0.9 | 9.4±1.3 |
| **Neutrophil (%)** | 14.6±5.1 | 15.4±6.1 | 15.0±2.8 | 14.6±3.2 | 18.0±4.8 | 16.0±4.6 |
| **Lymphocyte (%)** | 82.8±5.0 | 82.8±5.2 | 81.2±3.3 | 83.0±4.1 | 78.6±6.4 | 81.8±5.1 |
| **Monocyte(%)** | 2.0±1.2 | 1.0±1.2 | 1.4±1.1 | 0±1.3 | 2.0±1.87 | 1.2±0.8 |
| **Eosinophil (%)** | 0.6±0.5 | 0.8±0.8 | 0.8±0.8 | 1.2±0.8 | 1.4±0.9 | 1.0±0.7 |
| **Basophil (%)** | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 1.2±0.0 | 0.0±0.0 | 0.0±0.0 |
| **Reticuloyte(%)** | 0.9±0.1 | 1.0±0.2 | 1.0±0.12 | 0.0±0.16 | 1.0±0.16 | 1.0±0.2 |
| **PT (sec.)** | 10.1±1.1 | 11.4±1.2 | 11.0±1.7 | 1.1±2.1 | 14.8±5.6 | 14.3±1.2 |
| **APTT (sec.)** | 10.15±1.7 | 9.8±1.1 | 9.0±1.4 | 12.1±1.0 | 23.9±5.5 | 19.7±3.6 |

Data represented as Mean±SD of five animals. *= Value increased significantly differs at 95% level of significance ($p < 0.05$).

**Table 20b. Effect on Hematological parameters of female rats after 28 days of the oral administration of OciBest™**

| Parameters | Control Group | OSE 250 mg/kg | OSE 500 mg/kg | OSE 1000 mg/kg | Control Recovery Group 0 mg/kg | High Dose Reversible Group 1000 mg/kg |
|---|---|---|---|---|---|---|
| **RBC x 106(µl)** | 8.1±0.4 | 7.6±0.4 | 8.2±0.2 | 7.8±0.2 | 7.8±0.2 | 7.9±0.3 |
| **HCT( %)** | 39.3±1.3 | 36.5±2.4 | 38.4±0.6 | 37.4±1.2 | 36.5±0.7 | 36.5±1.7 |
| **MCV (µm3)** | 48.3±1.0 | 48.3±1.37 | 47.1±1.1 | 47.6±1.3 | 46.9±1.2 | 46.4 ±1.1 |
| **Hgb (g/dl)** | 14.2±0.5 | 13.4±0.8 | 14.8±0.2 | 14.7±0.7 | 14.8±0.3 | 15.0±0.8 |
| **MCH (pg)** | 17.4±0.3 | 17.8±0.3 | 18.1*±0.4 | 18.7*±0.5 | 19.0±0.3 | 19.1±0.7 |
| **MCHC (g/dl)** | 36.1±0.2 | 36.8±0.8 | 38.6*±0.7 | 39.3*±0.7 | 40.6±0.6 | 41.2±0.6 |
| **Platelet x$10^3$ (µl)** | 348.0±63.8 | 343.0±82.2 | 365.8±32.6 | 399.4±37.6 | 349.0±53.5 | 375.0±36.3 |
| **WBC x $10^3$ (µl)** | 7.7±1.2 | 6.5±1.5 | 6.1±1.0 | 8.1±2.7 | 9.5±3.4 | 9.0±1.5 |
| **Neutrophil (%)** | 14.0±4.8 | 14.8±3.9 | 12.8±3.1 | 16.2±2.3 | 13.6±5.0 | 13.8±3.0 |
| **Lymphocyte (%)** | 83.2±5.6 | 84.2±4.4 | 85.4±2.7 | 82.4±2.3 | 85.0±5.8 | 83.8±3.0 |
| **Monocyte(%)** | 1.4±0.9 | 0.6±0.5 | 0.6±0.9 | 0.8±0.8 | 0.6±0.5 | 1.4±0.5 |
| **Eosinophil (%)** | 1.4±0.5 | 0.4±0.5 | 1.2±0.8 | 0.6±0.9 | 0.8±0.8 | 1.0±0.7 |
| **Basophil (%)** | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 |
| **Reticuloyte(%)** | 1.0±0.1 | 1.0±0.2 | 1.0±0.2 | 1.0±0.2 | 1.1±0.1 | 1.1±0.2 |
| **PT (sec.)** | 10.5±1.2 | 9.2±1.9 | 10.5±2.1 | 10.3±0.7 | 13.6±3.2 | 15.3±2.2 |
| **APTT (sec.)** | 9.4±1.6 | 9.9±1.0 | 9.2±1.2 | 9.9±0.9 | 18.1±0.8 | 17.0 ±1.0 |

Data represented as Mean±SD of five animals.

### 8.6. Clinical biochemistry

All clinical biochemistry parameters of animals of different treated groups of both the sexes were comparable to their respective control groups (Table 21a and b). Significant increase in cholesterol (CHO) was observed in males dosed with 500 mg/kg/body weight of OciBest™ and decrease in AST and Chloride (CL) was observed in females at a dose of 500 mg/kg/body weight. Moreover, compared to the control groups, the females showed a decrease in GGT at a dose of 500 and 1000mg/kg/body weight of OciBest™ (Table 21a and b). The changes observed in above clinical chemistry parameters were non dose dependant and biologically insignificant, hence considered as not related to test item administration. All other parameters in animals of different treated groups of both the sexes were comparable to their respective control groups.

