EVALUATION OF EFFECTIVE HERBS AND FORMULATIONS FOR THE MANAGEMENT OF OSTEOARTHRITIS

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BY

MISS. PALLAVI SURYABHAN NIRMAL

UNDER THE GUIDANCE

OF

DR. SURESH D. JAGTAP

BHARATI VIDYAPEETH DEEMED UNIVERSITY
INTERACTIVE RESEARCH SCHOOL FOR HEALTH AFFAIRS (IRSHA)
PUNE, MAHARASHTRA, INDIA

JULY 2016

CERTIFICATE

This is to certify that the work incorporated in the thesis entitled **"Evaluation of effective herbs and formulations for the management of osteoarthritis"** for the degree of **'Doctor of Philosophy'** in the subject of Biotechnology under the faculty of Science has been carried out by **Miss. Pallavi Suryabhan Nirmal** in the Department of Herbal Medicine at Bharati Vidyapeeth Deemed University's Interactive Research School for Health Affairs (IRSHA), Pune during the period from August 2010 to July 2016, under the guidance of Dr. Suresh D. Jagtap.

Place: Pune
Date:

Dr. A. C. Mishra
Director

CERTIFICATION OF GUIDE

This is to certify that the work incorporated in the thesis entitled **"Evaluation of effective herbs and formulations for the management of osteoarthritis"** Submitted by **Miss. Pallavi Suryabhan Nirmal** for the degree of **'Doctor of Philosophy'** in the subject of Biotechnology under the faculty of Science has been carried out in the Department of Herbal Medicine, Bharati Vidyapeeth Deemed University's Interactive Research School for Health Affairs (IRSHA), Pune during the period from August 2010 to July 2016, under my direct guidance.

Place: Pune
Date:

Dr. Suresh D. Jagtap
Research guide

DECLARATION BY THE CANDIDATE

I hereby declare that the thesis entitled **"Evaluation of effective herbs and formulations 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 Science is piece of work carried out by me **under the supervision of Dr. Suresh D. Jagtap**. 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 inadvertently.

Place: Pune
Date:

Miss. Pallavi Suryabhan Nirmal
Research Student

……..This work is dedicated to my late father

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List of Abbreviations

Abbreviation	Full form
A	Assamese
AN	*Acacia nilotica*
B	Bengali
b. wt.	Bodyweight
BC	*Bombax ceiba*
bFGF	Basic fibroblast growth factor
BS	*Boswellia serrata*
CIOA	Collagenase induced osteoarthritis
cm, mts	Centimeter, meters
COX	Cyclooxygenase
DA	*Dashsamoolarishta*
E	English
ECM	Extracellular matrix
g	Gravity
G	Gujarati
GAG	Glycosaminoglycan
Gl	Gwalior
Gm, mg, µg. kg	Gram, milligram, microgram, Kilogram
H	Hindi
HC	Healthy control
IL	Interleukin
K	Kannada
Kk	Konkani
KV	Kilovolt
l, ml, µl	Litre, Milli litre, Micro litre
M	Malayalam
MA	Milliampere
Md	Mundari
min	Minute
Ml	Malayalam
Mm, µM	Millimolar, Micromolar
MMP	Matrix metalloproteinase

Mn	Manipuri
Mr	Marathi
NO	Nitric oxide
NSAID	Nonsteroidal anti-inflammatory drug
O	Oriya
OA	Osteoarthritis
P	Punjabi
p. o.	Per orally
PGE	Prostaglandin E
PL	*Piper longum*
PZ	Combination of *P. longum* and *Z. officinale*
RC	*Ricinus communis*
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RT	Room temperature
S	Sanskrit
SC	*Sida cordifolia*
SM	Synovial membrane
SP	Combination of *S. cordifolia* and *P. longum*
SPZ	Combination of *S. cordifolia*, *P. longum* and *Z. officinale*
Syn	Synonym
SZ	Combination of *S. cordifolia* and *Z. officinale*
T	Tamil
TC	*Triphala churna*
TG	'*Triphala Guggul*'
TIMP	Tissue inhibitors of metalloproteinase
Tl	Telugu
TNF-α	Tumor necrosis factor alpha
TT	*Tribulus terrestris*
U	Urdu
VEFG	Vascular endothelial growth factor
VN	*Vitex negundo*
ZO	*Zingiber officinale*

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Chapter 1
Introduction

1.1 Osteoarthritis (OA): Introduction and Prevalence

The word osteoarthritis (OA) is derived from Greek words; *osteon*, means bone, *arthron*, means joint and the suffix *-itis* means inflammation (Aigner and Schmitz, 2011). American College of Rheumatology defined OA as a "Heterogeneous group of conditions that leads to joint symptoms and signs which are associated with defective integrity of articular cartilage, in addition to related changes in the underlying bone at the joint margins". Until 1980s, OA was considered to be primarily a degenerative disorder and a natural occurrence of "wear-and-tear" on joints as a result of aging. The metabolically active role of the disease and the process of remodeling and repair of damaged tissue has changed thinking to the point that it is now possible to arrest the progress of disease or even reverse it. While, previously it was believed that OA involved only the joint, research has shown the disorder is one of the whole joint organs (Singh, 2010). Structural changes occur in subchondral bone and synovium, but the site of most prominent structural alterations is the cartilage. There is breakdown of the cartilage, fibrosis and hypertrophy of the synovial tissue and degeneration of menisci. Bone changes include osteophyte formation, bone remodeling and sclerosis of the subchondral bone (Pearle et al., 2005; Aigner and Schmitz, 2011; Charlie, 2013) (Fig. 1, source: Charlie, 2013).

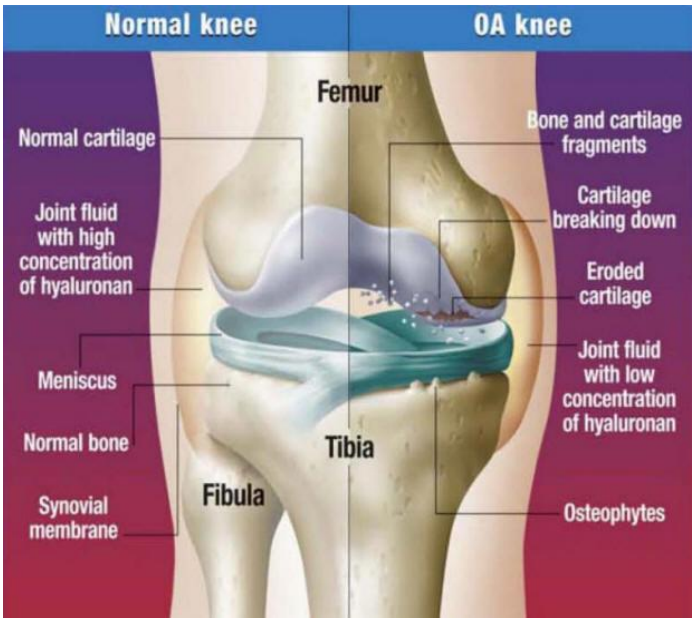

Figure 1: Cross sectional picture of healthy and osteoarthritic joint.

In current study, we have focused on knee OA, which is one of the most prevalent, commonly occurring progressive and chronic degenerative diseases amongst the elderly population in the world (Pelletier et al., 1999; Peat et al., 2001). With ageing and increasing obesity of the population, a growing incidence of OA is anticipated in the near future. OA is a complex and painful disease of the whole joint that reflects the imbalance between catabolic and anabolic mechanism of various tissues (Zhang et al., 2013). Typical radiolographical changes observed in OA are joint space narrowing, sclerosis of subchondral bone, and formation of cyst & osteophyte. Limitation of movement, crepitus, Joint pain, occasional effusion, tenderness and variable degrees of inflammation are clinical features of OA (Pelletier et al., 1999; Ishiguro et al., 2002).

OA affects about 3.6% of the global population (Guipu et al., 2014). However, the prevalence of OA in Indian population is 22–39% (Mahajan et al., 2005; Mujapara and Jarullah, 2009). The incidence of OA is higher in women than in men (Ishiguro et al., 2002) and worldwide estimates are that 9.6% of men and 18.0% of women aged 60 years and over have symptomatic OA (Woolf and Pfleger, 2003). For radiological knee OA these estimates are even higher, at a younger age also (45 years and over) prevalence is 14.1% for men and 22.8% for women (Schiphof, 2011). According to some population surveys, evidence of radiographic knee OA increases up to 80% for adults over the age of 65 (Neustadt, 2006). The World Health Organization (WHO) has estimated that 10% of the world's population over 60 years old suffers from OA; 80% of people with OA experience limitation of movement and 25% cannot perform major daily activities (Aaron and Racine, 2013). In the United States alone, more than 3, 50, 000 knee and hip replacements are performed each year (Arden and Nevitt, 2006). In 2011 there were 3, 06, 600 knee replacements performed in the U.S. (The health America report). This incidence of OA is on rise and is estimated to affect more than 25% of the adult U.S. population by 2030 (Hootman and Helmick, 2006). OA is a major cause of joint pain and disability throughout the world. It causes difficulty and discomfort in performing day to day activities and it is considered to be the 4th leading cause of "years lived with disability" (Symmons et al., 2006). As a result of mechanical and biological events in which inflammation causes joint destruction and inflammatory joint pain, ultimately leading to limited function or immobility of the joint (Ishiguro et al., 2002; Hougee,

2008; Schiphof, 2011). Due to severity and irreversibility of OA, it imposes a global burden of around 1 - 2.5% of total gross national products (Xie et al., 2008), thus it has become major and serious health and wellness concern worldwide. Imposing a tremendous social, clinical and economic burden with respect to socioeconomic factors, that reflects the current lack of effective medical therapies (Flannery, 2009).

1.2 Cartilage

Articular cartilage covers the surface of movable bones where they form joints. Its main function is to bear and absorb compressive load in joints (Hougee, 2008). Macroscopically, hyaline cartilage is white to yellowish, soft, unruffled overlay coat of the articulating joint surface (Aigner and Schmitz, 2011). Microscopically, cartilage consists of a collagen (90-95%), collagen fibrills and proteoglycan-rich hydrated extracellular matrix (ECM) with small number of cartilage cells, "chondrocytes" (Roach and Tilley, 2007; Hougee, 2008; Aigner and Schmitz, 2011). The major constituent (70–80%) of cartilage is water (Roach and Tilley, 2007). The principal component of cartilage is collagen type II, a fibrillar collagen that forms a meshwork of fibers within which proteoglycans, predominantly aggrecan molecules are located (Ishiguro et al., 2002; Roach and Tilley, 2007; Sutton et al., 2009). Proteoglycans consist of a negative charge and hence attract positive ions (Na+, K+). Because of the difference in osmotic balance within the cartilage, water is attracted towards it. Proteoglycans contains core protein in which glycosaminoglycans (GAG) chains are attached covalently (Ishiguro et al., 2002). The ability of the cartilage to stand compressive forces is aided by the sulfated GAGs such as chondroitin sulfate (Sutton et al., 2009). Altogether, this gives the cartilage its shock absorbing capacity (Hougee, 2008). The Synovial fluid functions as a source of nutrition for chondrocytes, it minimize friction during movement and also acts as a lubricant (Hougee, 2008). ECM is the functional element of the cartilage, its biochemical composition decides the mechanical properties of articular cartilage (Roach and Tilley, 2007; Aigner and Schmitz, 2011). These ECM constituents are bound together by glycoproteins such as cartilage oligomeric matrix protein (Sutton et al., 2009). However, it is the chondrocytes that are the "active players" in cartilage as well as the architects and designers of the matrix (Roach and Tilley, 2007; Mobasheri and Henrotin, 2011).

Cartilage is build up during development by chondrocytes which represents less than 5% of the total volume of cartilage but they are important for the maintenance of the cartilage (Hougee, 2008; Aigner and Schmitz, 2011). Chondrocytes are mostly surrounded by a specialized pericellular matrix that forms a biomechanical and biochemical interface between the rigid inter territorial matrix and the cells (Aigner and Schmitz, 2011). There is a constant but slow turnover of cartilage matrix in which chondrocyes produce cartilage matrix components which are important for maintaining high tensile strength as well as low compressibility under load, as well as matrix metalloproteinases (MMPs) that are proteolytic enzymes which breakdown collagens and proteoglycans (Ishiguro et al., 2002; Aigner and Schmitz, 2011). The changes of cartilage surface at various stages of OA can be observed and graded radiographically as well as at histologic level (Aigner and Schmitz, 2011). As cartilage is avascular, aneural and alymphatic; therefore, it cannot be the primary region for inflammation and pain (Hougee, 2008; Mobasheri and Henrotin, 2011).

1.3 Synovial joint and the Synovium

Synovial joints consist of five different tissue types i.e. bone, cartilage, synovial membrane, synovial fluid and tensile tissues such as ligament and tendon (Sutton et al., 2009; Mobasheri and Henrotin, 2011). Synovial joints also known as diarthroses. The joint contains synovial cells “Synoviocytes” and the space within it is filled with the lubricating synovial fluid called “joint cavity” (Sutton et al., 2009; Mobasheri and Henrotin, 2011). The synovial membrane (SM) is a specialized collagenous tissue that lines the joint. SM contains two layers, a lining layer directly next to the joint space “the synovial intima” and a supportive layer the ‘‘sub synovial’’ tissue. Synovium surface is folded with the villi. Synoviocytes are arranged loosely over three to four layers and do not lie on a basement membrane. There are three main types of synoviocytes, namely macrophages, fibroblasts and dendritic cells. The first two of these are most common and are known as ‘‘type A’’ and ‘‘type B’’, respectively. Macrophages possesses phagocytic activity. They contain lysosomes and a large golgi complex. Synovial fibroblasts produce collagens, fibronectin and hyaluronan for the synovial fluid. The SM secretes synovial fluid to lubricate and nourish the joint as well as removing unwanted substances (Sutton

et al., 2009). Beneath the surface of the SM, is a network of capillaries important for gas and nutrient exchange and the development of synovial inflammation (synovitis). Synovium traps synovial fluid and osmotically active load resistant molecules within its cavity (Mobasheri and Henrotin, 2011). Synovitis in OA is considered to be a secondary effect which results from the release of debris from the damaged cartilage (Aigner and Schmitz, 2011; Mobasheri and Henrotin, 2011). While rheumatoid arthritis is considered to originate from a synovial inflammatory autoimmune reaction with secondary cartilage destruction. In rheumatoid arthritis synoviocyte cells are secrete MMPs as well as catabolic cytokines (e.g., IL-1, TNF-α etc.), growth factors (VEGF, bFGF) and inducing inflammatory signaling pathways. Osteoarthritic synovium produces increased amounts of reactive oxygen species (ROS). Thus, the synovial reaction is of major importance to the symptoms of OA and also plays role in its progression (Aigner and Schmitz, 2011).

1.4 Pathophysiology of OA

Mechanism involved behind OA pathology is not clear, because etiology of the disease is multifactorial and overlapping pathophysiologic process affects the entire joint that is articular cartilage, subchondral bone, SM, menisci, ligaments, muscles etc. (Lambert et al., 2014). Many hypotheses regarding pathogenesis of OA have been proposed over the time, now molecular biology is a hope towards more targeted treatment of OA. The OA etiology includes mechanical, biochemical as well as genetic factors (Hougee, 2008). Normally, OA is divided into three broad stages. Stage I is the proteolytic breakdown of ECM. In stage II there is fibrillation of the cartilage along with the formation of osteophytes and breakdown products of the cartilage are released into the synovial fluid. During stage III, synovitis begins when synoviocytes phagocytose these products and produce inflammatory mediators like MMPs, which reaches to the chondrocytes in the cartilage matrix through the synovial fluid (Hougee, 2008; Henrotin et al., 2014) (Fig. 2, source: Henrotin et al., 2014). Synoviocytes as well as chondrocytes and infiltrating leukocytes, are the main cell types involved in the pathogenesis of OA (Sutton, 2009). Chondrocytes contribute to the inflammatory process by producing considerable amounts of inflammatory mediators (Amin et al., 1997; Attur et al., 1998;

Lee et al., 2013) and thereby enhancing the joint inflammation leading to pain, further degradation of cartilage and in the end to immobility of the joints (Hougee, 2008).