**Table 21a. Effect on biochemical parameters of male rats after 28 days of the oral administration of OciBest™**

| Parameters | Control Group | OSE 250 mg/kg | OSE 500 mg/kg | OSE 1000 mg/kg | Control Recovery Group 0 mg/kg | High Dose Reversible Group 1000 mg/kg |
|---|---|---|---|---|---|---|
| **ALT (IU/L)** | 34.5±5.1 | 34.1±4.9 | 36.4±2.7 | 35.8±5.1 | 33.3±3.7 | 35.2±5.8 |
| **AST (IU/L)** | 125.9±17.1 | 124.4±15.9 | 143.9±11.4 | 138.9±20.8 | 142.6±12.2 | 154.9±38.5 |
| **ALP (IU/L)** | 448.7±76.6 | 533.1±161.8 | 481.4±23.4 | 540.7±162.1 | 487.3±171.6 | 465.8±178.2 |
| **ALB (g/dl)** | 3.5±0.1 | 3.5±0.2 | 3.5±0.3 | 3.6±0.1 | 3.7±0.2 | 3.7±0.1 |
| **BUN (mg/dl)** | 14.8±1.0 | 16.4±1.4 | 18.7±5.9 | 14.4±2.3 | 14.7±1.5 | 15.2±1.8 |
| **UREA (mg/dl)** | 31.6±2.1 | 35.2±3.0 | 40.1±12.8 | 30.8±4.9 | 31.6±3.1 | 32.5±3.9 |
| **Na (mmol/L)** | 139.2±1.6 | 137.2±1.7 | 138.6±1.9 | 140.6±0.8 | 142.6±2.1 | 140.2±1.5 |
| **K (mmol/L)** | 4.4±0.2 | 4.4±0.2 | 4.7±0.4 | 4.8±0.4 | 4.8±0.2 | 4.7±0.4 |
| **Cl (mmol/L)** | 95.8±1.5 | 96.5±1.2 | 95.5±1.8 | 96.9±0.6 | 99.3±2.7 | 97.4±1.3 |
| **CAL (mg/dl)** | 10.6±0.2 | 10.5±0.3 | 10.7±0.5 | 10.8±0.7 | 9.9±0.7 | 10.7±1.2 |
| **CRE (mg/dl)** | 0.6±0.0 | 0.6±0.1 | 0.6±0.0 | 0.53±0.1 | 0.5±0.0 | 0.5±0.0 |
| **CHO(mg/dl)** | 52.3±5.0 | 50.2±6.7 | 64.3*±3.0 | 51.0±8.3 | 46.5±10.5 | 46.1±5.6 |
| **GGT (IU/L)** | 9.5±1.4 | 9.4±0.7 | 15.3±15.3 | 8.8±1.1 | 5.9±0.6 | 6.2±1.1 |
| **GLB (g/dl)** | 2.9±0.1 | 3.0±0.4 | 3.2±0.5 | 3.0±0.3 | 3.2±0.2 | 3.1±0.2 |
| **T.PRO (g/dl)** | 6.4±0.1 | 6.5±0.4 | 6.7±0.3 | 6.6±0.3 | 7.0±0.3 | 6.7±0.1 |
| **A:G** | 1.2±0.1 | 1.2±0.2 | 2.0±0.2 | 1.2±0.1 | 1.1±0.1 | 1.2±0.1 |
| **PHOS (mg/dl)** | 6.3±0.3 | 6.7±0.7 | 6.8±0.4 | 6.8±0.4 | 6.4±0.2 | 6.3±0.8 |
| **GLU (mg/dl)** | 81.8±19.2 | 82.9±5.1 | 83.4±6.4 | 79.9±5.4 | 89.1±12.3 | 88.0±6.2 |
| **T. BIL (mg/dl)** | 0.1±0.0 | 0.1±0.0 | 0.1±0.0 | 0.1±0.0 | 0.1±0.0 | 0.1±0.0 |
| **TGIR (mg/dl)** | 68.0±23.5 | 75.4±15.0 | 78.7±26.8 | 75.2±17.6 | 72.1±21.7 | 69.9±22.1 |

Data given as Mean±SD of five animals. *= Value increased significantly differ at 95% level of significance ($p < 0.05$).

**Table 21b. Effect on biochemical parameters of female rats after 28 days of the oral administration of OciBest™**

| Parameters | Control Group | OSE 250 mg/kg | OSE 500 mg/kg | OSE 1000 mg/kg | Control Recovery Group 0 mg/kg | High Dose Reversible Group 1000 mg/kg |
|---|---|---|---|---|---|---|
| **ALT (IU/L)** | 32.6±4.8 | 26.2±3.4 | 26.5±1.7 | 27.6±5.2 | 33.9±4.4 | 34.4±3.7 |
| **AST (IU/L)** | 143.2±31.0 | 109.5±23.4 | 102.3*±12.7 | 107.8±19.5 | 138.1±23.7 | 113.8±18.1 |
| **ALP (IU/L)** | 323.3±58.8 | 282.8±68.1 | 297.5±94.4 | 314.9±65.7 | 366.1±178.0 | 507.6±159.5 |
| **ALB (g/dl)** | 3.6±0.1 | 3.6±0.3 | 3.7±0.1 | 3.7±0.2 | 3.8±0.2 | 3.7±0.0 |
| **BUN (mg/dl)** | 18.0±0.5 | 17.5±2.6 | 18.4±3.3 | 17.1±0.7 | 20.6±2.6 | 20.7±3.1 |
| **UREA (mg/dl)** | 38.5±1.0 | 37.5±5.5 | 39.4±7.0 | 37.1±2.2 | 44.2±5.7 | 44.3±6.7 |
| **Na (mmol/L)** | 137.5±1.6 | 138.9±5.1 | 137.7±3.4 | 137.2±0.9 | 137.2±1.3 | 136.7±0.7 |
| **K (mmol/L)** | 4.3±0.4 | 4.6±0.5 | 4.1±0.4 | 4.4±0.3 | 4.6±0.4 | 4.7±0.2 |
| **Cl (mmol/L)** | 97.1±0.7 | 97.0±4.2 | 90.5*±2.8 | 92.7±1.7 | 96.2±1.9 | 95.8±1.0 |
| **CAL (mg/dl)** | 10.3±0.2 | 10.4±0.3 | 10.5±0.4 | 10.9±1.5 | 10.5±0.3 | 10.7±0.2 |
| **CRE (mg/dl)** | 0.7±0.1 | 0.6±0.0 | 0.6±0.0 | 0.5±0.1 | 0.5±0.0 | 0.5±0.0 |
| **CHO(mg/dl)** | 51.5±10.8 | 45.7±6.0 | 58.4±6.6 | 52.8±11.4 | 60.6±9.2 | 70.2±5.9 |
| **GGT (IU/L)** | 9.4±0.8 | 9.1±0.4 | 8.2*±0.3 | 7.5*±0.8 | 13.1±1.2 | 12.6±0.7 |
| **GLB (g/dl)** | 3.0±0.1 | 3.0±0.4 | 3.2±0.3 | 3.1±0.1 | 3.1±0.1 | 3.1±0.1 |
| **T.PRO (g/dl)** | 6.6±0.2 | 6.6±0.3 | 6.9±0.2 | 6.8±0.2 | 6.8±0.2 | 6.8±0.1 |
| **A:G** | 1.2±0.0 | 1.2±0.3 | 1.2±0.1 | 1.2±0.1 | 1.2±0.1 | 1.2±0.1 |
| **PHOS (mg/dl)** | 5.8±0.7 | 5.4±0.4 | 5.6±0.2 | 5.8±1.0 | 5.2±0.4 | 4.8±0.6 |
| **GLU (mg/dl)** | 97.5±7.6 | 112.1*±11.8 | 86.4±11.3 | 89.9±6.7 | 125.9±19.2 | 27.1±11.2 |
| **T. BIL (mg/dl)** | 0.2±0.0 | 0.2±0.0 | 0.2±0.0 | 0.2±0.0 | 0.2±0.1 | 0.2±0.0 |
| **TGIR (mg/dl)** | 45.2±9.7 | 38.2±8.3 | 42.3±7.9 | 48.8±12.3 | 37.5±4.4 | 36.5±5.6 |

Data given as Mean±SD of five animals.* = Value increased significantly differ at 95% level of significance ($p < 0.05$)

## 8.7. Urinalysis

The urine analysis revealed no adverse effects in any mice of either sex compared to the vehicle control group in the 28-day study. Urine parameters such as appearance, blood, nitrate, leukocyte, glucose, ketone, pH, protein and specific gravity did not show any significant difference among all the experimental animals of both the sexes and were comparable to animals of control group (Table 22a and b). Urine samples showed yellow and pale yellow colours and clear appearance in all the groups, which were comparable with the controls. Urine microscopic parameters in test groups of both the sexes were comparable with the control values. Urine microscopic observations showed presence of epithelial cells, red cells, pus cells, granular and epithelial casts, triple phosphate and calcium oxalate crystals occasional to few in animals of both the sexes (Table 23a and b).

**Table 22a. Effect on urine parameters of male rats after 28 days of the oral administration of OciBest™**

| Parameters | Control Group | OSE 250 mg/kg | OSE 500 mg/kg | OSE 1000 mg/kg | Control Recovery Group 0 mg/kg | High Dose Reversible Group 1000 mg/kg |
|---|---|---|---|---|---|---|
| **Volume** | 21.4±13.0 | 21.4±8.0 | 19.2±5.5 | 21.20±11.9 | 19.8±18.4 | 26.4±5.9 |
| **Blood** | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 |
| **Bilirubin** | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 |
| **Urobilinogen** | 0.1±0.0 | 0.1±0.0 | 0.1±0.0 | 0.1±0.0 | 0.1±0.0 | 0.1±0.0 |
| **Ketones** | 1.0±2.2 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 1.0±2.2 | 0.0±0.0 |
| **Protein** | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 2.0±4.5 | 0.0±0.0 | 0.0±0.0 |
| **Nitrite +ve** | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0± 0.0 | 1.0± 0.0 | 0.0± 0.0 |
| **-ve** | 5.0± 0.0 | 5.0± 0.0 | 5.0± 0.0 | 5.0± 0.0 | 4.0± 0.0 | 5.0 ± 0.0 |
| **Glucose** | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 |
| **pH** | 6.3±0.3 | 6.1±0.0 | 6.1±0.5 | 6.4±0.4 | 6.4±0.2 | 6.5±0.0 |
| **Sp. Gravity** | 1.0±0.0 | 1.0±6.2 | 1.0±0.0 | 1.0±0.0 | 1.0±0.0 | 1.0±0.0 |
| **Leucocytes** | 4.0±5.5 | 4.0±1.0 | 0.0±0.0 | 4.0±5.5 | 8.0±4.5 | 8.0±4.5 |

Data given as Mean±SD of five animals.

**Table 22b. Effect on urine parameters of female rats after 28 days of the oral administration of OciBest™**

| Parameters | Control 0 mg/kg | 250 mg/kg | 500 mg/kg | 1000 mg/kg | Control Recovery group 0 mg/kg | High Dose Reversible Group 1000 mg/kg |
|---|---|---|---|---|---|---|
| **Volume** | 16.8±5.76 | 25.40±13.85 | 16.8±7.29 | 21.40±7.40 | 13.20±7.19 | 15.30±4.60 |
| **Blood** | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 |
| **Bilirubin** | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 |
| **Urobilinogen** | 0.10±0.00 | 0.10±0.00 | 0.10±0.00 | 0.10±0.00 | 0.10±0.00 | 0.10±0.00 |
| **Ketones** | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 |
| **PrOSEin** | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 6.00±13.42 | 0.00± 0.00 |
| **Nitrite +ve** | 2.0± 0.00 | 2.0±0.00 | 2.0± 0.00 | 1.00± 0.00 | 1.00± 0.00 | 0.0± 0.00 |
| **-ve** | 3.00±0.00 | 3.0± 0.00 | 3.0± 0.00 | 4.00±0.00 | 4.00± 0.00 | 5.0±0.00 |
| **Glucose** | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 |
| **pH** | 6.2±0.27 | 6.14±0.65 | 6.4±0.82 | 6.30±1.04 | 7.50±1.17 | 6.70±0.45 |
| **Sp. Gravity** | 1.02±0.00 | 1.02±0.00 | 1.02±0.01 | 1.02±0.00 | 1.02±0.01 | 1.02±0.00 |
| **Leucocytes** | 2.00±4.47 | 0.00±0.00 | 4.00±5.48 | 2.00±4.47 | 6.00±5.48 | 4.00±5.48 |

Data given as Mean±SD of five animals.

**Table 23a. Effect on Urine microscopic parameters of male rats after 28 days of the oral administration of OciBest™**

| Parameters | | Control 0 mg/kg | 250 mg/kg | 500 mg/kg | 1000 mg/kg | Control Recovery Group 0 mg/kg | High Dose Reversible Group 1000 mg/kg |
|---|---|---|---|---|---|---|---|
| **Microscopic Observations** | | | | | | | |
| **Epithelial** | Absent | 0 | 0 | 0 | 0 | 0 | 0 |
| | 0-2 | 5 | 4 | 4 | 5 | 5 | 5 |
| | 3-6 | 0 | 1 | 1 | 0 | 0 | 0 |
| **Pus cells /h.p.f.** | Absent | 4 | 4 | 4 | 5 | 2 | 3 |
| | 0-2 | 1 | 1 | 1 | 0 | 3 | 2 |
| | 3-6 | 0 | 0 | 0 | 0 | 0 | 0 |
| **RBCs /h.p.f.** | Absent | 5 | 4 | 5 | 3 | 5 | 5 |
| | 0-2 | 0 | 1 | 0 | 2 | 0 | 0 |
| | 3-6 | 0 | 0 | 0 | 0 | 0 | 0 |
| **Casts /h.p.f.** | Absent | 0 | 1 | 2 | 1 | 0 | 0 |
| | Granular (0-2) | 2 | 0 | 2 | 1 | 0 | 2 |
| | Granular (3-6) | 0 | 0 | 0 | 0 | 0 | 0 |
| | Epithelial (0-2) | 3 | 4 | 1 | 3 | 5 | 3 |
| | Epithelial (3-6) | 0 | 0 | 0 | 0 | 0 | 0 |
| **Crystals/h.p.f.** | Absent | 0 | 0 | 0 | 0 | 0 | 0 |
| | Triple Phosphate (Occassional) | 2 | 2 | 3 | 0 | 1 | 0 |
| | Triple Phosphate (FEW) | 3 | 3 | 2 | 2 | 4 | 4 |
| | Triple Phosphate (MANY) | 0 | 0 | 0 | 3 | 0 | 0 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | Calcium Oxalate (OCC) | 0 | 0 | 0 | 0 | 0 | 1 |
| | Calcium Oxalate (FEW) | 0 | 0 | 1 | 1 | 0 | 1 |
| **Sperms/h.p.f.** | Absent | 4 | 4 | 3 | 5 | 4 | 4 |
| | OCC | 1 | 1 | 2 | 0 | 1 | 1 |
| | FEW | 0 | 0 | 0 | 0 | 0 | 0 |
| **Physical Observations** | | | | | | | |
| **Colour** | Yellow | 5 | 5 | 5 | 5 | 3 | 4 |
| | Pale Yellow | 0 | 0 | 0 | 0 | 2 | 1 |
| | Brown | 0 | 0 | 0 | 0 | 0 | 0 |
| **Appearance** | Clear | 5 | 5 | 5 | 5 | 5 | 5 |
| | Turbid | 0 | 0 | 0 | 0 | 0 | 0 |
| | Hazy | 0 | 0 | 0 | 0 | 0 | 0 |