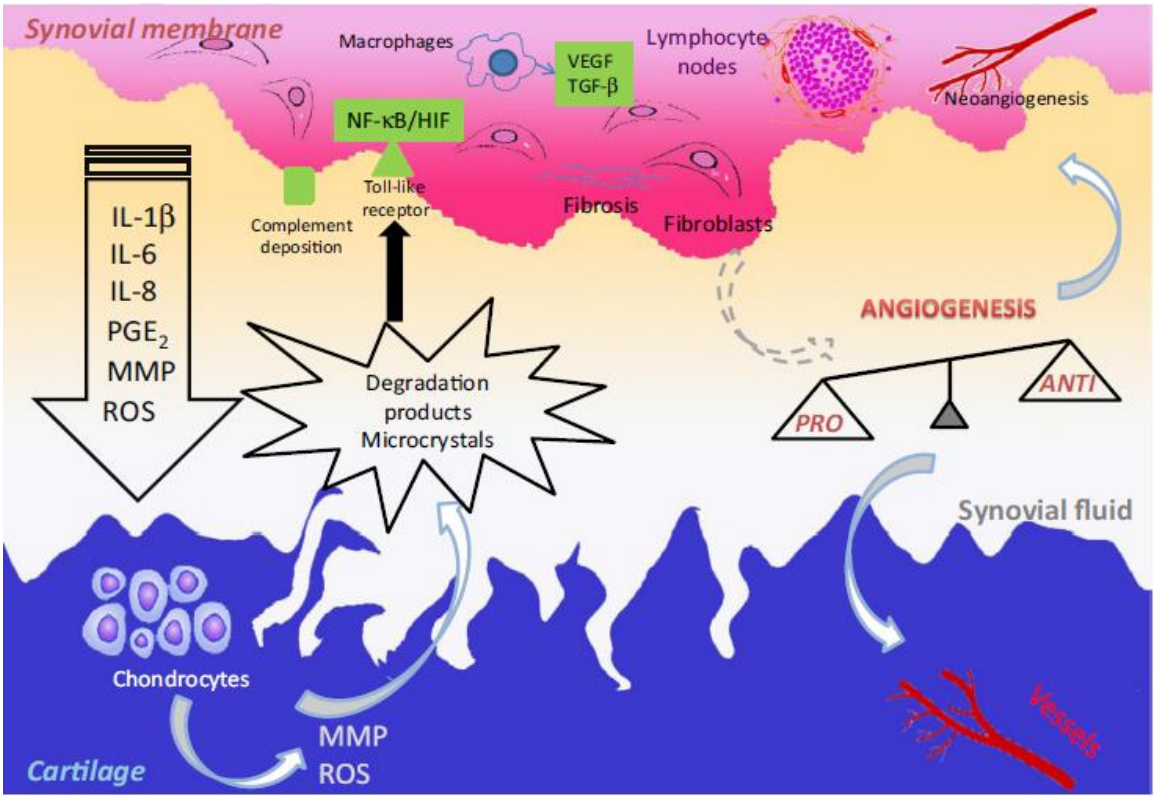

Figure 2: The complex interaction between articular cartilage and synovial membrane during OA.

Under normal conditions, chondrocytes maintains a dynamic equilibrium between synthesis and degradation of ECM components. Upregulation of catabolic processes and/or downregulation of anabolic processes have been found to play key roles in articular cartilage degeneration and thus development of OA. A disruption of matrix equilibrium leads to progressive loss of cartilage, fibrillation and clonal expansion of chondrocytes in the depleted regions, induction of oxidative stress and eventually, apoptosis of cells (Ishiguro et al., 2002). With progression of disease, there is usually an increase in both degradation and synthesis of ECM components within the joint, with an overall shift towards catabolism over anabolism. Clinically, degradation of the ECM results in the gradual impairment of articular cartilage, often accompanied by pain and

physical disability in the subchondral bone, typically in the form of sclerosis and osteophyte formation (Lee et al., 2013). Pathological changes in cartilage leads to disturbance of the balance between mechanical loading and direct cytokine/growth factor signals, which causes changes in gene expression. The inflammatory reaction in the disease is controlled by several soluble biochemical factors including prostanoids, cytokines and ROS produced by both synoviocytes and chondrocytes (Henrotin et al., 2003).

1.4.1 Reactive oxygen species and Reactive nitrogen species

The reactive oxygen species (ROS) have been described as key factors involved in cartilage pathogenesis of OA. They are produced by both, synovial tissue and chondrocytes (Henrotin et al., 2014). Oxidative damage constitutes a vicious circle between cartilage degradation and synovitis. Oxidative stress elicited by ROS, disturbs cartilage homeostasis and promotes catabolism via induction of cell death, breakdown of matrix components, upregulation of latent matrix-degrading enzyme production, inhibition of matrix synthesis and, oxidation of intracellular and extracellular molecules (Lee et al., 2013). During normal metabolism, ROS form as a natural byproducts. They control various aspects of biologic processes in addition to cell activation and proliferation. Particularly, low levels of ROS are reported to act as a secondary messenger in intracellular cell signaling in the regulation of the expression of a various gene products, which are cytokines, adhesion molecules, MMPs and matrix components. On the other hand, in pathologic conditions like inflammatory joint diseases, increased production of ROS in combination with reduction of antioxidants has been observed within the cells and thus involved in the progression of diseases. Such an imbalance between oxidants and antioxidants leads to “oxidative stress”. Thus ROS have been implicated along with metalloproteinases in the process of matrix and cell component degradation in OA (Aigner and Schmitz, 2011).

In addition to ROS, reactive nitrogen species (RNS) are also play significant role in cartilage degradation in OA (Pelletier et al., 1999; Aigner and Schmitz, 2011). Osteoarthritic cartilage produces a large amount of nitric oxide (NO) due to increased level of the inducible form of NO synthase (iNOS), the enzyme which produce NO

(Pelletier et al., 1999). NO found to inhibit the synthesis of cartilage matrix macromolecules and enhances MMP activity. Moreover elevated NO, reduces the synthesis of interleukin-1 receptor anatagonist (IL-1Ra) by chondrocytes. Further, an increased level of IL-1 with a decreased IL-1Ra level may leads to an overstimulation of osteoarthritic chondrocytes causing enhanced cartilage matrix degradation (Aigner and Schmitz, 2011).

Thus, RNS and ROS are exciting areas of future research in the pathogenesis and molecular biology of OA.

1.4.2 Cytokines

It is believed that the cytokines and growth factors are involved in the pathophysiology of OA. They are found to be associated with functional alterations in synovium, subchondral bone and cartilage. They are produced both spontaneously and after stimulation by the joint tissue. The major cytokines (Pro and anti-inflammatory) and antagonist said to be involved in OA include; interleukin 1 alpha (IL-1α), IL-1beta (IL-1β), IL-4, IL-6, IL-8, IL-10, IL-11, IL-13, IL-17, IL-18, leukemia inhibitory factor (LIF), tumor necrosis factor α (TNF-α) as well as IL-1Ra (Wojdasiewicz et al., 2014).

Several growth factors also show to be involved in OA, for example transforming growth factor β (TGF- β), fibroblast growth factor (FGF), platelet derived growth factor (PDGF) and insulin like growth factor (IGF). Growth factors like TGF- β, have a double effect, synthetic or catabolic.

The balance in between cytokine driven anabolic and catabolic processes decides the integrity of articular joint tissue. A shift in the balance between pro and anti-inflammatory cytokines is thought to contribute in OA pathogenesis. OA is characterized by progressive cartilage degradation, in which matrix integrity is not maintained and the homeostasis of catabolic, anabolic & anti-inflammatory cytokines or antagonist is disturbed (Pelletier et al., 1999).

1.4.2.1 Pro-inflammatory cytokines

Based on evidences, it is believed that pro-inflammatory cytokines are responsible for the catabolic process in OA (Hassanali and Oyoo, 2011), they are first produced by

the SM and diffused into the cartilage through the synovial fluid, where further they activate the chondrocyte cells to produce pro-inflammatory cytokines (Pelletier et al., 1999).

IL-1β and perhaps TNF-α are the prominent catabolic systems involved in the destruction of joint tissues and may leads to articular tissue damage. They are also responsible for increase in osteoclastic bone resorption, *in vitro* (Hassanali and Oyoo, 2011). IL-1β and TNF-α stimulate their own production and induce joint articular cells like chondrocytes and synoviocytes, to produce additional cytokines such as IL-8, IL-6, LIF, as well as stimulate proteases and prostaglandin E2 (PGE2) production (Pelletier et al., 1999). They have also been reported to inhibit collagen and proteoglycan synthesis, upregulate MMP expression as well as stimulate NO production (Sutton et al., 2009).

In OA, IL-6 increases the amount of inflammatory cells in the SM, which stimulates the proliferation of chondrocytes. Other IL-1 effects are, increased MMP synthesis and inhibition of proteoglycan production (Pelletier et al., 1999; Sutton et al., 2009; Hassanali and Oyoo, 2011). It also involved in the feedback mechanism that limits enzyme production by inducing the production of tissue inhibitor of matrix metalloproteinase's (TIMPs) (Sutton et al., 2009; Hassanali and Oyoo, 2011).

LIF, a cytokine of IL-6 family is up regulated in osteoarthritic SM and fluid, it is produced by chondrocytes in response to pro-inflammatory cytokines, which stimulates cartilage proteoglycan resorption, MMP synthesis and NO cellular production; however its role in OA has not yet been clearly defined. LIF enhances IL-1β & IL-8 expression in chondrocytes, and IL-1β & TNFα in synovial fibroblasts (Villiger et al., 1993). IL-1β and TNFα can upregulates LIF production in joint articular cells. LIF regulates the metabolism of cartilage & bone, and induces the resorption & the formation of bone. LIF can induce expression of collagenase & stromelysin by chondrocytes without disturbing the production of the specific TIMPs. Thus, LIF may be involved in the progression of cartilage destruction and inflammation in OA (Pelletier et al., 1999).

IL17 involved in the upregulation of various pro-inflammatory cytokines IL-1β, TNFα & IL-6 as well as MMP in target cells like macrophages. IL17 also increases the production of NO in chondrocytes (Pelletier et al., 1999; Sutton et al., 2009; Hassanali and Oyoo, 2011).

IL-8 is a potent chemotactic cytokine for polymorphonuclear neutrophils which stimulates their chemotaxis and generates reactive oxygen metabolites. It is synthesized by a variety of cells including monocytes/macrophages, chondrocytes and fibroblasts. TNFα can stimulate the release of IL-8 (Pelletier et al., 1999). It can enhance the release of cytokines in mononuclear cells which may further change the inflammatory reaction (Pelletier et al., 1999; Sutton et al., 2009). IL-18 expressed by macrophages and synovial fibroblasts, stimulates release of pro-inflammatory cytokines which induces NO synthesis as well as reduces expression of cartilage matrix components (Sutton et al., 2009). Chondrocytes express the IL-8 when stimulated by certain agents generated during response to cartilage injury (Pelletier et al., 1999).

IL-11 decreases the release of PGE2 from osteoarthritic synovial fibroblasts, suggest that contrary to the action of LIF, IL-11 can prevent the excessive ECM degeneration (Pelletier et al., 1999).

1.4.2.2 Anti-inflammatory cytokines and cytokine antagonist

Three anti-inflammatory cytokines viz. IL-4, IL-10 and IL-13 are expressed by synovial cells as well as chondrocytes (Pelletier et al., 1999; Sutton et al., 2009). IL-1Ra expressed by SM, is a competitive inhibitor of the IL-1β receptor which inhibits MMP production (Sutton et al., 2009). These cytokines decrease the production of IL-1β, TNFα and MMPs. They also upregulate IL-1Ra and inhibit of PGE2 release (Pelletier et al., 1999; Sutton et al., 2009). In addition, IL-4 reduces pro-inflammatory cytokine production, inflammation, vascularisation and cartilage degradation. It also Inhibits LIF synthesis as well as decreases osteoclast formation thereby, reducing bone resorption (Sutton et al., 2009).

1.4.3 Molecular etiopathology of OA

In OA, MMPs play significant role in the degradation of the matrix (Ishiguro et al., 2002; Pearle et al., 2005). MMPs consist of more than 20 proteolytic enzymes, each of which is the product of a different gene. Some MMPs are produced in large quantities by chondrocytes and synovial cells. They all share a structural similarity which includes a catalytic zinc-binding domain with a conserved sequence motif His Glu xxx xxx His

(Ishiguro et al., 2002). MMPs are divided into five subgroups, in terms of substrate specificity viz. collagenase, stromelysin, gelatinase, membrane type MMPs and others. Collagenases, particularly collagenase-1 (MMP-1) and collagenase-3 (MMP-13), are thought to be primarily involved in type II collagen degradation in OA. Stromelysin-1 (MMP-3) has shown to play a primary role in the degradation of proteoglycans and fibronectin (Pearle et al., 2005). It also activates other MMPs, and is thus considered to play a significant role in matrix degradation. In addition, 92 kDa type IV gelatinase (MMP-9) has various substrates, like type I, III, IV and V collagen, gelatin, proteoglycans, and elastin (Ishiguro et al., 2002; Pearle et al., 2005). It is reported that MMP-1, MMP-3 (stromelysin-1), MMP-2, MMP-8, MMP-9 (neutrophil collagenase) and MMP-13 (collagenase-3) play important roles in cartilage matrix destruction in OA (Ishiguro et al., 2002).

Other proteinases are classified as disintegrin and metalloprotease with thrombospondin repeats family (ADAM-TSx) that digest the aggrecan core protein, known as aggrecanase-1 and -2, respectively (Ishiguro et al., 2002).

Tissue inhibitors of metalloproteinases (TIMP)-1, TIMP-2, TIMP-3 and TIMP-4 possesse the ability to inhibit MMPs. TIMPs play vital role in the regulation of MMP activity and thus they are crucial in controlling destruction of cartilage. Therefore the balance between MMPs and TIMPs is very crucial in maintaining cartilage integrity (Pelletier et al., 1990). TIMP-3 is reported to inhibit aggrecanase activity, *in vitro*. TIMP-1 and TIMP-2 have growth-promoting activity, which may influence matrix turnover during OA. It is important that the level of latent MMP synthesis in OA cartilage significantly exceeds the up-regulation of TIMPs gene expression. Although TIMP-1 expression in chondrocytes was found to be higher than that by chondrocytes derived from normal cartilage however the amount and TIMP isoform produced by OA chondrocytes is not sufficient to inhibit the level of MMPs (Ishiguro et al., 2002) and once activated and not effectively regulated by TIMPs, MMPs degrade both the endogenous as well as newly synthesized ECM molecules, which are synthesized by chondrocytes and they leads to serious effect on cartilage. To replace the ECM molecules lost by MMP degradation; uncontrolled degradation may also sometimes induce a total loss of cartilage integration. The activity of MMPs is considered low in healthy cartilage. MMPs are produced by

chondrocyte and synoviocytes cells as latent pro-enzymes (Poole, 2000). Even though the loss of aggrecan molecules in articular is essential for the progression of OA, the final articular cartilage damage is inflicted by the loss of the collagen network. Collagenases like, MMP-1, -8, and -13 can only degrade the helical domain of collagen type II. However, the final dissolution may be occured by the activity of gelatinases that are also produced by chondrocyte cells. As damage progresses, denaturation and cleavage of collagen type II by collagenase occurs (Ishiguro et al., 2002).

Chondrocyte cells also synthesize the plasminogen activator (PA) which produces plasmin. Different forms of PA found in urine (uPA) and tissue (tPA) are (Ishiguro et al., 2002). Plasmin is known as a general MMP activator which can convert pro- MMP-3 to an active form of MMP-3. This active form in turn can activate procollagenases. The plasmin pathway is regulated by plasminogen activator inhibitors (PAI). In osteoarthritic cartilage, plasmin concentration is markedly higher than normal, and a remarkably higher level of activated MMPs is observed (Poole, 2000).

Thus, numerous activation pathways have been implicated in the OA process. These are the targets for therapeutic interventions. The goal of biologic therapies is to impede joint destruction via inhibition of catabolic activity and/or upregulation of anabolic activity, thereby slowing or preventing the progression of OA (Lee et al., 2013). This suggests that a therapeutic intervention which will inhibit specific MMP activity could be useful in the management of OA.

1.4.4 Synovitis in OA

Over the last decade, attention has turned to the importance of synovitis in OA. Synovitis has been proven to contribute to patient's symptoms (joint swelling and pain from inflammation), signs (effusion), and disease progression (Lambert et al., 2014). High prevalence of synovitis (95%) has been reported in OA patients. The prevalence and the severity of synovitis increase with advancing stage of OA. It's an indicator of pathology and a predictor of disease progression (Henrotin et al., 2014).

Synovial inflammation is characterized by increased number of lining cells in synovial layers made of synovial fibroblasts, and a mixed inflammatory infiltrate containing macrophages, T cells, and B cells. Macrophages drive inflammation and destructive

response and are responsible for the production of the major inflammatory mediators (Henrotin et al., 2014). They are also involved in osteophytes formation and synovial fibrosis through at least partially, the local secretion of TGF-β. Higher levels of mononuclear cell infiltration are found in SM of early OA stages (Benito et al., 2005). Innate immunity has been described as an early event of the OA synovitis. The T cell infiltrate in osteoarthritic SM is the site of type 1 helper T cell (Th1) differentiation and activation to produce Th1 cytokines, such as interferon γ (IFNγ). B cell infiltration is found in half of OA patients. Moreover, the presence of CXC-chemokine ligand (CXCL) 13, a potent chemoattractant of B cells, has been shown in OA synovial lymphoid aggregates (Henrotin et al., 2014).

The role of key intermediates in the cell signaling i.e., nuclear factor kappa B (NF-κB), c-Jun and p38, in the inflamed synovial tissue have been described. They have been found to be involved in the expression of most of the inflammatory mediators (Kapoor et al., 2011). Thus, NF-κB could also be a therapeutic target for the treatment of OA (Henrotin et al., 2014). Osteoarthritic SM, like rheumatic arthritis is hypoxic and hypoxia is responsible for cell activation and recruitment. It provokes the synthesis of hypoxia-inducible factors (HIF), which in turn are also induced by pro-inflammatory mediators. HIF-1 is a transcription factor that is constitutively expressed in SM cells. It gains transcriptional activity in hypoxic cells, leading to the expression of genes involved in angiogenesis, inflammation and tissue degradation (Henrotin et al., 2014).

1.5 Causes of OA

Multiple factors have been shown to affect the progression of OA which includes:

- Age - OA may begin at any age but typically occurs in older adult. With the increasing age, the water content of the cartilage decreases (as proteoglycan content diminishes), reducing cartilage vascularization and cartilage perfusion. Thus the cartilage becomes less resilient and susceptible to degradation (Goldring and Goldring, 2006).
- Gender - Females are more likely to develop OA than males and the symptoms get sever usually after the menopause (Mahajan et al., 2005).