**Table 23b. Urine microscopic observations in female rats treated with OciBest™**

| **Parameters** | | **Control 0 mg/kg** | **250 mg/kg** | **500 mg/kg** | **1000 mg/kg** | **Control Recovery group 0 mg/kg** | **High Dose Reversible Group 1000 mg/kg** |
|---|---|---|---|---|---|---|---|
| **Microscopic Observations** | | | | | | | |
| **Epithelial** | Absent | 0 | 0 | 0 | 0 | 0 | 0 |
| | 0-2 | 4 | 4 | 4 | 4 | 5 | 4 |
| | 3-6 | 1 | 1 | 1 | 1 | 0 | 1 |
| **Pus cells /h.p.f.** | Absent | 5 | 4 | 4 | 4 | 4 | 5 |
| | 0-2 | 0 | 1 | 1 | 1 | 1 | 0 |
| | 3-6 | 0 | 0 | 0 | 0 | 0 | 0 |
| **RBCs /h.p.f.** | Absent | 5 | 4 | 3 | 4 | 5 | 5 |
| | 0-2 | 0 | 1 | 2 | 1 | 0 | 0 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | 3-6 | 0 | 0 | 0 | 0 | 0 | 0 |
| **Casts /h.p.f.** | Absent | 0 | 0 | 0 | 0 | 2 | 0 |
| | Granular (0-2) | 0 | 0 | 2 | 0 | 1 | 1 |
| | Granular (3-6) | 1 | 1 | 1 | 1 | 0 | 0 |
| | Epithelial (0-2) | 0 | 0 | 0 | 0 | 2 | 4 |
| | Epithelial (3-6) | 4 | 5 | 2 | 4 | 0 | 0 |
| **Crystals/h.p.f.** | Absent | 0 | 0 | 0 | 0 | 0 | 0 |
| | Triple Phosphate (Occassional) | 0 | 0 | 0 | 0 | 2 | 2 |
| | Triple Phosphate (FEW) | 1 | 3 | 1 | 2 | 3 | 3 |
| | Triple Phosphate (MANY) | 3 | 2 | 2 | 3 | 0 | 0 |
| | Calcium Oxalate (OCC) | 0 | 0 | 0 | 0 | 1 | 0 |
| | Calcium Oxalate (FEW) | 1 | 1 | 1 | 0 | 0 | 0 |
| **Sperms/h.p.f.** | Absent | 0 | 1 | 1 | 0 | 5 | 5 |
| | OCC | 5 | 5 | 5 | 5 | 0 | 0 |
| | FEW | 0 | 0 | 0 | 0 | 0 | 0 |
| **Physical Observations** | | | | | | | |
| **Colour** | Yellow | 3 | 2 | 2 | 1 | 5 | 5 |
| | Pale Yellow | 2 | 3 | 3 | 4 | 0 | 0 |
| | Brown | 0 | 0 | 0 | 0 | 0 | 0 |
| **Appearance** | Clear | 5 | 5 | 5 | 5 | 5 | 5 |
| | Turbid | 0 | 0 | 0 | 0 | 0 | 0 |
| | Hazy | 0 | 0 | 0 | 0 | 0 | 0 |

### 8.8 Absolute, relative organ weight and histology

The organs like liver, kidney, heart, spleen, brain, epididymides, uterus and testis or ovary isolated in various groups did not reveal any abnormalities in their gross examinations and difference in their absolute (g) (Table 24a and b) and relative weights (%) (Table 25a and b) both in treated and control groups. In recovery groups, absolute and relative weights of all organs from treated animals were comparable to control group. However, a significant increase was observed in absolute organ weight (g) of adrenal from females dosed with 250 and 1000mg/kg/body weight of OciBest™ and in the relative organ weight (%) of adrenal from females dosed with 250 mg/kg/body weight of OciBest™.

**Table 24a. Effect on Absolute Organ Weight (g) of male rats after 28 days of the oral administration of OciBest™**

| Parameters | Control Group | OSE 250 mg/kg | OSE 500 mg/kg | OSE 1000 mg/kg | Control Recovery Group 0 mg/kg | High Dose Reversible Group 1000 mg/kg |
|---|---|---|---|---|---|---|
| **Body weight** | 224.6±47.0 | 227.6±37.3 | 235.4±17.4 | 230.8±26.7 | 272.8±44.5 | 281.0±27.8 |
| **Brain** | 1.8±0.3 | 1.8±0.1 | 1.9±0.0 | 0.2±0.1 | 1.9±0.1 | 1.8±0.1 |
| **Adrenals** | 0.0±0.0 | 0.1±0.0 | 0.1±0.0 | 0.1±0.0 | 0.1±0.0 | 0.1±0.0 |
| **Prostate/S.V** | 1.0±0.3 | 1.1±0.4 | 0.8±0.2 | 1.0±0.3 | 1.5±0.2 | 1.3±0.2 |
| **Testes** | 2.8±0.3 | 2.8±0.4 | 2.6±0.5 | 2.8±0.2 | 3.1±0.4 | 3.1±0.2 |
| **Epididymides** | 0.8±0.3 | 0.7±0.2 | 0.7±0.2 | 0.8±0.1 | 1.1±0.1 | 1.0±0.1 |
| **Heart** | 0.8±0.1 | 0.9±0.1 | 0.9±0.1 | 0.9±0.1 | 1.0±0.1 | 1.0±0.2 |
| **Liver** | 8.5±2.1 | 9.6±2.3 | 8.0±1.8 | 8.8±1.9 | 10.2±3.0 | 10.3±1.5 |
| **Kidneys** | 2.0±0.4 | 2.1±0.5 | 2.0±0.3 | 2.1±0.4 | 2.3±0.4 | 2.4±0.3 |
| **Spleen** | 0.4±0.1 | 0.4±0.1 | 0.5±0.1 | 0.5±0.1 | 0.5±0.1 | 0.6±0.1 |
| **Thymus** | 0.3±0.1 | 0.3±0.1 | 0.3±0.1 | 0.3±0.1 | 0.2±0.1 | 0.3±0.1 |

Data given as Mean±SD of five animals. S.V. = Seminal Vesicle

**Table 24b. Effect on Absolute Organ Weight (g) of female rats after 28 days of the oral administration of OciBest™**

| Parameters | Control Group | OSE 250 mg/kg | OSE 500 mg/kg | OSE 1000 mg/kg | Control Recovery Group 0 mg/kg | High Dose Reversible Group 1000 mg/kg |
|---|---|---|---|---|---|---|
| **Body weight** | 165.8±8.9 | 160.4±18.0 | 174.2±19.3 | 174.4±13.4 | 191.6±22.6 | 206.4±23.4 |
| **Brain** | 1.6±0.1 | 1.7±0.1 | 1.7±0.2 | 1.7±0.0 | 1.8±0.1 | 1.8±0.0 |
| **Adrenals** | 0.1±0.0 | 0.1±0.0 | 0.1±0.0 | 0.1±0.0 | 0.1±0.0 | 0.1±0.0 |
| **ovaries** | 0.2±0.0 | 0.2±0.0 | 0.1±0.0 | 0.2±0.0 | 0.2±0.0 | 0.2±0.1 |
| **Uterus** | 0.5±0.1 | 0.7±0.3 | 0.5±0.0 | 0.8±0.2 | 0.5±0.1 | 0.7±0.2 |
| **Heart** | 0.6±0.0 | 0.7±0.1 | 0.7±0.1 | 0.7±0.1 | 0.7±0.1 | 0.8±0.0 |
| **Liver** | 5.7±0.6 | 6.3±1.0 | 6.3±1.1 | 6.4±0.3 | 6.7±1.2 | 7.5±1.1 |
| **Kidneys** | 1.4±0.1 | 1.5±0.2 | 1.4±0.2 | 1.6±0.1 | 1.5±0.2 | 1.1±0.2 |
| **Spleen** | 0.3±0.0 | 0.4±0.1 | 0.3±0.1 | 0.3±0.1 | 0.4±0.0 | 0.4±0.1 |
| **Thymus** | 0.3±0.0 | 0.3±0.1 | 0.3±0.1 | 0.3±0.1 | 0.3±0.0 | 0.3±0.1 |

Data given as Mean±SD of five animals. S.V.= Seminal Vesicle and * = Value significantly differ at 95% level of significance ($p < 0.05$).