- Deformities of the bone(s) - Patients born with defective joints or cartilage have a significantly higher risk of eventually developing OA (Maguire, 2007).
- Joint injuries - Especially those resulting from an accident or some sports may increase the risk of developing OA (Peat et al., 2001).
- Obesity - Obese people have a higher risk of developing OA as their weight-bearing joints are under a greater strain compared to people of normal weight (Mahajan et al., 2005).
- Genetics - It is estimated that approximately 40% to 60% of cases of hand, hip and knee OA may have a genetic link (Mahajan et al., 2005).
- Repeated overuse of certain joints (Maguire, 2007).
- Lack of physical activity (Maguire, 2007).
- Nerve injury (Maguire, 2007).

1.6 Symptoms of OA

- Joint pain is the major symptom associated with OA pathophysiology which increases intensively upon excessive joint movement and weight bearing. Rest can give relief except in advance OA, as it can cause pain at night leading to loss of sleep. This pain is often deep, aching and not well localized.
- Inactivity and morning stiffness in joints; after excessive work or felt intermittently upon resuming activity, after periods of inactivity. Stiffness of ligaments and tendons becomes permanent with time.
- Crackling sound of the joints while movement when loose bodies are there.
- Acute inflammatory flares, characterized by local warmth, tenderness and effusion.
- Meniscal degeneration.
- Breakdown of cartilage.
- Reduced function and joint movement.
- Joint instability, buckling or giving way.
- Bony spurs or extra bone may form around the joint.
- Altered gait.
- Muscle atrophy or weakness.
- Joint effusion, swelling and inflammation, tenderness usually located over the joint.

- Oxidative stress (Ashraf, 2011).
- Radiographically visible changes in OA are, narrowing of the joint space with osteophyte growth at joint margins and advancement of the subchondral bone, fibrillation of the articular surface, fissures, thinning of the cartilage and tidemark duplication (Lorenz and Richter, 2006; Ashraf, 2011).
- Osteophytes could be considered as endogenous repair attempts in degenerating joints and may be a physiologic response to mechanical overloading (Aigner and Schmitz, 2011).
- Blood vessels cross the tidemark from the subchondral bone into the avascular articular cartilage. Synovial inflammation is observed accompanied by thickening of the synovial lining, angiogenesis and synovial effusion, all leading to joint swelling (Ashraf, 2011).

1.7 Treatments of OA

Generally the treatments of OA are divided into three catagories:

1. Non-pharmacological therapy
2. Pharmacological therapy
3. Surgical intervention

1. Non-pharmacological therapy

The treatment of OA generally starts with symptomatic therapy to combat pain (Singh, 2010). Therapy includes lifestyle modification, mainly exercise and weight reduction programs to manage the OA pain (Ghanekar and Raina, 2012). These treatments do not involve drugs and therefore reduce drug consumption and toxicity or even could help in delaying the need for joint replacement surgery (Ghanekar and Raina, 2012). Physiotherapy plays a key role and considered as the backbone of OA treatment. The main aim of treatment here is to increase strength of quadriceps muscle, reduce pain, to increase range of motion and to improve functions (Singh, 2010). Acupuncture, thermotherapy and sound wave diathermy have also been used for relieving the symptoms of OA (Ghanekar and Raina, 2012). Non-pharmacological therapy also includes patients education about the disease, do's and dont's, personalized self care,

weight reduction programmes, use of assisted devices and appropriate footwear (Singh, 2010).

2. Pharmacological therapy

The pharmacological therapy basically includes the use of oral analgesics and non-steroidal anti-inflammatory drugs (NSAID's). But the associated adverse effects of long use of these drugs on gastric system are also evident. In the patients with knee OA where, there is effusion and local signs of inflammation, the use of intra-articular corticosteroid injection is given (Singh, 2010). It is also evident that the excess use of intra-articular corticosteroid leads to further damage of the cartilage. Pharmacological therapy also includes following three types of drugs,

a) Symptom modifying drugs

   NSAID's and COX-2 inhibitors like celecoxib, etoricoxib, valdecoxib, acetaminophen, opoids etc. are used for knee OA (Mahajan et al., 2005; Felson, 2006).

b) Systematic slow acting

   Along with hyaluronic acid complementary and alternative medicine/ neutraceuticals Glucosamine and chondroitin sulfate are also widely used for treatment of OA (Felson, 2006).

c) Structural modifying OA drugs (SMOADS)/Chondroprotective drugs

   Tetracyclines are inhibitors of tissue MMPs. Minocycline and doxycycline have been shown to inhibit collagenase activity, prevent proteoglycan cell loss, cell death and deposition of type X-collagen matrix. Glycosaminoglycan polysulfuric acid, known as arteparon, work through reducing the collagenase activity. Similarly other agents like glycosaminoglycan peptide complex known as rumalon has shown to increase the levels of TIMP, while pentosan polysulfate (cartrofen) inhibits granulocyte elastase. However, larger clinical trials have yet to prove their structure modifying activity. Diancerin and its active metabolite rhein has the capability to inhibit IL-I beta in human synovium. It has improved pain score in patients of OA as well as it has been proposed as structure modifying drug for OA. Moreover disease modification potential of agents like glucosamine, hyaluronan, growth factors and

cytokine manipulation, gene therapy as well as chondrocyte and stem cell transplant needs further evaluation (Mahajan et al., 2005).

3. Surgical intervention

Patients with severe symptomatic OA and who failed to respond to any treatment should only be treated with surgery. It includes joint lavage, osteotomy, partial or complete joint replacements (Singh, 2010). It should be the last treatment of choice.

Current therapeutic agents for OA focus on symptomatic relief because pharmacological interventions that halt disease progression are not available. Allopathic medicines, even though are specific in their pharmacological and therapeutic actions, carry the risk of side effects and arthritic drugs are no exception (Ghanekar and Raina, 2012). The common side effects of anti-arthritic drugs are stomach pain, cramps or ulcers, gastrointestinal tract complaints, hypertension, decreased appetite, nausea, vomiting, diarrhea, constipation, heart burn etc., which are undesirable. They can make heart failure or kidney failure worse and can increase blood pressure and may increase risk of thrombosis (clotting). Surgical treatments include replacement of joints but such treatments are painful and moreover expensive. Economically backward people may not be able to afford it. Besides NSAIDs, alternative modalities include the use of various chondroprotective agents such as glucosamine and chondroitin sulphate (Mengshol et al., 2000). However, there are controversies regarding the therapeutic efficacy of these drugs. Thus unfortunately, current treatment options are ineffective and fail to address pathophysiological and biochemical mechanisms involved with cartilage degeneration and no disease-modifying OA drugs (DMOAD) are available for treatment of OA. Research is indicating that current drugs being used in OA may be producing short-term benefit, but actually accelerating the progression of the joint destruction.

Though a number of anti-inflammatory agents are being identified for their potential to relieve the pain, an effective formulation which can revert back the changes of inflammation is still unaddressed. Thus, it is necessary to find out alternative chondroprotective drugs which are routinely used in the traditional medicines that may play an important role in treatment of OA. In *Ayurveda*, OA is said to cause due to excessive intake of *vata* increasing food. Herbs like *Acacia nilotica* ssp. *Indica* (Bth) Brenan, *Bombax ceiba* L., *Boswellia serrata* Roxb., *Sida cordifolia* L., *Piper longum* L., *Zingiber officinale* Rosc., *Ricinus communis* L., *Vitex negundo* var. *incisa* (Lam.) C.B.Cl. and *Tribulus terrestris* L., *Withania somnifera,* (L.) Dunal, *Asparagus racemosus* Willd., *Emblica officinale* Gaertn., *Symplocos racemosa* Roxb., *Commiphora mukul* (Hook. ex Stocks) Engl., *Laccifer lacca* and formulations like '*Triphala Guggul*', '*Laksha Guggul*', *Dashamoolarishta* etc. are commonly used for the treatment of OA (Wilson et al., 2007; Jain et al., 2011; Siddiqui, 2011; Chhatre et al., 2014; Ladda and Kamthane, 2014).

1.8 Description of selected plants and formulations

1.8.1 *Acacia nilotica* ssp. *indica* (Bth) Brenan

Synonym: *Acacia arabica* (Lam.) Willd. var. *indica*

Family: Leguminosae

Vernacular names: Babul (E); Babul, Babur (H); Jaali (K); Karivelam (M); Barburah, Babula (S); Karuvelam, Kariram (T); Nallatumma (Tl); Babhul (Mr).

Habitat: Found in a variety of woodland types, wooded grassland and scrub escarpment, forests and low-lying forest, in deep soil.

Description: Grows 15-18 m in height. The bark is usually slaty green in young trees or almost black in mature trees. It contains deep longitudinal fissures in which the inner grey-pinkish slash is exposing which exude a reddish low quality gum. The leaves are bi pinnate, pinnae 3-10 pairs, 1.3-3.8 cm long, leaflets 10-20 pairs and 2-5 mm long. Thin, straight, light grey spines present in axillary pairs, generally 3-12 pairs, 5-7.5 cm long in young trees, and mature trees commonly without thorns. Flowers are in globulous heads and bright golden yellow. Pods are 7-15 cm long, green and tomentose when immature and greenish black when mature; indehiscent and deeply constricted between the seed which appears like a necklace. Seeds are 8-12 per pod, compressed, ovoid and dark brown shining with hard testa.

Flowering and Fruiting: March-December.

Distribution: The plant is common to Tropical Africa, Asia, Australia and America. It is naturally widespread in the drier areas of Africa; from Senegal to Egypt and down to South Africa and in Asia; from Arabia eastward to India, Burma and Sri Lanka. The largest tracts are found in Sind. It is distributed throughout the greater part of India in forest areas, roadsides, farmlands, tank foreshores, village grazing lands, agricultural fields, wastelands, bunds, along the national highways and railway lines. It has been widely planted on farms throughout the plains of the Indian subcontinent and is common in Maharashtra.

Reference: Oommen, 2000; Singh and Karthikeyan, 2001.

1.8.2 *Bombax ceiba* L.

Synonyms: *Bombax malabaricum* DC.

Family: Bombacaceae

Vernacular names: Semul (A); Shimul, Simul, Rokto-simul (B); Silk-Cotton Tree, Red silk cotton tree (E); Sawar, Shimalo, Shimur, Shemalo (G); Semal, Semul, Shembal, Pagun Kanti-senbal, Pagun (H); Kempuburuga, Booruga, Mullubooruga, Mullelava, Burugadamara (K); Mullilavu, Mullilabpoola, Ilavu, Elavu, Pulamaram (Ml); Sanval, Katesaval, Kantesaval, Saul, Simalo (Mr); Simble (P); Shalmali, Semul, Simul (S); Elavam, Illavarn, Mullilavau, Pulai, Ilavu (T); Buruga, Boorugachettu, Kondabooruga, Mundlaboorugachettu, Mulluburugacettu, Kondaburuga (Tl); Sembhal (U).

Habitat: It is found in the hotter parts of India, in forests upto 1350 m.

Description: A deciduous tree, upto 30 m in height; bark grey, covered with black, conical prickles. Leaves are digitate, crowded at the ends of branches. Leaflets are 5-7, elliptic-lanceolate to ovate-lanceolate, glabrous, 5-23 x 1.5-9 cm; Petioles long; Petiolules small. Flowers are bright red, crowded at the ends of branches, 8-14 cm across, appearing before the new leaves. Capsules are woody, ellipsoid, 10-12 cm long, 5-valved. Seed embedded in white, silky cotton, ovoid.

Flowering and fruiting: January-May.

Distribution: It is widely distributed throughout India, including the Andaman's, up to an altitude of 1500 m or even higher. In peninsular India, the tree is very common in the dry as well as moist, mixed deciduous forests. It is found in the mixed evergreen forests of West Bengal and Assam. The tree also grows sporadically in the mixed deciduous forests in the Sub-Himalayan region and lower valleys, also in bhabar tracts of Uttar Pradesh, Bihar and is common in Maharashtra.

Reference: Oommen, 2000; Singh and Karthikeyan, 2001.

1.8.3 *Boswellia serrata* Roxb.

Family: Burseraceae

Vernacular names: Indian Oblibanum Tree (E); Salai, Shallaki, Salga (H); Chitta, Guggul Mara, Maddi (K); Kunduruhkamaram (M); Salai, Sali, Dhupasali, Kurunda, Salaphali, Dhupali, Salai (Mr); Salai (O); Kundru, Ashramoorti, Guggula, Sallaki, Shallaki (S); Marattu-vellai, Kumancam, Kunturucam, Kunturu (T); Sallaki, Guggilamu, Anduga, Anduku (Tl).

Habitat: This plant grows in tropical dry deciduous forests and in very dry teak forests or in dry mixed deciduous forests. It is characteristically found on the slopes and ridges of hills, as well as on flat terrain, attaining a larger size on fertile soils.

Description: It is a moderate to large branching tree. Bark is very thin, greyish-green, ashy or reddish in colour with a chlorophyll layer beneath the thin outer layer, which peels off in thin, papery flakes. Leaves are deciduous, alternate towards the tops of branches, unequally pinnated; leaflets in about ten pairs with an odd one opposite, oblong, obtuse, serrated, pubescent and sometimes alternate. Flowers are white or pale rose on short pedicels in single axillary racemes shorter than the leaves. Fruit is capsular, three-angled three-celled, three-valved, septicidal, valves hard. Seeds are solitary in each cell surrounded by a broad membranaceous wing. Cotyledons intricately folded multifid.

Flowering and fruiting: January-August.

Distribution: The global distribution of this species is reported from Arabia to Indian subcontinent. It is found in India, Northern Africa and the Middle East. Within India its occurrence is conspicuous in the dry hills in Central, Northwest and Peninsular India. Also found in the drier parts of Peninsular India and is common in forests of Maharashtra.

Reference: Oommen, 2000; Singh and Karthikeyan, 2001.

1.8.4 *Piper longum* L.

Synonym: *Chavica roxburghii* Miq.

Family: Piperaceae

Vernacular names: Pipul (B); Indian Long Pepper (E); Lindi Pepper (G); Pipal, Pipli (H); Hippali, Thippali Balli (K); Tippali, Pippali (Ml); Pimpli, Pippal (Mr); Krishnapippali, Pippali, Kana (S); Tippili, Pippili (T); Pippallu, Pipili (Tl); Pipul (U).

Habitat: Grows usually in warmer places, mostly deciduous to evergreen forest, commonly under cultivation

Description: A slender sub-scandent herb, branchlets erect, straggling or sometimes climbing, hairless, with swollen nodes and creeping branches with roots at lower nodes. Leaves are alternate, variable in shape, usually egg shaped-heart-shaped, 7-15 x 4-6 cm, base heart-shaped and unequal, apex acute to acuminate, margin entire, hairless, lower leaves with long stalks and upper ones without stalk; lateral nerves 5-7 arising from the base. Fruiting spikes are cylindrically oblong, about 4 x 1 cm. Berries are globose, about 2 mm across, partly sunken in the rachis, compactly arranged, red turning black when ripe.

Flowering and fruiting: August-January.

Distribution: The species is distributed throughout in the Indo-Malaysian region and Sri Lanka. It is a native of North East India. Within India its presence is reported from the lower hills of Central to Eastern Himalayas as well as Western Ghats of Kerala and Tamil Nadu. Found in the hotter parts of India, from the central Himalayas to Assam, Khasi and Mikir hills, lower hills of West Bengal and evergreen forests of Western Ghats from Konkan to Kerala, and also reported from Car Nicobar Islands and is occasional in Maharashtra. In Maharashtra this plant is also under cultivation.

Reference: Oommen, 2000; Singh et al., 2001.

1.8.5 *Ricinus communis* L.

Family: Euphorbiaceae

Vernacular names: Era-gach (A); Veranda (B); Castor bean, Castor oil plant, Wonder tree (E); Diveli (G); Divelio, Diveli, Erand, Erandi (H); Oudla (K); Chittamankku (Ml); Kege (Mn); Eranda, Gandharva hasta (S); Amanakku, Vilakkennai, Kottaimuttu (T); Erand (Mr).

Habitat: Grows nearly throughout India in dry and arid environments, on waste lands and cultivated frequently.

Description: It is a shrub that sometimes looks like a small tree. Perennial, branched, 1–3 m tall with succulent stems; leaves are herbaceous, alternate, orbicular, palmately compound, 1–6 cm broad, with 6–11 toothed lobes, glabrous. Flowers are numerous in long inflorescences. The fruit is a globose capsule 2.5 cm in diameter, on an elongated pedicel, and is usually spiny. Seeds are usually 3-ovoid, tick-like and shiny, 0.5–1.5 cm long, veined or finely dotted to large splotches.

Flowering and fruiting: November-December.

Distribution: Globally, the species is native to tropical Africa. In India, it is found in Karnataka (Chikmagalur, Hassan, Mysore, Shimoga), in all districts Kerala and Tamil Nadu. It is Cultivated chiefly in Andhra Pradesh, Maharashtra, Karnataka and Orrisa. Commonly found in Maharashtra on buds and west places.

Reference: Singh et al., 2001; Gupta and Singh, 2015.

1.8.6 *Sida cordifolia* L.

Family: Malvaceae

Vernacular names: Swetberela, Brela (B); Country mallow (E); Mahabala, Khapat (G); Kharanti (Gl); Kungyi, Bariyar (H); Hettuthi, Hettugigada (K); Kobirsir bhaji, Muttava (Kk); Marang, Lupaaraba (Md); Kurunhott, Vellurum (Ml); Chikana, Khiranti (Mr); Badianaula, Bisvokopari (O); Kowar, Simak (P); Bala, Burrayra (S); Nilatutti, Puniar tutti (T); Tellantisa, Tellagorra (Tl).

Habitat: Grows throughout India in moist places, common on roadside. Found in plains from the coast to 1000 m.

Description: Erect and branched subshrubs upto 1.5 m tall. Plant is branched, softly hairy and with much stellete, hair nearly all over and subpersistant. Stem is green, densely tomentose with minute stellate and spreading simple hairs. Leaves are 1-2 inch long, cordate or subacute, not acuminate, crenate or dentate; petioles are half to one and half inch long. Flower is yellow, peduncles, axillary, jointed much above the panicles, upper flowers nearly sessile and fasciculate towards the tip of the branches forming subspicate inflorescence. Fruit is one fourth to one third inch in diameter.

Flowering and fruiting: September-December.

Distribution: Found in Sri Lanka, also throughout the tropical and subtropical regions of India up to an elevation of 1800 mts.; in Himachal Pradesh, Bengal, Gujrat, Andra Pradesh, Assam, Jammu and Kashmir, Tamil Nadu, Uttar Pradesh, Karnataka, Kerala are chief regions of occurrence and commonly in Maharashtra.

Reference: Singh and Karthikeyan, 2001; Jain et al., 2011.

1.8.7 *Tribulus terrestris* L.

Family: Zygophyllaceae

Vernacular names: Gokhurkata (A); Gokhru kanta, Gokhri (B); Punctue vine, Yellow vine, Caltrop (E); Bethagokharu, Nanagokharu, Mithagokharu (G); Hussuk, Gokharu, Chhotagokharu, Gokshri (H); Michikand, Neggilu, Negalu, Neggilamullu (K); Nerinnil, Nerungil (M); Nerinjil (Ml); Sharatte, Sarata (Mr); Gokhyura (O); Bhakhra (P); Goksurah, Trikantaka (S); Nerinjil, Nerinchi, Nerucil, Kamaraci (T); Cinnpalleru, Palleru kaya, Palleru, Sannaneggilugida (Tl); Gokhru, Khar-e-khasak-khurd (U).

Habitat: Commonly grown in the dry and hotter parts of the country, recently cultivated

Description: It is a small prostrate, annual or biennial, procumbent herb grows upto 90 cm in length. Leaves are opposite, compound with lanceolate stipules. Leaflets are oblique at base. Flowers are yellow in colour, predicelled, solitary and axillary. Fruit is dry woody sub-globose schizocarp and easily recognized by the spines present all over the surface. Seeds are many (or 4 to 6) in each of the five parts of the fruit, which separates on maturity.

Flowering and fruiting: Throughout the year.

Distribution: Found in South Europe, South Asia, Africa and Australia. It is native to the Mediterranean region. A species of the tropical regions found to be distributed throughout India from sea level to 3500 m and is common in Maharashtra.

Reference: Oommen, 2000; Singh and Karthikeyan, 2001.

1.8.8 *Vitex negundo* var. *incisa* (Lam.) C.B.Cl.

Family: Verbenaceae

Vernacular names: Pochotia (A); Nirgundi, Samalu, Nishinda (B); Indian privet, Five-leaved chaste tree, Horseshoe (E); Punjabastusta (F); Nagoda, Shamalic (G); Nirgundi, Sambhalu, Samhalu, Sinduar, Sinuar, Mendi (H); Bile-nekki (K); Indrani (Ml); Linghad, Nigad, Nirgundi, Tellavavili (Mr); Banna, Marwan, Maura, Mawa, Swanjan Torbanna (P); Nirgundi, Sephalika, Svetasurasa, Vrikshaha (S); Chinduvaram, Nirnochchi, Nochchi, Notchi, Vellai-Nochchi (T); Sindhuvara, Nalla-Vavili, Tella-vavili (Tl).

Habitat: It thrives in humid places or along water courses in wastelands and mixed open forests, Planted as a hedge.

Description: It is a woody, aromatic shrub growing to a small tree. It grows 6-28 feet in height. It commonly bears tri- or penta-foliate leaves on quadrangular branches, which gives rise to bluish-purple coloured flowers in branched tomentose cymes. Flowers are rectangular and 2-8 inches long. Fruit is small, drupe, one fourth inches diameter, blackish when ripe. Bark is thin, smooth and brownish.

Flowering and fruiting: April-December.

Distribution: It is native to tropical Eastern and Southern Africa and Asia. Found in Afghanistan, Bangladesh, Bhutan, Cambodia, China, India, Indonesia, Japan, Korea, Kenya, Madagascar, Malaysia, Mozambique, Myanmar, Nepal, Pakistan, the Philippines, Sri Lanka, Taiwan, Tanzania, Thailand and Vietnam. It is grown commercially as a crop in parts of Asia, Europe, North America and the West Indies. Throughout India, it is found in warmer zones; ascending to 900 m in the North Western Himalaya and common on hedges in Maharashtra.

Reference: Singh et al., 2001; Rana and Rana, 2014.

1.8.9 *Zingiber officinale* Rosc.

Family: Zingiberaceae

Vernacular names: Spice Ginger, Canton Ginger, Halia, Ginger (E); Sunth, Adu (G); Adrak, Sonth (H); Alla, Shunthi (K); Incji, Andrakam, Chukka, Erukizhangu, Enchi (Ml); Aale, Sunth (Mr); Ada (O); Sunthi, Srungavera, Ardraka, Vishvabheshaja, Nagara (S); Ingee, Inji (T); Shonti, Allam, Allamu, Allamu Chettu (Tl); Adrak, Adi (U).

Habitat: Humid, partly-shaded habitats in the tropics and subtropics; commonly cultivated.

Description: It is an herbaceous, rhizomatous perennial plant. The rhizomes are aromatic, thick-lobed, pale yellowish, differing in shape and size in the different cultivated types. Digitately branched rhizome produces stems up to 1.50 m in height with linear lanceolate sheathing leaves (5–30 cm long and 8–20 mm wide). The herb develops several lateral shoots in clumps which begin to dry when the plant matures. The leaves are narrow, distichous, linear- lanceolate, dark green. The flowers are in spikes, greenish yellow with a small dark purple or purplish black tip.

Flowering and fruiting: September-December

Distribution: The plant is native to South-East Asia. It is cultivated in the tropical regions in both the Eastern and Western hemispheres. It is commercially grown in Africa, China, India, and Jamaica; India is the world's largest producer. It is mainly cultivated in Kerala, Andhra Pradesh, Uttar Pradesh, West Bengal, and Maharashtra.

Reference: Sharma et al., 1996; Kumar et al., 2011.

1.8.10 *Dashamoolarishta*

Dahsamoolarishta is a fermented liquid (arishta) traditional formulation prepared from *Dashamoola* (ten roots), which is an equal proportion of combination of roots of following ten plants: *Aegle marmelos* (L.) Corr. (*Bilva*; Family: Rutaceae), *Premna optusifolia* R. Br. (*Agnimantha*; Family: Verbenaceae), *Gmelina arborea* Roxb. (*Gambhari*; Family: Verbenaceae), *Oroxylam indicum* (*Shyonak*; Family: Bignoniaceae), *Stereospermum colais* Mabb. (*Patala*; Family: Bignoniaceae), *Desmodium gangeticum* (L.) DC. (*Shalparni*; Family: Fabaceae), *Solanum anguivi* Lam. (*Bruhati*; Family: Solanaceae), *Solanum virginianum* L. (*Laghukantakari*; Family: Solanaceae), *Tribulus terrestris* L. (*Gokshur*; Family: Zygophyllaceae), *Uraria picta* (Jacq.) Desv. ex DC. (*Prishniparni*; Family: Fabaceae).

Along with these ten plants, additional 62 ingredients which also include some plants are mixed in a proportion and kept further for fermentation.

1.8.11 *Triphala*

Triphala is a traditional herbal formulation, consisting equal parts of three medicinal plant's fruits namely *Terminalia chebula* Retz. (Haritaki; Family: Combretaceae), *Terminalia belerica* (Gaertn.) Roxb. (Vibhitaki; Family: Combretaceae) and *Embellica officinalis* Gaertn. or *Phyllanthus emblica* L. (Amalaki; Family: Euphorbiaceae). In this study we have used *Triphala churna*, which is 1:1:1 combination of powders of these three dried fruits.

1.8.12 '*Triphala Guggul*'

'*Triphala Guggul*' is a *Guggul* based traditional formulation. *Guggul* (Myrrh) oleoresin is obtained from the *Commiphora mukul* (Family: Burseraceae) tree. '*Triphala Guggul*' is prepared by taking *Triphala*, *Piper longum* fruit and *Guggul* resin in 1:1:5 combinations.

1.9 Images of plants and their parts used

Figure 3: *Acacia nilotica* ssp. *indica* (Bth) Brenan habit and gum

Figure 4: *Bombax ceiba* L. habit and gum

Figure 5: *Boswellia serrata* Roxb. habit and gum

Figure 6: *Piper longum* L. habit and inflorescence

Figure 7: *Ricinus communis* L. habit and leaves

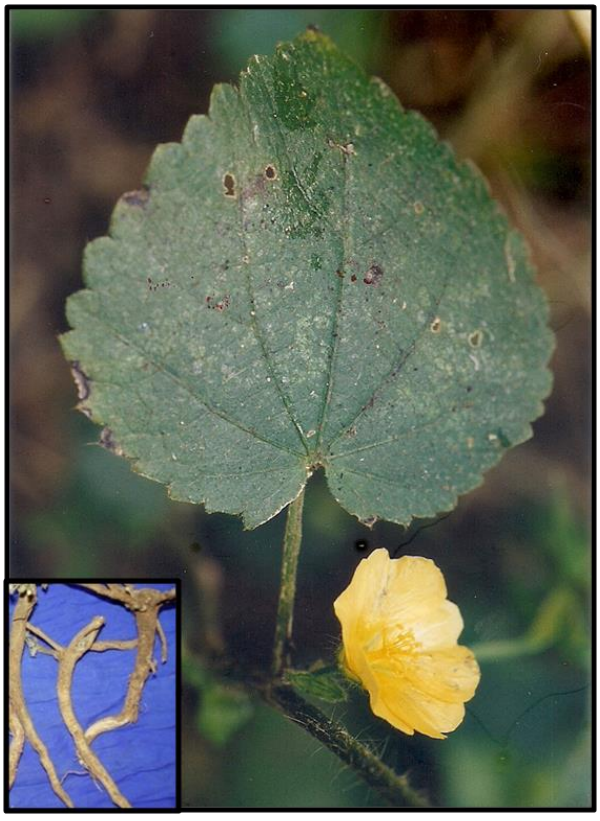

Figure 8: *Sida cordifolia* L. habit and roots

Figure 9: *Tribulus terrestris* L. habit and fruits

Figure 10: *Vitex negundo* var. *incisa* (Lam.) C.B.Cl. habit and leaves

Figure 11: *Zingiber officinale* Rosc. cultivation and dry rhizomes

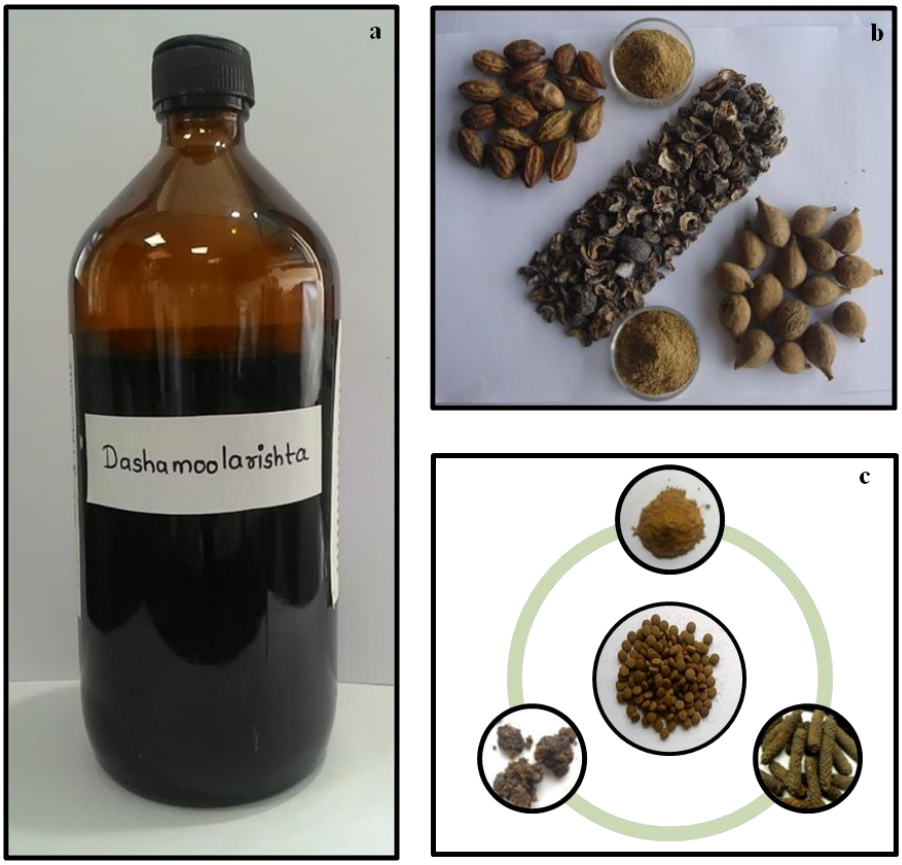

Figure 12: Formulations. (a) Dashamoolarishta shown on left side; (b) Triphala powder is a combination of fruits of *T. chebula* [upper left corner], *T. belerica* [lower right corner] and *E. officinalis* [middle image]; (c) '*Triphala Guggul*' is a combination of *Triphala*, *Guggul* gum and fruits of *P. longum*.

1.9 Genesis of thesis

According to WHO, OA is the second commnest musculoskeletal problem in the world population. Treatment of OA is becoming a major medical issue, as the age increases. Despite the availability of conventional drugs like NSAIDs for OA, their efficacy is limited in a proportion of patients coupled with their high cost and severe adverse effects. It has necessitated the search for newer chondroprotective drugs for this debilitating disease. Thus, it is imperative to explore a potential drug that may help to reduce inflammation, protect cartilage damage, offer relief from disease symptoms and restore patient activity levels and improve joint functions, with minimal side effects. Such a combination of properties is seen in traditional remedies. The ancient documents made by Charaka, Sushrutha and many other contributors suggests that the ancient science of life, provides a safe alternative treatment using herbal drugs with least or no side effects to treat arthritic pain. OA patients are therefore increasingly using complementary and alternative medicines (CAM) that primarily is thought to be chondroprotective, either in the form of nutraceuticals or in the form of herbals, and their different combinations. However, there is inadequate scientific evidence to support the clinical efficacy of many of these alternative medicines. Thus choosing an herbal remedy for the treatment of OA can open a new area of research and relief for all the people suffering from OA. Currently, research drive is focused towards regeneration of cartilage through disease modifying drugs (Schiphof, 2011). Herbal medicine is the basis of various traditional medicine systems around the world. Plants are the source of ~25% of currently used crude drugs, with another 25% obtained from chemically altered natural products (Ahmed et al., 2005a). Now a day, the research on herbal medicines is at peak and more attention is being focused towards elucidation of their underlying molecular mechanisms.

1.10 Hypothesis of thesis

Traditional herbal intervention can be a better and safer alternative for the management of osteoarthritis.

Chapter 2
Objectives

Considering the problems with NSAIDs to treat OA and potential of complementary and alternative medicines (CAM) in the form of herbals, there is a need to generate scientific evidence to explore for possible treatments from these alternative medicines. Hence with these views, the objectives for present study are as follows:

1. To collect and process plant material.
2. To comparatively evaluate anti-osteoarthritic potential of selected herbs using *in vivo* model.
3. To comparatively evaluate anti-osteoarthritic potential of selected gums and formulations using *in vivo* model.
4. To develop and comparatively evaluate anti-osteoarthritic potential of selected new combination formulations using *in vivo* model.
5. To evaluate anti-arthritic effects of new combination formulation using HIG-82, rabbit synoviocytes.

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Chapter 3
Review of literature

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The socioeconomic impact of OA is high on society. Due to aging and obesity, cost for the management of OA will expect to increase in the future. More than 10% of the population above 60 years is affected by OA, worldwide (Hunter et al., 2014). Current treatments for OA are limited. In addition to physiotherapy, exercise and weight loss; pharmacological treatments with local (intra-articular) injections of corticosteroids and/or systemic administration of analgesics and non-steroidal anti-inflammatory drugs (NSAIDs), are restricted to symptomatic relief. Analgesics and NSAIDs, which are commonly prescribed to OA patients, generally decrease pain and improve function, but have no demonstrated beneficial effect on chondroprotection or OA prevention and modification (Thysen et al., 2015). In most severe cases, osteotomy (a surgical intervention that change the load pattern in the joint by altering leg alignment) or joint prosthesis (replacement of the joint with an artificial device) appear to be the only options to partially restore joint functionality and thus improve quality of life. Long-term use of available pharmacological agents to relieve OA symptoms is associated with substantial side effects (Leong et al., 2013).

The goal of OA management is to search for new therapeutic strategies that could prevent, reduce or stop the progression of the disease or resolve the existing damage to the joint. However, the development of such interventions is a complex and challenging due to the multifactorial complexity of the disease (Bijlsma et al., 2011; Martel-Pelletier et al., 2012). There is currently no cure for OA and there are no therapies which slow or arrests OA progression (Martel-Pelletier et al., 2012). Given the nature of OA and limitations to its conventional medical management, life-long treatment will likely be required to arrest or slow its progression, also improve symptoms, and are safe for clinical use. Thus, it is necessary to find out alternative chondroprotective drugs which are routinely used in traditional systems which play a significant role in treatment of OA with minimum or without imparting any side effects and help in management of these disease in the era of modern medicine.