**Table 25a.Effect on Relative Organ Weight of male rats after 28 days of the oral administration of OciBest™**

| Parameters | Control 0 mg/kg | 250 mg/kg | 500 mg/kg | 1000 mg/kg | Control Recovery Group 0 mg/kg | High Dose Reversible Group 1000 mg/kg |
|---|---|---|---|---|---|---|
| **Brain** | 0.8±0.1 | 0.8±0.1 | 0.8±0.0 | 0.7±0.1 | 0.7±0.1 | 0.7±0.1 |
| **Adrenals** | 0.3±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 |
| **Prostate/S.V** | 57.1±12.4 | 58.8±19.5 | 40.0±8.8 | 59.4±13.2 | 0.5±0.1 | 0.5±0.1 |
| **Testes** | 1.3±0.2 | 1.2±0.15 | 1.1±0.2 | 1.2±0.1 | 1.1±0.1 | 1.1±0.1 |
| **Epididymidis** | 0.3±0.1 | 0.3±0.1 | 0.3±0.1 | 0.3±0.0 | 0.34±0.0 | 0.3±0.1 |
| **Heart** | 0.4±0.1 | 0.4±0.0 | 0.3±0.0 | 0.4±0.0 | 0.4±0.0 | 0.3±0.1 |
| **Liver** | 3.8±0.3 | 4.2±0.5 | 3.4±0.6 | 3.9±0.9 | 0.4±0.4 | 3.7±0.5 |
| **Kidneys** | 0.9±0.1 | 0.9±0.1 | 0.8±0.1 | 0.9±0.1 | 0.9±0.0 | 0.8±0.1 |
| **Spleen** | 0.2±0.0 | 0.2±0.0 | 0.2±0.0 | 0.2±0.0 | 0.2±0.0 | 0.2±0.0 |
| **Thymus** | 0.1±0.0 | 0.1±0.0 | 0.2±0.0 | 0.1±0.0 | 0.1±0.0 | 0.1±0.1 |

Data given as Mean±SD of five animals. S.V= Seminal Vesicle

**Table 25b.Effect on Relative Organ Weight of female rats after 28 days of the oral administration of OciBest™**

| Parameters | Control 0 mg/kg | 250 mg/kg | 500 mg/kg | 1000 mg/kg | Control Recovery Group 0 mg/kg | High Dose Reversible Group 1000 mg/kg |
|---|---|---|---|---|---|---|
| **Brain** | 1.0±0.2 | 1.1±0.153 | 1.0±0.1 | 1.0±0.0 | 1.0±0.1 | 0.6±0.1 |
| **Adrenals** | 0.0±0.0 | 0.1*±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 |
| **Ovaries** | 0.1±0.0 | 0.1±0.0 | 0.1±0.0 | 0.1±0.0 | 0.0±0.0 | - |
| **Uterus** | 0.3±0.1 | 0.4±0.2 | 0.3±0.0 | 0.4±0.2 | 0.3±0.0 | - |
| **Epididymidis** | - | - | - | - | - | 0.4±0.0 |
| **Heart** | 0.4±0.0 | 0.4±0.1 | 0.4±0.0 | 0.4±0.0 | 0.4±0.0 | 0.4±0.0 |
| **Liver** | 3.4±0.2 | 4.0±0.5 | 0.3±0.6 | 3.7±0.2 | 3.5±0.3 | 4.0±0.5 |
| **Kidneys** | 0.8±0.0 | 0.9±0.2 | 0.8±0.0 | 0.9±0.1 | 0.8±0.0 | 0.8±0.1 |
| **Spleen** | 0.2±0.0 | 0.2±0.1 | 0.2±0.0 | 0.2±0.0 | 0.2±0.0 | 0.2±0.0 |
| **Thymus** | 0.2±0.0 | 0.2±0.1 | 0.2±0.1 | 0.2±0.0 | 0.2±0.0 | 0.1±0.0 |

Data given as Mean±SD of five animals. S.V= Seminal Vesicle and * = Value increased significantly differs at 95% level of significance ($p < 0.05$).

## 8.9 Histopathological Findings

Histopathological examination revealed minimal to moderate mononuclear cell infiltration in the liver, trachea and lung. In lung, mild peri-vascular MNC infiltration was observed in 3 of 5 male and 1 of 5 female rats of the control groups. At a higher dose of 1000 mg/kg, 1 of 5 male and 2 of 5 female rats showed mild peri-vascular MNC infiltration. Mild peri-bronchiolar MNC infiltration was observed in 1 of 5 male and 2 of 5 male rats dosed with 0 and 1000 mg/kg of OciBest$^{TM}$, respectively. 1 of 5 female and male rats of control group showed severe peri-bronchiolar MNC infiltration and moderate poly-morphonuclear cell infiltration was observed in 1 of 5 male rats dosed with 500 mg/kg of OciBest$^{TM}$. In trachea, 1 of 5 female rats of the control group and 1 of 5 male and female rats dosed with 1000 mg/kg of OciBest$^{TM}$ showed minimal MNC infiltration where as in liver, minimal MNC infiltration and moderate MNC infiltration was observed in 1 of 5 male rats of the control group (Table 26a and b).

**Table 26a. Effect on Histopathological parameters of male rats treated with OciBest™**

| | | | Control 0 mg/kg | 250 mg/kg | 500 mg/kg | 1000 mg/kg |
|---|---|---|---|---|---|---|
| **Name of organ** | **Lesion & Severity Grade** | | **No. of animals showing lesion** | | | |
| **Lung** | **PV MNC infiltration** | + | - | - | - | - |
| | | **1+** | 3 | - | - | 1 |
| | | **2+** | - | - | - | - |
| | **PB MNC infiltration** | + | - | - | - | - |
| | | **1+** | 1 | - | - | 2 |
| | | **3+** | - | - | - | - |
| | **PMNC infiltration** | **2+** | - | - | 1 | - |
| | | **3+** | - | - | 1 | - |
| **Trachea** | **MNC infiltration** | + | - | - | - | 1 |
| | | **1+** | - | - | - | - |
| | | **2+** | - | - | - | - |
| **Liver** | **MNC infiltration** | + | 1 | - | - | - |
| | | **1+** | 1 | - | - | - |

Organs with abnormality alone are counted whereas normal organs not considered in above table.

+= Minimal, 1+=Mild, 2+ =Moderate, 3+ = Severe, N= Number of animals in the group, MNC = Mononuclear Cell, PMNC= Poly-morphonuclear Cell, PV = Perivascular and PB = Peribrochial.

**Table 26b. Effect on Histopathological parameters of female rats treated with OciBest™**

| | | | Control 0 mg/kg | 250 mg/kg | 500 mg/kg | 1000 mg/kg |
|---|---|---|---|---|---|---|
| **Name of organ** | **Lesion & Severity Grade** | | **No. of animals showing lesion** | | | |
| **Lung** | **PV MNC infiltration** | **+** | - | - | - | 1 |
| | | **1+** | 1 | - | - | 2 |
| | | **2+** | - | - | - | - |
| | **PB MNC infiltration** | **+** | - | - | - | - |
| | | **1+** | - | - | - | - |
| | | **3+** | 1 | - | - | - |
| | **PMNC infiltration** | **2+** | - | - | - | - |
| | | **3+** | - | - | - | - |
| **Trachea** | **MNC infiltration** | **+** | 1 | - | - | 1 |
| | | **1+** | - | - | - | - |
| | | **2+** | - | - | - | - |
| **Liver** | **MNC infiltration** | **+** | - | - | - | - |
| | | **1+** | - | - | - | - |

Organs with abnormality alone are counted whereas normal organs not considered in above table.