Traditional herbal medicines have the potential to provide a solution to this problem. Now a day's plant based products are in greater demand because they are perhaps considerably safe and efficacious even when taken over a long period of time. About 47 % of the adults use non-prescribed alternative medications (including food supplements

and nutraceuticals) for the management of OA (Leong et al., 2013). In *Ayurveda*, the disease *Sandhigatavata* is described under *Vatavyadhi* in all the *Samhitas* and *Sangraha Granthas*. OA of modern medical science is considered to be its close equivalent (Yadavaji, 2001). It is said to be caused by the excessive intake of *vata vrudhi kara ahara* like *katu*, *tikta* and *kashaya rasa pradhana dravya* and *ativyayama* or *abhighata* (injuries) (Gandagi and Patil, 2015). *Ayurveda* mentions use of various plants and formulations for treatment of OA. Herbs like *Acacia nilotica* ssp. *Indica* (Bth) Brenan, *Bombax ceiba* L., *Boswellia serrata* Roxb., *Sida cordifolia* L., *Piper longum* L., *Zingiber officinale* Rosc., *Ricinus communis* L., *Vitex negundo* var. *incisa* (Lam.) C.B.Cl., *Tribulus terrestris* L., *Withania somnifera*, (L.) Dunal, *Asparagus racemosus* Willd., *Emblica officinale* Gaertn., *Symplocos racemosa* Roxb., *Commiphora mukul* (Hook. ex Stocks) Engl., *Laccifer lacca* and formulations like *Triphala*, '*Triphala Guggul*', '*Laksha Guggul*', *Dashamoolarishta* etc. are commonly used for the treatment of OA by practitioners (Wilson et al., 2007; Jain et al., 2011; Siddiqui, 2011; Chhatre et al., 2014; Ladda and Kamthane, 2014).

Present review of literature has summarized the effects and potential mechanism of action of herbs and formulations for OA treatment. Furthermore, we have described about the herbal products prescribed for OA treatment. For this study, we have focused on herbs and formulations which are routinely prescribed by the physicians and have strong *in vitro* and pre-clinical evidence for treating OA, but are not well studied for their mechanism of action. The plants selected for this study are *Acacia nilotica, Bombax ceiba, Boswellia serrata, Piper longum, Ricinus communis, Sida cordifolia, Tribulus terrestris, Vitex negundo* and *Zingiber officinale*. Formulations are *Dashamoolarishta*, *Triphala* and '*Triphala Guggul*'.

3.1 National and international reports on activities of herbs and formulations with respect to OA

Anemarrhena asphodeloides Bunge (Family: Agavaceae) is a Chinese herb and its anti-inflammatory and protective effect in osteoarthritic cartilage has been reported (Kang et al., 2010). *In vitro* study on Avocado soybean unsaponifiables (ASU) showed that it has decreased the spontaneous production of stromelysin, prostaglandin E2

(PGE2), cytokines etc. by chondrocytes, and partially reversed the IL-1β effects like induced production of MMP-3, IL-6 and IL-8. ASU also enhanced the incorporation of newly synthesized prostaglandins in the cartilage matrix. It increased the basal synthesis of aggrecan as well (Henrotin et al., 1996). Appleboom et al. (2001) stated that ASU significantly decreased the pain in OA patients. *Camellia sinesis* (L.) Kuntze (Family: Theaceae) used to produce tea, has been shown to inhibit iNOS, COX-2 and NF-kB pathways (Singh et al., 2003). In collagen induced arthritis (CIA) in mice, it has shown marked inhibition in inflammatory mediator's viz. COX2, interferon-γ and TNF-α (Haqqi et al., 1999). Pretreatment of human osteoarthritis chondrocytes with green tea catechins, significantly inhibited the expression of MMPs, *in vitro* and they have also inhibited the degradation of proteoglycan & type II collagen and selectively inhibited the aggrecanases, ADAMTS-1, -4 & -5 in human cartilage (Adcocks et al., 2002; Vankemmelbeke et al., 2003; Ahmed et al., 2004). *C. sinesis* polyphenols showed increased bone mineral density and serum osteocalcin level, thus indicating its role in bone health (Shen et al., 2011). Capsaicin is an amide isolated from genus Capsicum, showed greater improvements in OA patients, compared to placebo (Deal et al., 1991). In a clinical trial, Chicory root improved pain and relieved joint stiffness in OA; it inhibited production of COX-2, iNOS, TNFα and NF-κB (Olsen et al., 2010). *Clerodendrum phlomidis* L. f. (Family: Verbinaceae) reported to reduce the swelling associated with OA (Kilimozhi et al., 2009). Curcumin is a diarylheptanoid from Turmeric, reported to inhibit the incorporation of arachidonic acid into membrane lipids, leukotriene B4 and leukotriene C4 synthesis, PGE2 production as well as the secretion of collagenase, hyaluronidase & elastase by macrophages (Wallace, 2002). It also inhibited IL-1β-induced upregulation of MMP-3, decrease in type II collagen synthesis was also blocked in human chondrocyte cells (Shakibaei et al., 2007; Henrotin et al., 2010). Similarly, it inhibited collagenase and stromelysin expression in HIG-28 rabbit chondrocytes (Jackson et al., 2006). *In vitro* studies on OA showed that curcumin acts through suppression of NF-κB mediated IL-1β/TNFα catabolic signaling pathways, inhibiting production of MMP-3, -9 & -13, and inhibiting the JNK, AP-1 & NF-κB pathways (Liacini et al. 2002, 2003; Shakibaei et al., 2007; Mathy-Hartert et al., 2009). *Harpagophytum procumbens* (Burch.) DC. ex Meisn. (Family: Pedaliacea) is found in South Africa. Main active

compound, harpagoside from the plant has shown to inhibit the IL-1β induced production of MMP-1, -3 & -9 in human chondrocyte cells (Schulze-Tanzil et al., 2004). In a randomized placebo trial, it also significantly reduced pain in OA patients, compared to placebo (Lecomte and Costa, 1992). *Humulus lupulus* L. (Family: Cannabaceae) is widely found in Northern hemisphere. *In vivo* studies have reported its potential to inhibit PGE2 and COX-2 production in OA (Hougee et al., 2006). *Lonicera japonica* Thunb. (Family: Caprifoliaceae) is another Chinese herb, which has shown anti-inflammatory activity in *in vivo* OA models (Kwak et al., 2003; Kang et al., 2010). *Passiflora edulis* Sims (Family: Passifloraceae) found in almost all parts of world. Flavonoid from the plant attenuated inflammation through inhibition of regulatory enzymes of arachidonic acid metabolism (Arjmandi et al., 2004; Hooshmand et al., 2007). *In vivo* studies have reported that *Perna canaliculus* (Family: Mytilidae) alleviated the pain associated with OA (Hielm-Bjorkman et al., 2009). *Punica granatum* L. fruit extract suppressed joint inflammation and damage in CIA in mice (Shukla et al., 2008). It is also reported to inhibit IL-1β induced expression of MMP-1, -3 & -13 mRNA as well as proteins in OA chondrocyte cells (Ahmed et al., 2005b). Gil et al. (2000) have demonstrated reduced expression of MAPK and NFκB in OA chondrocyte cells, *in vitro* with *Punica granatum* L. (Family: Punicaceae) treatment. *Rosa canina* L. (Family: Rosaceae) is widely cultivated in Europe, Northwest Africa and Western Asia. Rosehip powder is extracted from fruits of this plant. Hyben vital is a phytomedicinal preparation of powder, has shown safety and efficacy in the OA treatment (Rein et al., 2004). It has also been reported to inhibit the production of NO & PGE2, and reduce the secretion of cytokines (TNF-α, IL-1β), chemokines and various MMPs such as MMP-1, -3 & -13 in OA (Schwager et al., 2011). A meta-analysis of randomized controlled trials showed rosehip powder reduced pain and led to reduced use of analgesics in OA patients (Chrubasik et al., 2006; Christensen et al., 2008). *Tripterygium wilfordii* Hook. f. (Family: Celastraceae) showed reduction in the expression of COX-2, MMP-3, -13, PGE2, AP-1 and NFκB in OA chondrocyte cells, *in vitro* (Sylvester et al., 2001). *Uncaria tomentosa* (Willd. ex Schult.) DC. (Family: Rubiaceae) is a medicinal plant from the Amazon river basin. A number of *in vitro* and *in vivo* studies have showed its role in inhibiting TNFα and NF-κB in OA (Piscoya et al., 2001). Clinical studies have demonstrated its use in

improving the joint function in OA (Miller et al., 2005). *Urtica dioica* L. (Family: Uricaceae) is widely found in European, Asian and African countries. Clinical studies have demonstrated its use in providing mild to moderate relief in OA symptoms (Randall et al., 1999). *Salix* L. bark (Family Salicaceae) is widely found in European, Asian and African countries. *In vivo* studies have reported the potential of bark in reducing NO, IL-1β, TNF-α and IL-6 in OA (Sharma et al., 2011). The extract was also capable of reducing pain and improvement of movements in OA (Schmid et al., 2001). *Withania somniefera* (L.) Dunal (Family: Solanaceae) root extract exhibited a significant decreased levels of glycosaminoglycans (GAGs) released from cartilage explants in a subset of OA patients (Nethery et al., 1992).

Even though the active phytochemical constituents of individual plants have been well established, they usually present in minute amount and always are insufficient to achieve the desirable therapeutic efficacy. So, when these plants of varying potency combined, may theoretically produce a greater result as compared to individual use of the plant. This phenomenon of positive herb-herb interaction is known as synergism (Parasuraman et al., 2014). In a clinical trial of OA, a polyherbal formulation Gitadyl has shown reduction in symptoms (Ryttig et al., 1991). Another polyherbal formulation Phtodolor has shown pain reduction in OA trial (Long et al., 2001). In a study carried out in Korea on GCSB-5, which is a purified extract from a mixture of 6 oriental herbs. *In vitro* and *in vivo* study reported its potential for the protection of articular cartilage against progression of OA through inhibition of MMPs, inflammatory mediators and NF-*κ*B activation (Kim et al., 2012). Zheng et al. (2013) reported pharmacological action of active compound from Duhuo Jisheng decoction (DHJSD), is a widely used traditional Chinese formulation against OA. TLPL/AY/03/2008, an herbal formulation contains *Boswellia serrata, Commiphora mukul, Withania somnifera, Vitex negundo, Ricinus communis, Nyctanthes arbortristis* L. and *Zingiber officinale*; developed and manufactured by Tulip Lab Private Limited, India. It significantly reduced joint pain, improved joint function and mobility in subjects suffering from knee OA and it is safe for use (Nipanikar et al., 2013). Recently, prospective, open labeled, randomized active controlled study carried out in Maharashtra for one year. Wherein, herbal formulation containing extracts of *Boswellia serrata, Alpinia galangal, Commiphora wightii, Glycyrrhiza glabra, Tinospora cordifolia* and

Tribulus terrestris, offered better symptomatic relief of pain than paracetamol in OA (Patil and Murthy, 2016).

Various plant based products are now prescribed for OA, like Ostolief is a polyherbal formulation of Charak Pharma Pvt. Ltd., Mumbai that contains ingredients like *B. serrata, W. somnifera, T. terrestris, T. cordifolia, S. cordifolia* and *Strychnos nux vomica*. In clinical trial, Ostolief emerges as a disease-modifying therapy with potent anti-inflammatory and analgesic activity (Langade et al., 2006). Rumalaya forte is another polyherbal formulation of the Himalaya Drug Company, Bangalore. It contains herbs, such as powders of *Boswellia serrata, Commiphora wightii, Alpinia galanga, Glycyrrhiza glabra* and extracts of *Tribulus terrestris* & *Tinospora cordifolia*. Its efficacy and safety in long-term use and management of OA is well documented. Reosto is also a polyherbal formulation of the Himalaya Drug Company, Bangalore. It contains herbs, such as powders of *Commiphora wightii, Vanda roxburghii, Terminalia arjuna, W. somnifera, S. cordifolia* and *Kukkutandatvak* bhasma. Its efficacy and safety in osteoporosis is also established (Nachinolcar et al., 2007).

The work on OA was initiated at our organization (IRSHA) in 2007 under the support of CSIR-NMITLI program. In which Sumantran et al. (2007a) reported the chondroprotective activity of aqueous extract of fruit powder of *Emblica officinalis* (Family: Euphobiaceae). Wherein, the extract had shown strong inhibition in collagenase type 2, on human cartilage explants, *in vitro* and caused a significant long-term decrease in levels of GAGs release. They also showed '*Triphala Guggulu*' inhibits two major enzymes, hyaluronidase and collagenase type 2 which degrades cartilage in OA. Thus, it has shown chondroprotection through inhibition of hyaluronidase and/or collagenase. Aqueous extract of *W. somniefera* (Family: Solanaceae) root powder also showed chondroprotective activity by strongly inhibiting the activities of the collagenase type II and gelatinase enzymes *in vitro,* on OA cartilage explants (Sumantran et al., 2007c). Furthermore, the effects of aqueous extracts of *W. somnifera* root and glucosamine sulphate were tested on the levels of nitric oxide and GAG secreted by knee cartilage from chronic OA patients and both drugs showed anti-inflammatory activity (Sumantran et al., 2008). In another study they have evaluated the *in vitro* anti-arthritic effects of new *Ayurvedic* formulation containing *Zingiber officinale* root, *Tinospora cordifolia* stem,

Phyllanthus emblica fruit and oleoresin of *Boswellia serrate*, which showed significant inhibition in the release of GAGs and aggrecan by cartilage explants from OA patients (Sumantran et al., 2011).

Taking these leads further and based on review of literature, it has decided to study with 9 herbs and 3 formulations which are described below.

3.2 Selected herbs and formulations

Review of literature of selected plants is described below with respect to their *Ayurvedic*, ethnobotany and pharmacology uses. Ethnobotany uses is devided according to plant parts. In pharmacological activities; *in vitro*, *in vivo* and clinical activity with respect to anti-oxidant, anti-inflammatory followed by anti-arthritic activities are mentioned

3.2.1 *Acacia nilotica* ssp. *indica* (Bth) Brenan

Traditional uses

Properties and actions of *A. nilotica* mentioned in *Ayurvedic* texts are: *Rasa* (Test based on activity)-*Kasaya*; *Guna* (Properties)-*Guru* (heavy to digest), *Ruksha, Visada*; *Veerya* (potency)-*Sita*; *Vipaka* (taste after digestion based on activity)-*Katu* (pungent); *Karma* (Pharmacological actions)-*Grahi*. *Kaphahara*, *Visaghna*. *KaphaPitta Samak, Rakta Rodhak, Bran Ropan, Sthamvan, Sankoshak, Krimighan, Sanhan, Grahi, Rakt Pitta Samak, Mutral, Daah Pra Saman* (Kapoor, 2000).

A. nilotica bark and seeds are used as a source of tannins (Bargali and Bargali, 2009). The juice of bark mixed with milk, is dropped into the eye for conjunctivitis. The burnt bark and burnt almond shell both pulverized and mixed with salt to make a good tooth-powder (Farzana et al., 2014). The bark is also used in treating hemorrhages, wounds, ulcers, chronic dysentery, colds, diarrhea, tuberculosis, skin diseases, bronchitis, seminal weakness, oral ulcers and leprosy (Bargali and Bargali, 2009). Decoction of bark is largely used as an astringent douche in gonorrhoea, cystitis, vaginitis, leucorrhoea, prolapse of the uterus & piles, gargle and mouth wash. Extract of seeds are used for general body vigour (Farzana et al., 2014). The Resins repel insects and water (Malviya et al., 2011). The gum is reported to be used to treat cough, asthma, diarrhoea, dysentery,

haemorrhages, leprosy, burns, colic, intermittent fever and general debility (Oommen et al., 2000). It is also used as suspending and emulsifying agent and in preparation of many formulations. The gum is expectorant, antipyretic and cures lung troubles. It is administered in the form of mucilage in diarrhea, dysentery and diabetes mellitus. Powdered gum mixed with the white of an egg, is applied on burns and scalds. Fried in ghee, the gum is useful as a nutritive tonic and aphrodisiac in cases of sexual debility (Farzana et al., 2014). Leaves are astringent, tonic to the liver and the brain, antipyretic, enriches the blood. Tender leaves beaten into a pulp are used as a gargle in spongy gums, sore throat and as wash in hemorrhagic ulcers and wound. The tender leaves infusion used as an astringent and remedy for diarrhoea and dysentery (Farzana et al., 2014). Bruised tender leaves formed into a poultice and applied to ulcers act as stimulant and astringent. Also promotes and strengthens the vision and cure eye diseases. The pods have molluscicidal and algicidal properties (Malviya et al., 2011). Pods decoction is beneficial in urogenital diseases and prevents premature ejaculation. It is an astringent and injected to allay irritation in acute gonorrhoea and leucorrhoea. The pods are used for impotency and in dry cough. Powder of root is useful in leucorrhoea, wound healing and in burning sensation. The fruits are found to be useful in diarrhoea, dysentery and diabetes (Farzana et al., 2014). Roots are used as an aphrodisiac. Flowers used to treat syphilis lesions (Bargali and Bargali, 2009; Malviya et al., 2011).

Pharmacological uses

Different extracts of bark showed 44-90 % inhibition of oxidation of linoleic acid and 49-87 % DPPH radical scavenging activity. Bark extract also increased the antioxidant enzymes viz., catalase, superoxide dismutase, glutathione-S transferase and glutathione peroxidase activities in the liver of N nitrosodiethylamine-administered rats (Sultana et al., 2007). *Acacia* species are rich source of polyphenolic compounds, known to have strong antioxidant properties that help in prevention and therapy of various oxidative stress related diseases (Singh et al., 2009; Duganath, 2010). Singh et al. (2009) evaluated eight different green pod extracts for effective and strong *in vitro* & *in vivo* metal chelations and free radical scavenging activity. Different compounds like umbelliferone from the stem bark, keampherol from methanol extract, have been reported to possess potent antioxidant activity (Singh et al., 2008; Singh and Singh, 2008; Singh et al., 2010).