+= Minimal, 1+=Mild, 2+ =Moderate, 3+ = Severe, N= Number of animals in the group, MNC = Mononuclear Cell, PMNC= Poly-morphonuclear Cell, PV = Perivascular and PB = Peribrochial.

# SECTION-9

# DISCUSSION

## 9. Discussion

In the present study, we compared the anti-inflammatory activity of aqueous and methanolic extracts of *C. cassia*, *C. zeylanicum, O. basilicum* and *O. sanctum* in RAW264.7, SW1353 and human primary chondrocytes to correlate their efficacy in terms of management of OA.

We found that in LPS activated RAW264.7 cells, the methanolic extracts of cinnamon ($CC_M$, $CZ_M$) attenuated NO release more significantly than aqueous extracts ($CC_W$, $CZ_W$). In an earlier study, it had been reported that the water extract of CC could not inhibit LPS-induced NO production in RAW 264.7 cells at 100 µg/ml concentration (Ho and Tsai, 2004). Interestingly, we found that at 100 µg/ml dose, $CC_W$ significantly inhibited LPS-induced NO production in RAW 264.7 cells. The difference in these results could be attributed to the method of preparation of the extracts, source variation, time of collection of the material and so on that may affect the presence of phytoactives in the extract, which contribute to their biological activity. It was further observed that the methanolic extracts ($CC_M$ and $CZ_M$) effectively decreased $PGE_2$ production in RAW264.7, SW1353 and human primary chondrocytes compared to the aqueous extracts. However, $CZ_M$ was found to be more effective than CC in reducing $PGE_2$ production. The water extract of CC was earlier shown to decrease $PGE_2$ production by almost 34% at 100 µg/ml concentration in RAW 264.7 cells, (Ho and Tsai, 2004) whereas our study showed almost 80% reduction in $PGE_2$ production at the same concentration of the extract. Moreover, we have analysed the effect of the extracts on $PGE_2$ production in SWI353 and primary chondrocytes as well. OA cartilage is known to spontaneously release more $PGE_2$ than the normal cartilage (Goldring and Berenbaum, 2004; Dave *et al*., 2000). Thus, blocking of $PGE_2$ production by cinnamon in OA could be a promising strategy in preventing cartilage degradation and chondrocyte apoptosis. In SW1353, the methanolic extracts of CC and CZ, reduced LTB4 levels more effectively than the aqueous extracts. In human primary chondrocytes, $CC_W$

and $CC_M$ induced an enhanced decrease in LTB4 levels that decreased below the basal values. Since LTB4 is involved in a number of important cellular processes in the body (Afonso *et al.,* 2012), its downregulation below the basal level may lead to severe complications (Monteiro *et al.,* 2011; Crooks and Stockley, 1988; Sala *et al.,* 1988). However, $CZ_M$ effectively reduced LTB4 levels than $CC_W$. Thus, CZ seems to be better option than CC in terms of LTB4 inhibition as it did not reduce LTB4 below the basal levels. In human primary chondrocytes, the methanolic extracts of cinnamon ($CC_M$ and $CZ_M$) effectively reduced the levels of MMPs 2, 9 and 13 compared to the aqueous extracts ($CC_W$, $CZ_W$). **Thus, based on the above results, the methanolic extracts ($CC_M$, $CZ_M$) of both the varieties of cinnamon were found to be more effective than the aqueous extracts in terms of $PGE_2$, LTB4 and MMP inhibition. However, on comparing the two species of cinnamon, CZ was found to be more effective than CC and thus could be considered for its potential therapeutic application in the management of inflammatory conditions associated with OA.**

In case of *O. basilicum*, $OB_W$ decreased NO and iNOS levels more significantly than $OB_M$. The production of NO through iNOS pathway is regulated at transcriptional and posttranscriptional level (Aktan, 2004). Thus the aqueous extract of *O. basilicum* could be explored for alleviation of NO to reduce inflammation associated with osteoarthritis. The anti-inflammatory activity of $OB_W$ and $OB_M$ was further compared by analyzing their effect on the expression of COX-2 and NFkB proteins in RAW264.7. Compared to LPS-treated control cells, there was a significant dose-dependent decrease in COX-2 and total NFkB expression in both $OB_M$ and $OB_W$ treated cells. Overexpression of NFkB has been shown to play an important role in the pathogenesis of OA. It regulates the expression of pro-inflammatory enzymes, including iNOS and COX-2 (Marcu *et al.,* 2010). Various phytochemicals have been shown to inhibit COX-2 expression through blocking of NFκB activation (Salminen *et al.,* 2012). Since $OB_W$ reduced NFκB more significantly than $OB_M$, it has a good potential in the

management of OA related inflammation.We further compared the effect of $OB_W$ and $OB_M$ on $PGE_2$ levels, involved in cartilage degradation and chondrocyte apoptosis (Futani *et al.,* 2002). $OB_W$ was more effective than $OB_M$ in reducing $PGE_2$ levels. OA cartilage spontaneously releases more $PGE_2$ than the normal cartilage (Sun, 2010). Thus, regulation of $PGE_2$ production by $OB_W$ could be a promising strategy in preventing cartilage degradation. $OB_W$ and $OB_M$ were further compared for their potential to modulate IL-1β induced LTB4 production in SW1353 and chondrocytes. It is important to note that in chondrocytes, $OB_M$ decreased LTB4 levels below the basal values that may otherwise lead to severe complications and hence needs careful evaluation. Thus, $OB_W$ could be a better therapeutic option for modulating the pathophysiological conditions associated with OA. We further compared the effect of $OB_W$ and $OB_M$ on IL-1β induced MMP production in chondrocytes. Interestingly, in chondrocytes, $OB_W$ induced significant decrease in the production of MMPs. Thus, by modulating the expression of these MMPs by $OB_W$, the continued degradation of articular cartilage could be prevented. **All these data suggested that compared to the methanolic extract, the aqueous extract of *O. basilicum* could be explored for its potential applications in the management of inflammatory conditions in OA.**

In our study, we have also compared the efficacy of aqueous ($OS_W$) and methanolic ($OS_M$) extracts of *O. sanctum* in modulating the expression of proinflammatory molecules in the management of OA. Both $OS_W$ and $OS_M$ effectively modulated IL-1β induced $PGE_2$ and LTB4 levels, compared to the positive control, *B. serrata*. Owing to the contribution of LTB4 in the pathogenesis of many inflammatory processes, it represents an important target for therapeutic regulation (Pingping *et al.,* 2015; Chmiel *et al.,* 2002). We observed highly significant inhibition of $PGE_2$ and LTB4, which is possibly one of the mechanisms of anti-inflammatory actions of $OS_W$ and $OS_M$. The results were compared to that of *Boswellia serrata*, a well known drug for the management of OA. Interestingly, *O. sanctum* showed more

significant decrease in $PGE_2$ levels in human chondrosarcoma cell line SW1353 compared to the positive control *B. serrata*.

**As all the tested herbals cannot be taken further, we focused our further studies with *O. sanctum* (OS). Moreover, OS has strong traditional support, is easily availabile and exhibited potent anti-inflammatory activity. So it was selected for future in vitro and *in vivo* studies.**

The literature survey revealed that *O. sanctum* contained chemical constituents which are responsible for its varied pharmacological and medicinal properties (Jamal, 2011; Rahman *et al*., 2011; Temimi *et al*., 2015). Thus, in our further studies we mixed the aqueous and methanolic extracts of *O. sanctum* in different proportions, to find out whether the mixture would further decrease the expression of pro-inflammatory molecules at lower concentrations. Three different preparations/LOTs of *O. sanctum* were prepared (LOT001, LOT002 and LOT02).

Compared to other LOTs, LOT02 showed a significant decrease in $PGE_2$ and $LTB_4$ levels at lower concentrations. Interestingly, LOT02 decreased the expression of IL-1β, TNF-α and COX-2 proteins in SW1353 significantly compared to IL-1β stimulated control cells. Inflammatory cytokines such as IL-1β and TNF-α, secreted from inflamed synovium, are major mediators of disturbed chondrocyte function and cartilage degeneration (Wojdasiewicz *et al.,* 2014). These inflammatory cytokines are also involved in up-regulation of iNOS and cyclo-oxygenase-2 (COX-2) in chondrocytes and lead to the release of nitric oxide (NO) and PGE2. LOT02 not only reduced the expression of IL-1β, TNF-α and COX-2, but also reduced the intracellular nitric oxide levels. Thus, LOT02 could be used as an anti-inflammatory agent for chondrocyte protection.

LOT02 was further evaluated for its anti inflammatory effect on human primary chondrocytes. It significantly reduced IL-1β induced $PGE_2$ and $LTB_4$ levels. Moreover, in cell viability assay, we found that compared to IL-1β stimulated control cells, LOT02 induced proliferation of cells, thereby indicating that it could help in maintaining chondrocyte homeostasis.

It was found to reduce the hyaluronidase activity and proteoglycan loss from the chondrocytes. Hyaluronidase are family of enzymes that degrade hyaluronan or hyaluronic acid (HA), critical component of the extracellular matrix of articular cartilage (Unterman *et al.,* 2012). HA also serves as a scaffold for the aggregation of cartilage proteoglycan and facilitates the anchorage of these proteoglycan aggregates to the chondrocyte cell surface (Unterman *et al.,* 2012). **All these results suggested that LOT02 modulated the inflammatory markers involved in cartilage degradation and could be used as a chondroprotective agent for the management of OA.**

**Since LOT02 showed interesting results in the cell lines and primary chondrocytes, we further evaluated its safety in subacute toxicity study in Wistar rats.** The clinical use of herbal drugs without any standard dosage together with lack of adequate scientific evidence has raised concerns regarding their toxicity status. The assessment of safety and toxicity of herbal medicines should be done before their human consumption. Thus, toxicity evaluation of herbal medicines is carried out in various experimental animal models to predict their safety and for selecting a safe dose for future human use (Akbarsha *et al.,* 1998; Sethi *et al.,* 2010; Pragya *et al.,* 2012). *O. sanctum*, is being used as medicinal herb for thousands of years without any known adverse effects. There are many reports highlighting the efficacy and safety of *O. sanctum*, but limited scientific reports have been published about its safety. Recently, in acute oral toxicity, LOT02 (now called as OciBest$^{TM}$) was found to be safe up to a high dose of 5 g/kg and did not show any abnormal symptoms or toxic effect in the treated rats (Chandrasekaran *et al.,* 2013).

Subacute toxicity study in Wistar rats did not show any change in clinical, ophthalmological and haematological parameters with 1000 mg/kg OciBest$^{TM}$, when administered for 28 days. Ophthalmological examination did not reveal any test item related changes in both the eyes of any of the experimental animals. The hematological system is an important indicator of

the physiological and pathological status of animals or humans (Etim NseAbasi, 2014). It is highly sensitive to toxic compounds and small changes in the hematological system could have higher predictive value for drug associated toxicity (Etim NseAbasi, 2014). All the haematological parameters in animals of different treated groups of both the sexes were comparable to their respective control groups. No alterations were observed in recovery groups of animals in both the sexes. Moreover, the rats under high dose reversible group did not show any significant difference in haematological parameters. Subacute exposure of rat to mid and high doses of the OciBest$^{TM}$ produced small and transient changes in some hematological parameters. The changes observed were biologically insignificant and could not be considered to test item administration.

Clinical biochemistry is mainly performed to evaluate the effect of drugs on hepatic and renal functions as well as on glucose and total cholesterol levels. Liver is the major site for metabolism including drugs and is the site of cholesterol synthesis and degradation. The transaminases, aspartate aminotransferase (AST), and alanine amino transferase (ALT) are the known enzymes which play an important role in liver function and are used as biomarkers for predicting possible toxicity (Burtis *et al.,* 2008). Any damage to the parenchymal liver cells results in elevations of these transaminases in the blood. The administration of OciBest$^{TM}$ to the treated groups did not show any changes in the biochemical parameters and were comparable to their respective control groups. Moreover, both AST and ALT did not show any treatment related increase even at 1000 mg/kg dose compared to the control group. However, some changes in GGT AST and CL parameters were observed at the mid and high doses of OciBest$^{TM}$ in the female rates. Since these changes were not dose dependant, they were biologically insignificant and hence considered not to be related to test item administration.

Blood urea and creatinine are important markers of renal toxicity. There are several reports of kidney toxicity related to the use of

phytotherapeutic drugs since kidneys eliminate many drugs and their metabolites (Perrone *et al.,* 1992). Our data showed that OciBest™ did not produce any harmful effect on the kidney functions of the treated rats. Urine parameters did not show any significant difference among all the experimental animals of both the sexes and were comparable to animals of control group.

Organ weight is an important index of physiological and pathological status of animals. OciBest™ did not show any harmful effect on the absolute and relative organ weights of treated rats of either sex. However, an increase in absolute organ weight (g) of adrenal in females treated with low and high dose of OciBest™ was observed. However, the absence of any gross and microscopic findings revealed that the change in the weight of adrenal was not because of the tested drug. OciBest™did not induce any pathological changes in heart, brain, thymus, spleen and reproductive organs such as testis/uterus and epididymis/ovaries of the mice. Mild consolidation of lungs was observed internally. However, histopathological analysis of lungs did not reveal any microscopic changes and was considered incidental / spontaneous in nature, which could not be attributed to the treatment with OciBest™. Thus, OciBest™ (LOT02), could be considered to be non-toxic and safe for its future clinical applications.

# SECTION-10

# SUMMARY

## 10. Summary

We have compared the anti-inflammatory activity of aqueous and methanolic extracts of *C.cassia* (CC), *C.zeylanicum* (CZ), *O. basilicum* (OB) and *O. sanctum* (OS) in mouse macrophage (RAW264.7), human chondrosarcoma (SW1353) cells and human primary chondrocytes to correlate their efficacy in terms of management of osteoarthritis (OA).

The methanolic extracts ($CC_M$, $CZ_M$) of both the varieties (*C. cassia* and *C. zeylanicum*) of cinnamon were found to be more effective than the aqueous extracts in terms of $PGE_2$, LTB4 and MMP inhibition.

In RAW264.7, the aqueous extract of *O. basilicum* ($OB_W$) decreased NO and $PGE_2$ production more effectively compared to the methanolic extract of *O. basilicum* ($OB_M$)**.** Our results are in line with the earlier reports suggesting the potential of methanolic extract of the whole plant of *O. basilicum* to decrease NO and iNOS mRNA expression in RAW264.7 (Selvakkumar *et al*. 2007). Interestingly, decrease in NO was accompanied by a corresponding decrease in iNOS protein expression. $OB_W$ decreased total NFκB and COX-2 proteins significantly, compared to $OB_M$. Similarly, in SW1353 and chondrocytes, $OB_w$ decreased $PGE_2$ and LTB4 production appreciably, compared to $OB_M$. In chondrocytes, $OB_W$ reduced the production of MMP-2, MMP-9 and MMP-13 significantly, than $OB_M$.