It was reported that fruit, flower, pod, bark extracts, various plant root and leaf extracts possesses potent anti-oxidant activity (Sundaram and Mitra, 2007; Kalaivani and Mathew, 2010; Abuelgassim, 2013; Rasool et al., 2013; Abdel-Faridetal., 2014). *A. nilotica* aqueous extract showed anti-inflammatory activity in carrageenan-induced paw edema (Dafallah and Al-Mustafa, 1996). Similarly, androstene, isolated from aerial parts of the plant showed dose-dependent anti-inflammatory activity in TPA-induced mouse ear edema (Chaubal et al., 2003). Cassane, a diterpene niloticane obtained from ethyl acetate bark extract showed inhibitory effect against cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) (Eldeen et al., 2010). Aqueous pod extracts also showed inhibition in carrageenan-induced paw edema and cotton pellet-induced granuloma models in rats (Sokeng et al., 2013). Khan et al. (2015) showed methanol extract of pods, aqueous leaf & bark extract possesses anti-inflammatory activity.

3.2.2 *Bombax ceiba* L.

Traditional uses

Properties and actions of *B. ceiba* mentioned in *Ayurvedic* texts are: *Rasa-Madhura, Kashaya*; *Guna-Snigdha* (Unctuous); *Veerya*-S*heeta* (cooling); *Vipak-Madhura* (sweet). *Graahi, Vrishya, Balya, Raktsthambhana, Vedana Asthapana, Rasaayani, Pramehaghna, Pravakika-Shamana, Daahadhaman* (Kapoor, 2000).

Ethnobotanically, it has stimulant, astringent, haemostatic, aphrodisiac, diuretic, anti-diarrhoeal, cardiotonic, emetic, demulcent, anti-dysentric, alternative and anti-pyretic properties (Jain et al., 2009). Leaf is used in glandular swellings, rheumatism, antidysentric, haematinis, menorrhagia, leucorrhoea, anaemia and infertility. Flower is used in haematuria, anaemia, lecucorrhoea, haemorrhoids, hydrocoele, gonorrhoea, menstrual disorders & leucorrhoea, boils & sores, splenomegaly, internal bleeding, cancer, colitis, premature ejaculation, snakebite, permanent sterilization, diuretic and laxative. Fruit is used as anti-fertility agent, in uterus protrusion, leucorrhoea etc. Fruit and heartwood are anti-diabetic, anti-diarrhoeal and used in snakebite. Seeds are used in chicken pox and small pox. Spines are used in skin troubles, acne and headache (Jain et al., 2009; Antil et al., 2013). Stem and bark are used in treatment of bacterial, protozoal and viral infection and digestive disturbances, boil, heatburn, heart tonic, kidney stone,

spermatorrhoea & weakness, headache, dislocated bones, snakebite, scorpion, centipede and spider stings. Gum is used in asthma, giardiasis, bleeding piles, diarrhoea & dysentery, dental caries, aphrodisiac and in scabies. Roots are used in diarrhoea, dysentery, boils, burns, diabetes, impotency & as aphrodisiac, scorpion string, snakebite, sex tonic, urinary troubles, brain tonic, gonorrhoea, syphilis, bedwetting, leucorrhoea and spermatorrhoea (Jain et al., 2009).

Pharmacological uses

Dar et al. (2005) isolated mangiferin, 2-beta-D-glucopyranosyl-1, 3, 6, 7- tetrahydroxy-9H-xanthen-9-one, from methanolic extracts of *Bombax ceiba* leaves and demonstrated strong antioxidant activity using DPPH assay. Gandhare et al. (2010) evaluated the antioxidant potential of bark. Several concentrations of ethanolic and aqueous extracts were compared for their antioxidant activity in different *in vitro* models and it was observed that free radicals were scavenged by the extracts in a concentration dependent manner. Ethanol extracts of resin and methanol extracts of *B. ceiba* possesses anti-oxidant activity (Vieira et al., 2009; Rao et al., 2015). Kumar (2011), showed the anti-oxidizing property of ethyl acetate soluble fraction of the gynoecium part of *B. ceiba* and also revealed that ethyl acetate soluble fraction of *B. ceiba* possesses remarkable anti-inflammatory property. Later on, in *in-vitro* anti-inflammatory activity assessed by human red blood corpuscles membrane, *B. ceiba* extracts showed significant activity compared to diclofenac (Anandarajagopal et al., 2013). Methanol extract of *B. malabaricum* leaves demonstrated significant anti-inflammatory activity in dose dependent fashion and it is because of reduced secretion of prostaglandins due to inhibition of the production of one of the principal mediators for inflammation like nitric oxide (Hossain et al., 2013).

3.2.3 *Boswellia serrata* Roxb.

Traditional uses

Properties and actions of *B. serrata* mentioned in *Ayurvedic* texts are: *Rasa-Kasaya*, *Tikta*, *Madhur* (sweet); *Guna-Laghu* (light), *Ruksha*; *Veerya-Sheeta*; *Vipak-*

Katu. *Kapha Pitta Samak, Sotha Har, Bran Sodhan, Bran Ropan, Chachusya, Dipan, Pachan, Grahi, Vata Anuloman, Hirdya, Mutral, Jwaraghan* (Kapoor, 2000).

The gummy exudate from *Boswellia* is grouped with other gum resins and referred to collectively as *guggals*. In addition to its beneficial use for arthritis, this gummy resin is also mentioned in traditional texts as an effective remedy for diarrhoea, dysentery, ringworm, boils, fevers (antipyretic), skin & blood diseases, cardiovascular diseases, mouth sores, bad throat, bronchitis, asthma, cough, vaginal discharges, hair-loss, jaundice, hemorrhoids, syphilitic diseases, irregular menses and stimulation of liver. It is also diaphoretic, astringent, diuretic and acts both as internal and external stimulant (Siddiqui, 2011). Gum and bark are employed in drug formulations reported to be used in the treatment of ulcers, cystic breast, piles, skin diseases, diarrhea and dysentery (Oommen et al., 2000).

Pharmacological uses

B. serrata contains anti-inflammatory triterpenoids called boswellic acids (BA), reported as a inhibitor of human leukocyte elastase-a member of serine proteases subfamily, which can hydrolyze collagen IV and elastin of the extracellular matrix (Safayhi and Ammon, 1997). Acetyl-11-Keto-β-Boswellic Acid (AKBA) is a naturally occurring pentacyclic triterpene isolated from the gum resin exudate from the stem of *B. serrata*, which shown inhibition of NF-κB activation by TNFα, IL-1β, LPS etc. (Takada et al., 2006). An *in vivo* study evaluated effect of *B. serrata* extract and ketoprofen, on glycosaminoglycan (GAG) metabolism. In which *B. serrata* significantly reduced the degradation of GAGs compared to controls (Reddy et al., 1989; Blain et al., 2010). 5-Loxin® is a novel *B. serrata* extract contain at least 30 % AKBA using a selective enrichment process. Extract reported to inhibit the 5-lipoxygenase in 90-days in a placebo-controlled clinical trial. A significant reduction of MMP-3 in synovial fluid was also observed. Thus 5-Loxin reduces pain as well as significantly improves physical functioning in patients with OA and is safe for use (Sengupta et al. 2008). Another novel composition of *B. serrata* gum extract, Aflapin has demonstrated anti-inflammatory and anti-arthritic activity in a rat model. Aflapin showed significant protection from IL-1β-induced death of human primary chondrocytes, improved GAGs production and inhibited MMP-3 production. In patients with knee OA, Aflapin was found to be more effective

than Loxin alone (Sengupta et al. 2010, 2011). In a double-blind, placebo-controlled trial, patients were given either 1000 mg *Boswellia* daily or placebo in three divided doses for eight weeks. Patients in the *Boswellia* group experienced a significant ($p<0.001$) decrease in pain, swelling and increases in range of motion in knee OA patients, compared to placebo (Kimmatkar et al., 2003). In a randomized, prospective, open label, comparative study effect of *Salai* extract was compared with valdecoxib in patients of OA of knee for six months, concluded in terms of safety, efficacy and duration of action, showed *Salai* extract superior to valdecoxib (Sontakke et al., 2007). A clinical study receiving *B. serrata* extract reported to decrease knee pain, increased knee flexion and increased walking distance. The frequency of swelling in the knee joint was also decreased (Ghosh et al., 2015). In another double blind placebo controlled, crossover study, *B. serrata* used in combination with other herbs. This treatment showed significant decrease in pain severity ($p<0.001$) and disability scores ($p<0.05$), compared to placebo (Kulkarni et al., 1991). In a randomized trial of multiplant *Ayurvedic* drugs containing *B. serrata* revealed the potential efficacy in the symptomatic treatment of knee OA (Chopra et al., 2004). Akhtar et al. (2011) studied the efficacy of herbal-laucine mix (HLM) which contains *B. serrata* as one of the constituent. HLM had strongly inhibited iNOS, MMP-9 & -13 expressions and NO production in IL-1β stimulated OA chondrocyte cells, *in vitro*. HLM also showed protection to cartilage by inhibiting GAG release from human cartilage explants, *in vitro*. These inhibitory effects were mediated by inhibiting the activation of NF-κB in human OA chondrocyte cells. Sumantran et al. (2011) evaluated *in vitro* anti-arthritic activity of new formulation containing *B. serrata* and the data strongly suggest that oleoresin of *B. serrata* plays a crucial role in the chondroprotective activity of this formulation. The *Gugguls*-gum-resin possesses anti-rheumatic activity (Siddiqui, 2011; Akhtar and Haqqi, 2012).

3.2.4 *Piper longum* L.

Traditional uses

Properties and actions of *P. longum* mentioned in *Ayurvedic* texts are: *Rasa-katu*; *Guna-laghu, snigdha* (unctuous), *Teekshna*; *Veerya-Sheeta*; *Vipak-Madur*. *KaphaVat*

Samak, Pitta Samak, Dipan, Vatanuloman, Swashar, Mutral, Jawaraghana, Khustaghana, Rasayan, Balya (Kapoor, 2000).

An equal part of powdered seeds of *Embelia ribes*, fruit of *P. longum* and borax powder has been used as an *Ayurvedic* contraceptive (Chopra et al., 1956). Immature spikes, roots, seeds and dried unripe fruits are used as an alternative to tonic; long pepper tonic acts as a valuable alternative tonic in paraplegia, chronic cough, enlargement of the spleen and abdominal viscera. The roasted fruits are beaten up with honey and give to treat rheumatism. The dried immature fruit and the root are extensively used in acute and chronic bronchitis. The unripe fruit is sweetish, cooling and useful in biliousness. The ripe fruit is sweet, pungent, a stomachic, aphrodisiac, alternative, laxative, anti-diaeehoeic, anti-dysentric, is useful in *Vata* & *Kapha*, asthma, bronchitis, abdominal complaints, fevers, leucoderma, urinary discharges, tumours, piles, diseases of the spleen, pains, inflammation, leprosy, insomnia, jaundice, hiccoughs and tubercular glands. The dried spikes are acrid, mildly thermogenic, stomachic, aphrodisiac, carminative, expectorant, febrifuge, tonic, laxative, digestive, emollient and antiseptic. They are useful in anorexia, dyspepsia, flatulent colic, asthma, bronchitis, hiccoughs, gastropathy and epilepsy (Selvam, 2008). The roots are bitter, thermogenic, tonic, diuretic, purgative, expectorant, anthelmintic, stomachic, digestive and emmenagogue, they are useful in vitiated conditions of *Vata*, gout, lumbago, dyspepsia, apoplexy, stomachalgia and splenopathy. Roots are also used for the treatment of heart disease. A decoction of the roots is given for relief in swellings of the joints of cattle in the north-western Himalayan regions (Chopra et al., 1956). An infusion of the root is prescribed after parturition, to help in the expulsion of the placenta (Khushbu, 2011).

Pharmacological uses

P. longum extract possesses free radical scavenging activity (Joy et al., 2010; Yadav et al., 2014). Antioxidant activities of various fruit extracts were evaluated by using *in vitro* antioxidant assay models like phosphomolybdenum and reducing power assay. The percentage of antioxidant activity by phospho-molybdenum assay was in the order acetone>ethanol>water>petroleum ether. The results showed that the fruits have antioxidative activity (Anu et al., 2013). According to Stohr et al. (2001) *Piper* extracts and piperine possesses inhibitory activities on prostaglandin and leukotrienes, COX-1;

thus exhibit anti-inflammatory activity (Kumari et al., 2012). The aqueous extract of seeds showed inhibition in paw swelling in freund's complete adjuvant induced arthritis in rats by inhibiting the adherence of neutrophils to endothelial monolayer by suppressing the TNF-α induced expression of intercellular adhesion molecule-1, vascular cell adhesion molecule-1, E-selectin and also inhibited the NF-κB (Yende et al., 2010). Bae et al. (2010) showed that the chloroform extract expressed the anti-inflammatory activity by inhibiting adhesions of neutrophils to endothelial monolayer by blocking the TNFα induced expression of CAMs and E-selectin through inhibition of NF-κB in endothelial cells. At the same time the extract exhibited antioxidant activity which further contributes to the anti-inflammatory activity (Bae et al., 2010). Piperine showed anti-inflammatory activity through inhibition of LPS-induced endotoxin shock via inhibition of IFN production. Fruit oil of *P. longum* has shown to be useful for joint pain (Wilson et al., 2007). Bang et al. (2009) showed that piperine has anti-arthritic activity as it significantly inhibited the production of IL-6, PGE2 and MMP-13 in IL-1β stimulated fibroblast like synoviocytes. Majoon Yahya bin Khalid is a formulation containing *P. longum* as one of the ingredient; which is effective in reducing joint pain, stiffness and restriction of joint movements in open label evaluation study of primary knee OA (Khalid et al., 2015).

3.2.5 *Ricinus communis* L.

Traditional uses

Properties and actions of *R. communis* mentioned in *Ayurvedic* texts are: *Guna-Snigdha, Tiksna, Suksma*; *Karma-Rechana, Vrishya*. *Upayokta* are *Pliharoga, Udavarta, Vastishoola, Gulma, Antravruddhi, Katishoola, Vatarakta, Kasa* (Gandagi and Patil, 2015).

The plant is commonly called as Castor and its oil is obtained from seeds. Castor oil is a cathartic and along with quinine sulphate it is given to induce labour. It is used as a contraceptive and externally for dermatitis and eye ailments (Rana et al., 2012). Seeds are used in paralysis, for wound healing and also applied in hemorrhoids. Oil is a laxative and vermicide; used in treatment of rheumatic arthritis, acute diarrhoea, paralysis, epilepsy, distention of the uterus, used as ear drops to hardened cerumen and also used for warts (Ladda and Kamthane, 2014). The seed is cathartic and aphrodisiac, also used

to reduce gouty & rheumatic swellings and inflammation of the breasts during lactation (Gupta and Singh, 2015). Leaves are said to give relief in caries and are applied over guinea-worm sores to extract the worm, used to wash the skin ulcer, in headache, in boils, on sores & swellings, in flatulence in children, used as an emetic in poisoning by narcotics like opium as well as lotion for the eye. They are also applied over the breast for milk stimulation (Rana et al., 2012; Ladda and Kamthane, 2014). The leaves of plant are active against mycobacteria & yeast, and are occasionally fed to cattle (Rana et al., 2012). Bark is used as dressing for ulcers and sores. Flowers are useful in glandular tumors, anal troubles, vaginal pain as well as in arthralgia. Fruit is an appetizer useful in tumors and pain, *Vata*, piles, diseases of the liver and spleen. The root bark is used as a purgative and alternative in chronic enlargements and skin diseases. Roots are used for toothache, in inflammations, pains, asicites, fever, glands, asthma, eructations, bronchitis, leprosy, diseases of the rectum, and for headache (Ladda and Kamthane, 2014).

Pharmacological uses

Seed, stem and various leaf extracts possesses free radical scavenging potential (Gupta et al., 2006; Kumar et al., 2010; Ganiyat, 2012; Nemudzivhadi and Masoko, 2014). Oloyede (2012) reported that responsible chemical constituents of antioxidant activity are Methyl ricinoleate, Ricinoleic acid, 12 octadecadienoic acid and methyl ester. Leaves contain major phenolic compounds possessing anti-oxidant activity (Taur et al., 2011). Methanol extract of aerial parts possesses significant radicals scavenging abilities on DPPH, NO, and superoxide radicals (Iqbal et al., 2012). The methanol extract of leaves had shown the anti-inflammatory activities in both the acute and sub-chronic models in rats (Ilavarsan et al., 2006; Saini et al., 2010). Possible pharmacological mechanism involved in anti-inflammatory action is related to the inhibition of the Phospholipase A2 (Valderramas et al., 2008). Anti-inflammatory and free radical scavenging activities of the methanolic extract of roots of *R. communis* was studied in rats. The methanolic extract at doses 250 and 500 mg/kg p. o. exhibited significant ($P<0.001$) anti-inflammatory activity in carrageenan induced hind paw edema model. The extract at the dose of 500 mg/kg p. o. also exhibited significant ($P<0.001$) anti-inflammatory activity in cotton pellet granuloma model. The methanolic extract showed significant free radical scavenging activity (Rana et al., 2012). Decoction of roots possesses anti-inflammatory activity (Doshi et al., 2014).

Study carried out by Ziaei et al. (2016) demonstrated that based on the different anti-inflammatory and analgesic evaluations, OA induced by intra-articular injection of MIA in rats were reduced with topical application of a mixture of *Lawsonia inermis* and *R. communis* extracts.