In SW1353, the aqueous ($OS_W$) and methanolic ($OS_M$) extracts of *O. sanctum* were found to be equally effective in terms of $PGE_2$ and LTB4 inhibition. Interestingly, both the extracts of *O. sanctum* induced more significant decrease in $PGE_2$ levels in human chondrosarcoma cell line SW1353 compared to the positive control *B. serrata* ($BS_W$). Later on, different combinations (LOT001, LOT002 and LOT02) of *O. sanctum* methanolic and water extracts were made, out of which LOT02 showed a significant decrease in $PGE_2$ and $LTB_4$ levels at lower concentrations. LOT02 significantly decreased the expressions of IL-1β, TNF-α and COX-2 proteins in SW1353 cells compared to IL-1β stimulated control cells. In

chondrocytes, LOT02 significantly reduced the hyaluronidase activity and proteoglycan release, thereby indicating that it exhibited chondroprotective activity. Since all the extacts could not be taken further, so *O. sanctum* LOT02, was taken for *in vivo* studies.

The rats treated with LOT02 (OciBest$^{TM}$) did not show any change in body weight, food and water consumption, motor activity, sensory reactivity and foot splay measurements. There were no significant changes in hematological, pathological and biochemical parameters; and histopathology of tissues (liver, kidney, spleen, heart, and testis/ovary) among rats of either sex. At a dose of 1000 mg/kg/body weight of LOT02, a significant increase in mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) was observed in the male and female rats, which was comparable to their respective male and female control groups. Urine parameters (appearance, blood, nitrate, leukocyte, glucose, ketone, pH, protein and specific gravity) in both the male and female rats were comparable to their respective controls. In addition, no changes were observed in the vital organs of rats at macroscopic and microscopic levels. Our results showed that oral administration of OciBest$^{TM}$ was not toxic to the male and female Wistar rats upto the highest dose tested, thereby suggesting its clinical usefulness in future.

# SECTION-11

# CONCLUSIONS

## 11. Conclusions

- ✓ In the present work, the anti-inflammatory potential of aqueous and methanolic extracts of four different medicinal plants, such as *Cinnamomum cassia, C. zeylanicum, Ocimum sanctum and O. basilicum* was evaluated on RAW264.7, SW1353 and chondrocytes.
- ✓ The aqueous and methanolic extracts of *C. cassia, C. zeylanicum* and *O. basilicum*, were non-toxic to Raw264.7, SW1353 as well as human chondrocytes at 0.1, 1, 10 and 100 µg/ml doses.
- ✓ The aqueous and methanolic extracts of *O. sanctum* were non-toxic to Raw264.7, SW1353 as well as human chondrocytes at 0.1, 1 and 10 µg/ml doses.
- ✓ The methanolic extracts (CCM, CZM) of both the varieties of cinnamon were found to be more effective than the aqueous extracts in terms of $PGE_2$, LTB4 and MMP inhibition in the tested cell lines. However, on comparing the two species of cinnamon, CZ was found to be more potent than CC.
- ✓ As compared to methanolic extract, the aqueous extract of *O.basilicum* significantly modulated NO, iNOS, $PGE_2$, LTB4 and MMP levels in RAW264.7, SW1353 and human primary chondrocytes.
- ✓ *O. sanctum* methanolic and water extracts were found to be equally effective in reducing $PGE_2$ and LTB4 levels. So, *O. sanctum* aqueous and methanolic extracts were mixed in different ratios as 1:1 (LOT001), 1:2 (LOT002) and 1:4 (LOT02).
- ✓ LOT02 showed a significant decrease in $PGE_2$ and $LTB_4$ levels at lower concentrations. LOT02 significantly decreased the expression of IL-1β, TNF-α and COX-2 proteins in SW1353 cells.
- ✓ In chondrocytes, LOT02 significantly reduced the hyaluronidase and proteoglycan activity, thereby indicating its chondroprotective potential.
- ✓ In the sub-acute toxicity study, LOT02 named as OciBest$^{TM}$ showed no adverse effects in the experimental animals upto 1000 mg/kg dose.

# SECTION-12

# LIMITATIONS OF THE STUDY

## 12. Limitations of the study

- The study needs to be performed in osteoarthritis model [complete Freund's Adjuvant (CFA) induced OA model] so as to find out whether OciBest$^{TM}$ could prevent the degradation of cartilage during OA.

- In future, clinical studies are required for its acceptance as a drug in the management of Osteoarthritis.

# SECTION-13

# FUTURE PROSPECTS

## 13. Future Prospects

The drug OCIBest$^{TM}$ developed from the above study could be tested in clinical trials in OA patients. This would further validate our experimental studies and would translate our finding from bench to bedside

# SECTION-14

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# SECTION-15

# STUDENTS PROFILE

## 15. Students Profile

**Research publications emanated from the project:**

- Kaul-Ghanekar R, **Raina P**. (2012) "Potential of Nutraceuticals and Medicinal Plants in the Management of Osteoarthritis" Acta Biologica Indica 1(1):27-46.

- **Raina P**, Chandrasekara CV, Aggarwal A, Wagh N, Kaul-Ghanekar R. (2015) "Comparative analysis of anti-inflammatory activity of aqueous and methanolic extracts of *C. cassia* and *C. zeylanicum* in RAW264.7, SW1353 and human primary chondrocytes" International journal of Pharmacy and Pharmaceutical Sciences (Accepted; I.F 0.55).

- **Raina P**, Chandrasekaran CV, Deepak M, Aggarwal A and Kaul-Ghanekar R. (2015) "Evaluation of subacute toxicity of methanolic/aqueous preparation of aerial parts of O.sanctum in Wistar rats: clinical, hematological, biochemical and histopathological studies" Journal of Ethanopharmacology (Accepted; I.F 2.9).

**Communicated manuscripts:**

- **Raina P**, Chandrasekara CV, Deepak M, Aggarwal A, Wagh N, Kaul-Ghanekar R. Comparative analysis of anti-inflammatory activity of aqueous and methanolic extracts of *Ocimum basilicum* in RAW264.7, SW1353 and human primary chondrocytes.

**Manuscripts Under preparation:**

- **Raina P**, Chandrasekara CV, Deepak M, Aggarwal A, Wagh N, Kaul-Ghanekar R. Evaluation of anti-inflammatory potential of *Ocimum sanctum* in SW1353 and human primary chondrocytes.

**Other publications**

- Choudhari AS, **Raina P**, Deshpande M, Zawar A, Bodhankar S, Kaul-Ghanekar R. (2013) "Evaluating the anti-inflammatory potential of *Tectaria cicutarium* L. root extracts in vitro as well as *in vivo*" Journal of Ethanopharmacology 150: 215-222.

- Suryavanshi S, Kadam KS, **Raina P**, Nimbargi R, Pandit VA, Kaul-Ghanekar R. (2015) "Evaluation of acute and sub-acute toxicity of a standardized polyherbal formulation (HC9): an *in vivo* study". International journal of Pharmacy and Pharmaceutical Sciences (Accepted; I.F 0.55).

- **Book chapters in Taylor and Francis publications Ltd., a book on Gynaecological disorders.**

- Kaul-Ghanekar R, Suryavanshi S, **Raina P (2012).** Early detection biomarkers in breast cancer.ISSN: 978-1-46-658428-0.

- Kaul-Ghanekar R, Suryavanshi S, **Raina P (2012).** Non-invasive biomarkers in breast cancer. ISSN: 978-1-46-658428-0.