3.2.6 *Sida cordifolia* L.

Traditional uses

Properties and actions of *S. cordifolia* mentioned in *Ayurvedic* texts are: *Bhavamishra* mentioned four varieties those are *Bala, Atibala, Nagabala, Mahabala*; *Rasa-Madhura*; *Gunas-Laghu, Snigdha, Pischila*; *Virya-Sheeta*; *Vipaka-Madhura*; *Karma-Balya, Brumhana* (nourishing). *Doshaghnata* are *VataPittahara* (Gandagi and Patil, 2015). *VatPitta Samak, Badana Sthapan, Sotha Har, Balya, Bat Har, Sanehan, Anuloman, Grahi, Hirdya, Rakt Pit Samak, Sukral, Balya Brigham, Oja Bardhak* etc. (Kapoor, 2000).

S. cordifolia has been proven to be effective in balancing *Vata dosha* and its oil is used both externally and internally for arthritis (Jain et al., 2011). The roots, leaves and stems are utilized as traditional medicines in chronic dysentery, gonorrhea and asthma. It is also indicated for piles, to induce/promote aphrodisia and as a remedy for neurodegenerative diseases, including Parkinson's disease (Galal et al., 2015). The seeds of *S. cordifolia* are traditionally used as aphrodisiac and also indicated in the treatment of gonorrhea, cystisis, piles, colic and tenesumus. The roots are administered as a curative agent for nervous disorders such as facial paralysis and hemiplegia, as well as in urinary disorders. The root bark is exploited as stomachic, demulcent, tonic, astringent, bitter, diuretic, aromatic and as antiviral agent (Galal et al., 2015).

Pharmacological uses

Dhalwal et al. (2005) studied that leaf, stem, root and whole plant extracts of *S. cordifolia*. They exhibited effective reducing power and free-radical scavenging activity. The highest antioxidant activity was observed in the root extract. The anti-inflammatory activity of ethyl acetate and alcohol extracts of *S. cordifolia* was studied in rats, in which both, aerial and root extract showed dose dependent activity (Diwan and Kulkarni, 1983).

In a study, it is demonstrated that *S. cordifolia* can increase pain tolerance and appears to have anti-inflammatory properties (Kanth and Diwan, 1999). The aqueous extract of leaves showed a significant inhibition of carrageenan-induced rat paw edema, showing anti-inflammatory activity (Franzotti et al., 2000). A new alkaloid, 1, 2, 3, 9-tetrahydro-pyrrolo [2,1-b] quinazolin-3-ylamine isolated from *S. cordifolia* showed anti-inflammatory activity in carrageenan induced rat paw edema model (Sutradhar et al., 2007). *S. cordifolia* has shown to inhibit COX leading to the inhibition of PGE synthesis (Anilkumar, 2010). Polireddy (2015) reported anti-arthritic activity of ethanolic extract of *S. cordifolia* in freund's adjuvant induced rat model.

3.2.7 *Tribulus terrestris* L.

Traditional uses

Properties and actions of *T. terrestris* mentioned in *Ayurvedic* texts are: *Rasa-Madhura*; *Guna-Guru*, *Snigdha*; *Veerya-Sheeta*; *Vipaka-Madhura*; *Karma-Brumhana*, *Vatanut* (pacifies *Vata-dsha*), *Vrusya* (aphrodisiac), *Ashmarihara* (removes urinary stone), *Vastishodhana* (cures bladder ailmenst) (Chhatre et al., 2014).

It is a vital constituent of '*Gokshuradi Guggul*', a potent *Ayurvedic* medicine used to support proper functioning of the genitourinary tract and to remove the urinary stones. It has been used for centuries in *Ayurveda* to treat impotence, venereal diseases and sexual debility. In addition to all these applications, the *Ayurvedic* Pharmacopoeia of India attributes cardiotonic properties to the root and fruit (Chhatre et al., 2014). Traditionally, *T. terrestris* is used as a tonic, aphrodisiac, anti-arthritic, palliative, astringent, stomachic, anti-hypertensive, diuretic, lithotriptic and urinary disinfectant (Wilson et al., 2007; Chhatre et al., 2014). The roots and fruits are sweet, cooling, diuretic, aphrodisiac, emollient, appetiser, digestive, anthelmintic, expectorant, anodyne, anti-inflammatory, alterant, laxative, cardiotonic, styptic, lithontriptic and tonic. They are useful in strangury, dysuria, vitiated conditions of *Vata* and *Pitta*, renal and vesical calculi, anorexia, dyspepsia, helminthiasis, cough, asthama, consumptions, inflammations, cardiopathy, haemoptysis, spermatorrhoea, anaemia, scabies, ophthalmia and general weakness (Chopra et al., 2011). The dried fruits are very effective in most of the genitourinary tract disorders (Chhatre et al., 2014). The fruits are extensively used since

ancient times as aphrodisiac (Fatima et al., 2015). The leaves are reported to be used to treat gonorrhoea, affection of urinary calculi, inflammations, skin diseases and verminosis (Ukani et al., 1997; Chhatre et al., 2014). Seeds are useful in epistaxis. The stem has astringent property and its infusion is useful in gonorrhoea (Ukani et al., 1997; Chhatre et al., 2014). The ash of the whole plant is supposed to be good for external application in rheumatoid arthritis. The roots are aperients, demulcent and tonic (Ukani et al., 1997; Chhatre et al., 2014).

Pharmacological uses

T. terrestris contains steroids, saponins, estradiol, flavonoids, alkaloids, unsaturated fatty acids, vitamins, tannins, resins, nitrate potassium, aspartic acid and glutamic acid. Dried fruits contain semi drying oil, peroxides, diastase, traces of glucosides, resins, protein and large amount of inorganic matters (Fatima et al., 2015). *T. terrestris* is a natural powerful anti-oxidant (Bhattacharjee, 2004). In a study carried out on Korean herbal medicine, *T. terrestris* showed potent inhibition of COX-2 activity (Hong et al., 2002; Srivastava et al., 2005). The methanolic extract also showed a dose-dependent inhibition of rat paw volume in carrageenan-induced inflammation in rats (Baburao et al., 2009). The ethanolic extract inhibits the expression of COX-2 and inducible nitric oxide synthase (iNOS) in lipopolysaccharide-stimulated RAW264.7 cells. It also suppressed the expression of pro-inflammatory cytokines like TNF-α and IL-4 in macrophage cell line. Thus, the ethanolic extract inhibits the expression of mediators related to inflammation and expression of inflammatory cytokines, which has a beneficial effect on various inflammatory conditions (Oh et al., 2012). Mishra et al. (2013) studied anti-arthritic activity of methanolic extract of fruit using freund's complete adjuvant induced arthritis in rats. *T. terrestris* inhibited leukocyte migration which may have beneficial effect for joint preservation.

3.2.8 *Vitex negundo* var. *incisa* (Lam.) C.B.Cl.

Traditional uses

Properties and actions of *V. negundo* mentioned in *Ayurvedic* texts are: *Rasa-Madhura, Tikta, Katu*; *Virya-Usna*; *Guna-Laghu*; *Vipaka-Katu*; *Karma-VataKapha hara*,

Caksushya, *Keshya*, *Krimigna*, *Vrunaropana*; *Upayokta-Gandamala*, *Kasa swasa*, *Vatavyadhi* (Gandagi and Patil, 2015).

V. negundo is reported to use as stomachic, astringent, anthelmintic, promotes the growth of hair, useful in disease of the eye, dysmenorrheal, leucoderma, inflammation, enlargement of the spleen, biliousness, bronchitis, asthma, painful teething of children. It has expectorant, carminative, antiseptic, digestive, antipyretic, anodyne, alterant, diuretic, emmenagogue, ophthalmic, depurative, vulnerary, tonic and rejuvenating properties (Sharma et al., 2005). It is also prescribed as a vermifuge. People sleep on pillows stuffed with *V. negundo* leaves to dispel catarrh and headache (Vishwanathan and basavraju, 2010). Crushed leaf poultice is applied to cure headaches, neck gland sores, tubercular neck swellings and sinusitis. Essential oil of the leaves is also effective in treatment of venereal diseases and other syphilitic skin disorders. The leaves are aromatic, tonic and vermifuge (Ladda and Magdum, 2012). The leaves are discutient and are also use to treat swelling of joints accured from acute rheumatism and from suppressed gonorrhoea. The juice of the leaves used in removing foetid discharges and worms from ulcers (Nadkarni, 2002; Kirtikar and Basu, 2008). Leaves and bark are useful in scorpion stings. Seeds are useful in eye diseases as an *anjan* (Sharma et al., 2005). A tincture of the root-bark provides relief from irritability of bladder and rheumatism (Vishwanathan and basavraju, 2010). Roots are used as an antidote to snake venom, also considered tonic, expectorant and febrifuge (Nadkarni, 2002; Sharma et al., 2005; Kirtikar and Basu, 2008). Roots are used in otalgia, dyspepsia, arthritis, colic, rheumatism, leprosy, wounds, verminosis, flatulence, urinary disorders, dysentery, ulcers, bronchitis, cough, leprosy, malarial fever, skin diseases, haemorrhoids, dysmenorrhea and general debility. The flowers are useful in diarrhoea, fever, cholera, hepatopathy, cardiac disorders and haemorrhages. The dried fruit acts as a vermifuge. Fruit is nervine, emmenagogue and cephalic (Nadkarni, 2002; Kirtikar and Basu, 2008).

Pharmacological uses

Leaves combat oxidative stress by reducing lipid peroxidation (Tandon and Gupta, 2005; Vishwanathan and Basavaraju, 2010). Derivatives from the seeds like Vitedoin A, Vitedoin B possess anti-oxidant potential (Zheng et al., 1999; Zheng and Luo, 1999; Onu et al., 2004). A compound, Vitexin also possesses anti-oxidant activity

(Tondon and Gupta, 2005). Tiwari and Tripathi (2007) demonstrated antioxidant property of different fractions of *V. negundo* in various *in vitro* systems. Dharmasiri et al. (2003) investigated anti-inflammatory activity of the aqueous extract of leaves using carrageenan-induced and formaldehyde-induced rat paw edema. The early phase of carrageenan-induced rat paw edema was significantly suppressed. Chawla et al. (1992) reported anti-inflammatory potential of chloroform extract of seeds in carrageenan induced rat paw edema model. Bark, seeds, seed oil and essential oil from *V. negundo* also has anti-inflammatory activity (Rao et al., 1977; Ahmad et al., 1989; Chawla et al., 1991; Jana et al., 1999; Nyiligira et al., 2004). Singh et al. (2009) reported anti-inflammatory activity of ethanolic extract of roots. *V. negundo* leaves possess anti-arthritic activity with evidence of inhibition for secretory PLA2 through molecular docking (Tandon, 2005; Vyawahare et al., 2008; Vinuchakkaravarthy et al., 2011). Leaves extract also showed significant anti-arthritic activity against freund's complete adjuvant induced arthritis in rats (Petchi et al., 2011). Agnuside, compound isolated from the leaf extract has suppressed inflammatory mediators (PGE2 and LTB4) and T-cell-mediated cytokines [IL-2, TNF-α, interferon- γ (IFN-γ), IL-4, IL-10 and IL-17] (Pandey et al., 2012). Anti-inflammatory potential from the ethanolic extract of *V. negundo* and its leaves also reported in carrageenan-induced rat paw edema and cotton pellet granuloma models (Tandon and Gupta, 2006; Saravana et al., 2013). Seeds possesses potential therapeutic effect on adjuvant induced arthritis in rats by decreasing the levels of TNF-α, IL-1β & IL-6 and increasing that of IL-10 in serum as well as down-regulating the levels of COX-2 and 5-LOX (Zheng et al., 2014).

3.2.9 *Zingiber officinale* Rosc.

Traditional uses

Properties and actions of *Z. officinale* mentioned in *Ayurvedic* texts are: *Rasa-Katu*; *Guna-Guru*, *Ruksha*, *Teekshna*; *Virya-Ushna*; *Vipaka-Madhura*; *Doshaghnata-Kapha Shamaka*; *Karma-Vata Kapha hara*, *Deepana* and *Bhedana* (Gandagi and Patil, 2015).

Z. officinale is a common spice with several ethnomedicinal and nutritional values. It has been used traditionally for treating musculoskeletal disorders (Kumar et al., 2011).

Rhizome of *Z. officinale* is one of the most common constituents of diets worldwide. It is used in conditions viz. headaches, nausea, motion sickness, vascular conditions, vomiting, arthritis, cold, as an antifungal & antimicrobial etc. Ginger is claimed to treat cold extremities, warm the body and improve a weak and tardy pulse (Akhtar and Haqqi, 2012). Fresh ginger has been used for cold-induced disease, asthma, cough, colic, heart palpitation, swellings, dyspepsia, loss of appetite and rheumatism. A glue of powdered dried ginger was applied to the temples to mitigate headache. The rhizomes of ginger are used as spice in food and beverages and in traditional medicine as carminative, antipyrexia and treatment of waist pain rheumatism and bronchitis. It is used for the treatment of gastrointestinal disorders and piles (Ghosh, 2011). Besides these, ginger is very often used to cure many illness such as indigestion, tastelessness, loss of appetite, flatulence, intestinal, allergic reactions, acute and chronic cough, common fever, allergic rhinitis, sinusitis, acute chronic bronchitis, respiratory troubles, pain backache or any kind of muscular catch, painful tooth and swelled gum etc. (Kumar et al., 2011).

Pharmacological uses

Ginger extract showed anti-inflammatory potential by decreasing carrageenan-induced edema in rats (Jana et al., 1999). The major constituents of rhizomes are volatile oils, linoleic acid, oleoresin (gingerol) and trace elements like magnesium, potassium and phosphorus. 6-gingerol, active compound from its rhizome inhibit NF-κB, activator protein-1, TNFα, IL-12, inducible NO synthase and COX-2 (Srivastsava et al., 2005; Pragasam et al., 2011). 6-gingerol reported to inhibit LPS-induced iNOS expression & production of NO and other RNS in macrophages. It also blocked peroxynitrite-induced oxidation and nitration reactions, *in vitro* (Ippoushi et al., 2003). An *in vitro* study reported that ginger (rhizomes) extract was effectively inhibited production of PGE2, TNF-α and COX-2 expression in human synoviocyte cells by regulating NF-κB activation and degradation of its inhibitor (Thomson et al., 2002). The beneficial effects of ginger were attributed to its ability to inhibit COX and LOX pathways resulting in the blockade of PGE2 and LTB4 production in affected joints and it is found to be safe for use (Wallace, 2002; Hong et al., 2004). *Z. officinale* decreases IL-1, NO and PGE2 and inhibit leukotriene B4 production in osteoarthritic cartilage (Shen et al., 2003; Anilkumar,

2010), it has been reported to decrease the IL-1β-induced expression of TNFα and TNFα induced production of COX-2, and activation of NF-κB in synoviocytes (Frondoza et al., 2004). Plant also inhibited production of TNFα in human osteoarthritic synoviocytes and chondrocytes (Setty and Sigal, 2005). Sumantran et al. (2011) showed *in vitro* biochemical evidence of anti-arthritic potential of a standardized multiherbal *Ayurvedic* formulation containing *Z. officinale* as a key ingredient. In a randomized, double-blind, placebo-controlled, crossover study in patients with OA, patients in the ginger extract group showed statistically significant effect in the first period of treatment before crossover (Bliddal et al., 2000). Ginger alleviates pain and associated symptoms in patients suffering from OA. In another randomized, double-blind, placebo-controlled trial revealed effects of ginger extract on knee OA, 63% of the patients in the ginger extract group experienced reduction in knee pain on standing versus 50% in the placebo group. In this study, Ginger extract has been used as an alternative to NSAID therapy and has shown positive results (Altman and Marcussen, 2001). *Z. officinale* inhibits biotransformation of arachidonic acid. In clinical study on knee OA using formulation containing *Z. officinale* as one of its ingredients, there was significant reduction in pain score (Chopra et al., 2004). Similarly, in a double-blind, randomized, placebo controlled clinical trial; Ginger extract and ibuprofen were significantly more effective than the placebo in the symptomatic treatment of OA, while there was no significant difference between the ginger extract and ibuprofen groups (Haghighi et al., 2005).

3.2.10 *Dashamoolarishta*

Traditional uses

Properties and actions mentioned in *Ayurvedic* texts are: *Grahani* (malabsorption syndrome), *Aruchi* (anorexia), *Shwasa* (respiratory diseases, wheezing, asthma, *Kasa* (cough, cold), *Gulma* (bloating, abdominal tumor), *Bhagandara* (anal fistula), *Vatavyadhi* (vata imbalance disorders, neurological disorders), *Kshaya* (tissue depletion, chronic respiratory disorders), *Chardi* (vomiting), *Panduroga* (anemia), *Kamala* (liver disorders, jaundice), *Kushta* (skin disorders), *Arsha* (piles), *Meha* (urinary disorders, diabetes), *Mandagni* (low digestion strength), *Udara* (ascites), *Sharkara* (urinary gravels), *Ashmari* (urinary calculi), *Mutrakrichra* (difficulty in urination), *Dhatukshaya* (in emaciated, lean

and weak person), *Krushaanaam pushtijanana* (improves nourishment in lean and weak), *Vandhyaanaam* garbhada (useful in female infertility), *Shukraprada* (Improves sperm and semen quality and quantity), *Balaprada* (improves immunity, strength) (Sharangdhara Samhita).

Dashamoolarishta is used in indigestion, lack of taste, respiratory conditions, fistula, in diseases of *Vata* imbalance, vomiting, anemia, liver diseases, skin diseases, haemorrhoids, urinary tract conditions, cough and jaundice. It is used as a general health tonic. *Dasamoolarishta* is also used for ladies having problem with conception and pregnancy. It also improves immunity and strength (Sharangdhara Samhita).

Pharmacological uses

Aqueous extract of *Dashamoola* showed anti-inflammatory effect on carrageenan induced rat paw edema and cotton pellet implantation comparable to diclofenac (Singh et al., 2011). *Dashamoolarishta* is arishta dosage form of *Dashamoola*. *Dashamoolarishta* showed significant antioxidant activity and antiinflammatory potential in the carrageenan induced rat paw edema model providing rationale of their use in inflammatory conditions (Pawar et al., 2013). Anti-inflammatory activity of *Dashamoolarishta* was revealed in carrageenan-induced inflammation, model of peritonitis and in cotton pellet granuloma, it showed significant ($p<0.001$) anti-inflammatory activity by paw edema reduction in rats, decrease in proteins in peritoneal fluid ($p<0.001$) and decrease in granuloma weight ($p<0.05$) as compared to respective vehicle control groups (Parekar et al., 2015).

3.2.11 *Triphala*

Traditional uses

Properties and actions mentioned in *Ayurvedic* texts are: *Rasa-Kasaya*. *Guna-Ruksha, Sara*. *Virya-Anusna*; *Vipaka-Madhura*; *Doshaghnata-Tridoshasamaka*; *Karma-Chaksusys*, *Dipana*, *Vrishya*, *Prameha*, *Kustha*, *Vishamajwarnashaka*, *Medohara* (Chouhan et al., 2013).

Triphala is used for gastric disorders such as digestion problems, poor food assimilation, cleansing of colon, constipation & tonifier of the GI tract & colon, cardiovascular disorders, high blood pressure, serum cholesterol reduction, ophthalmic problems, liver

dysfunction, inflammation and complications of the large intestine. It is also used as a blood purifier, to improve the mental faculties and is reported to possess anti-inflammatory, analgesic, anti-arthritic, hypoglycaemic and anti-aging properties (Chouhan et al., 2013).

Pharmacological uses

Triphala is a natural antioxidant and thus important in preventing or slowing the progression of aging and age-associated oxidative stress-related degenerative diseases. It exhibited DPPH, H_2O_2 and very strong superoxide anion scavenging activity. It significantly inhibited generation of NO· and HO· radicals (Babu et al., 2013). *Triphala* has a promising anti-inflammatory effect in the inflamed paw of arthritis-induced rats (Kalaiselvan and Rasool, 2015). *Triphala* shown anti-arthritic activity, the physical and biochemical changes observed in arthritic animals were altered significantly to near normal conditions after *Triphala* treatment. In another study, the efficacy of *Triphala* on monosodium urate crystal-induced inflammation in mice was studies, where significant inhibition in paw volume, levels of lysosymal enzymes, LPO and inflammatory mediator TNF-α was found (Chouhan et al., 2013).

3.2.12 '*Triphala Guggul*'

Traditional uses

Properties and actions mentioned in *Ayurvedic* texts are: *Lekhana*-scrapes toxins, *Shodhana*-blood purifier, *Bhagandar*-treats fistula-in-ano, *Shothaghna*-alleviates oedema, *Arshaghna*- treats haemorrhoids (Mehra et al., 2011).

'*Triphala Guggul*' is used as a cholesterolemic, laxative, carminative, alterative and anti-inflammatory. In *Ayurveda*, myrrh oleoresin from the *Commiphora mukul* tree, referred to as *Guggulu*, is being used as an important component of anti-arthritic drugs. *Guggulu* destroys *ama* (bodily wastes and toxins), enhances body metabolism & strength and rejuvenates the body (Singh et al., 2003). *C. mukul* include as an anti-inflammatory, antispasmodic, carminative, emmenagogue, hypoglycemia, antiseptic, astringent, sedative, anthelmintic, diuretic, *Guggulu* reduced the stikiness of platelets, use as *Rasayana* (Rejuvenation), *Balya* (increase vitality and strength of the body), *Vrushya*

(aphrodisiac) (Chavan et al., 2015). Purana *Guggul* (*Guggulu* older than a year) is used against *Picchilguna* (sticky quality) of *Kaphadosha*. *Guggulu* along with *Indrayava* (seeds of *Holarrheana antidysenterica*), *Aluaa* (Aloe vera) and *Guda* (jaggery) is used as intestinal antiseptic in *Atisara* (Diarrhea), *Pravahika* (Amebic dysentery), *Kshayaj Atisara* (Diarrhea related to wasting). In *Ammenorhea*, *Guggul* is given with *Kasis* (Ferrous sulphate) and *Aeluva* (Aloe vera). If cause of *Vandhyatva* (Infertility) is *ShwetPradar* (Leucorrhea) then *Guggul* is given with *Rasona* (Garlic). It acts as *Vranashodhak* (clearing of wound) and *Vranaropak* (healing of wound). *Guggulu* is also used for the treatment of Medoroga (Obesity) (Chavan et al., 2015).

Pharmacological uses

The ethyl acetate extract of *C. mukul* exhibited good reducing power and anti-lipid peroxidation activities (Dubey et al., 2009). Chavan et al. (2015) reported anti-inflammatory action of guggusterones. Thus, inhibition of hyaluronidase and/or collagenase, accounts for its reported chondroprotective activity. It has been reported in a clinical trial that *C. mukul* capsules when administered to patients with OA of the knee, led to significant improvement without side-effects (Singh et al., 2003). Sumantran et al. (2007b) showed that '*Triphala Guggulu*' inhibits two major enzymes, hyaluronidase and collagenase which degrade cartilage in OA. *Guggul* reduced the thickness of the joint swelling during the course of drug treatment, indicating that gum *Guggul* has a beneficial role in experimental arthritis. Gum *Guggul* appeared to be a relatively safe and effective supplement to reduce symptoms of OA after 1 month of treatment and significantly improve the WOMAC (Western Ontario and McMaster Osteoarthritis Index) total score and continued to further improve on long term uses and even after 2 months of treatment no side effects were reported during the trial (Patwardhan et al., 2010).

On the basis of review of literature, in the present study, traditional medicinal herbs including *A. nilotica*, *B. ceiba*, *B. serrata*, *P. longum*, *R. communis*, *S. cordifolia*, *T. terrestris*, *V. negundo*, *Z. officinale* and formulations viz. *Dashamoolarishta*, *Triphala*, and '*Triphala Guggul*' were investigated comparatively for their efficacy and mechanism of action on reducing inflammation as well as on cartilage protection using *in vivo*

studies. Furthermore, using prioritized herbs, new combination formulation was developed and it was also validated using *in vitro* cell line studies.

3.3 *In vivo* models for OA

OA is a chronic disease and therefore acute inflammatory pain models (e.g., following intraplantar or intra-articular injection of carrageenan) have limited translational relevance to human OA (Chapman, 2016). Although, the animal models used may not have fuller relevance to various types of human OA, potential plants have to be tested in currently used different models for efficacy and mechanism of actions because they shed a lot of light on their medicinal value. There are numerous classical animal models of OA involving a number of animal species, rabbits, dogs, sheep and rodent (rats and mice). Spontaneous/naturally occurring models of OA (Dunkin-Hartley guinea pig, STR/ort and STR/1N mouse strains) are more like human OA with slower onset and progression. Osteoarthritic pathology is seen in these animals by 6 months. These are viewed as effective screening models but like human OA, these models are not very sensitive when identifying the initiating events and they are time consuming (Ashraf, 2011).

Genetically modified animals (knock out or transgenic mice) are a major tool to investigate genetic predispositions/contributions to OA. They are well defined and allow insight into mechanisms of tissue formation and maintenance, matrix assembly, signaling proteins and molecular interactions in OA but their usefulness for the study of the pathophysiology of OA is debatable. Each genetic model describes a distinct molecular entity making it difficult to study the effects of different treatment approaches. Longer time is needed for OA to develop in genetic models compared to surgical and enzymatic models that are also faster and consistent (Ashraf, 2011).

Papain, sodium monoiodoacetate (MIA) and collagenase are some of the chemicals employed to induce OA in animals. They eliminate the need for surgery and avoid possible infection issues in some animals. Their advantages are ease of induction and reproducibility. Chemically induced models are less invasive than surgical models and have a unique pathophysiology which has no correlation to that of post-traumatic OA. Therefore, these models are mainly used to study the mechanism of pain and its use as a

target for drug therapy. Papain is a proteolytic enzyme which was historically used in OA induction and it breaks down proteoglycans (Kuyinu et al., 2016). However, the use of papain for an OA model is becoming increasingly rare. Instead, the most commonly used compound in OA study today is MIA. The MIA model has emerged as a good model to study osteoarthritic pain and the effects of analgesic drugs because it is reproducible and mimics pathological changes and experience of pain as seen in human OA. Intra-articular injection of MIA produces progressive joint degeneration through inhibition of glycolysis and subsequent chondrocyte death that develops over several weeks. Similar to human OA, joint pathology is characterized by chondrocyte necrosis resulting in decreased thickness of the articular cartilage and fibrillation of the cartilage surface, exposure of the underlying subchondral bone, swelling and reduction in bone mineral content and density (Kobayashi et al., 2003). However, since iodoacetate is a metabolic poison, chondrocyte cell death in the model is extensive, unlike that observed in human OA (Ashraf, 2011).

The collagen type II of knee joint network is extremely stable. Intra-articular injection of collagenase is a structurally relevant model, as it induces articular degeneration both by digesting collagen and proteoglycans from cartilage and by causing articular instability, thereby reproducing some of the main events associated with OA onset and development (Kim and Cheon, 2012; Adaes et al., 2014). Highly purified bacterial collagenase was injected into the knee joints and lesions develop after 1 to 4 weeks. Correlations between the degree of joint instability, the amount of cartilage degradation and the size of osteophytes were observed. Synovial activation is important for the induction of joint pathology and like in human OA, macrophages are the predominant cell type in the inflamed synovium of mice with collagenase-induced OA, while no polymorphonuclear leukocytes are observed during the chronic phase of disease (Kim and Cheon, 2012). Yeh et al. (2008) demonstrated that intra-articular administration of collagenase induced osteoarthritic changes including degradation of cartilage, synovial inflammation, remodelling of subchondral bone, and osteophyte formation in the facet joints of rats. Collagenase injection into the facet joint could not only directly destroy the cartilage but also induce an inflammatory reaction of the synovium and increased cartilage degradation. Subchondral bone changes developed from 1 week after collagenase injection (Yeh et al., 2008). 50 U of collagenase induced severe OA-like changes 1 week

after surgery (Yeh et al., 2008). Huh et al. (2008) reported anti-osteoarthritic potential of Korean herb *Siegesbeckia pubescens* in a rabbit collagenase induced model by studying various biomarkers from cartilage, synovium and synovial fluid. Asiria et al. (2014) evaluated the effect of herbal extract using collagenase induced OA in rats and demonstrated that intra-articular administration of collagenase induced osteoarthritic changes included highly destructed chondrocytes with pyknotic nuclei with no clear lacunae, shrunken cytoplasm that contained many vacuoles and were surrounded by a faint cartilage capsule. Most of the cells had atrophic nuclei. Extensive cartilage destruction and disappearance of chondrocytes were seen in collagenase-treated rats (Asiria et al., 2014).

Surgical models (structural alteration to the tendons muscles or ligaments) of mechanical instability represent the chronic traumatic form of OA. Mechanical instability can be induced using various surgical methods such as Anterior Cruciate Ligament Transection (ACLT) and menisectomy. The disadvantage of the surgical models is that only knee damage can be studied (Ashraf, 2011).

It has been reported that in animal models like dogs, cats and sheep's their large size, the lack of availability of biochemical reagents to study the molecular dynamics in affected joints and time required for disease induction, has reduced the usefulness of these models in evaluating potential disease modifying agents. Smaller animals like rats, mice and guinea pigs thus enable low cost studies to be carried out due to their small size and shorter time frame of OA development (Ashraf, 2011). Mouse has very thin cartilage, rabbits have different histology from humans and guinea pigs are having sedentary lifestyle (Gregory et al., 2012). Murine and rat models of knee OA exhibit weight-bearing asymmetry, resembling the tendency for patients to avoid weight bearing on an osteoarthritic knee. In addition, these models exhibit lowered hind-paw withdrawal thresholds to punctate mechanical stimulation, resembling reduced mechanical pain thresholds that have been observed distant to the arthritic joint in people with knee OA (Chapman, 2016). Mice and rats are comparatively low-cost laboratory animals and are easy to house & handle. However unlike mice, naturally occurring OA is extremely uncommon in rats and transgenic rat models of OA are not yet available (Thysen et al., 2015). Rats possess a thicker cartilage with a complex zonal structure, which makes

partial and full cartilage lesions possible to reproduce using chemical or surgical methods (Thysen et al., 2015).

Thus based on review of literature we have selected collagenase induced osteoarthritis (CIOA) rat model for comparative evaluation of anti-osteoarthritic activity of selected plants and formulation.

3.4 *In vitro* model used for the present study

High prevalence of synovial membrane inflammation (synovitis) has been reported in OA (Henrotin et al., 2014). Synovitis was found in 95% of OA patients. The prevalence and the severity of synovitis increase with advancing stage of OA. It's an indicator of pathology and a predictor of disease progression (Henrotin et al., 2014).

Depending upon limited size of the tissue, biological and biochemical investigation of specific cells becomes difficult and complicated. Main limitation of cell line study is to isolate specific cells from the host animal and to grow them under controlled conditions *in vitro*. Furthermore, cultures of normal, diploid cells undergo senesce during serial passaging *in vitro* (Hayilick, 1965). Hence, it is necessary to establish a cell line, growing indefinitely in culture without losing the properties of experimental interest (Georgescu et al., 1988). Therefore, instead of using primary cultures, we have used rabbit synoviocytes sarcoma cell line (HIG-82) for our further *in vitro* studies. A group from New York demonstrated gene expression of the matrix-degrading enzyme collagenase-1 in rabbit synoviocytes and human fibroblasts (Suzuki et al., 1997). Turner-Brannen et al. (2011) showed the ability of an innate immune-modulatory IDR-peptide to influence the IL-1β-induced regulatory pathways and selectively to suppress inflammatory responses in synovial fibroblasts. This study provided a rationale for examining the use of IDR-peptides as potential therapeutic candidates for chronic inflammatory diseases such as inflammatory arthritis. Li et al. (2013) showed ant-arthritic activity of a compound extracted from traditional Chinese plant using LPS stimulated fibroblast like synoviocytes (FLS). Results indicated that the compound suppressed LPS-stimulated FLS migration and invasion by inhibiting MMP-9 expression and activity. It showed inhibition in the transcriptional activity of MMP-9 by suppressing the binding activity of NF-kB in the MMP-9 promoter, and suppressed the TLR4/MyD88/NF-kB pathway.

Lipopolysaccharide (LPS), a cell wall constituent of gram-negative bacteria, is released during bacterial lysis and exerts a direct effect on tumor cell proliferation, invasion and metastasis *in vitro* and *in vivo*. Toll-like receptor 4 (TLR4), the receptor for LPS, also expressed in FLSs, is important in the regulation of immune responses and is involved in inflammation-induced cell motility.

Thus, based on review of literature we have selected HIG-82, rabbit synoviocytes for our study and synovial inflammation was stimulated using LPS.

Chapter 4
Material and Methods

4.1 To collect and process plant material

Initially, Herbs and formulations which are traditionally used to treat OA were listed out. Based on review of literature, nine herbs and three formulations were selected for the study. All plants were authenticated at Ayurved College, Bharati Vidyapeeth Deemed University, Pune. Formulations were prepared by Ayurved College, Bharati Vidyapeeth Deemed University, Pune.

4.2 To comparatively evaluate anti-osteoarthritic potential of selected herbs using *in vivo* model

The study was divided into two parts for nine selected plants. Part one includes comparative evaluation of six plants and part two include, comparative evaluation of three selected gums. In this objective comparative evaluation of anti-osteoarthritic potential of *S. cordifolia, P. longum, Z. officinale, R. communis, V. negundo* and *T. terrestris* were carried out using collagenase type II-induced osteoarthritis (CIOA) rat model and further prioritized herbs were evaluated for their mechanism of action using qPCR studies by studying various OA biomarkers.

4.2.1 Collagenase type II-induced osteoarthritis (CIOA) rat model

Institutional animal ethics committee approval for the experimental protocol was obtained before initiation of the study (BVDUMC/CPCSEA/1962/2011-12). Guidelines laid down by the CPCSEA were observed throughout the study for animal handling and experimentation. Rats were obtained from National Institute of Biosciences (Pune, India).

4.2.1.1 Experimental animals

54 female wistar rats weighing between 180-300 gm were used for the experiment. They were housed for two weeks in solid bottomed polypropylene cages for acclimatization before use, maintained under standard conditions and fed standard rat chaw with water *ad libitum*.

4.2.1.2 Drug and dosage

The dose was selected based on the human dose mentioned in the *Ayurvedic* literature and was calculated for animal use, based on the body surface area ratio (Methekar et al., 2012). Powders of herbs were administered orally at the dose of 270 mg/kg b. wt. p. o. in the form of suspension prepared in water. Standard drug indomethacin (purchased from local pharmacy, manufactured by Jagsonpal pharmaceuticals Ltd, India) was given at the dose of 3 mg/kg b. wt. p. o. in 0.5% sodium-carboxy methyl cellulose (Rasool and Varalakshmi, 2007).

4.2.1.3 Treatment

Rats were divided into nine groups, consisting of 6 animals in each. Treatment was given once per day orally through feeding needles from day 14^{th} to 34^{th} of the experiment.

4.2.1.4 Induction of OA

Rats were anesthetized with diethyl ether (Merck, India). The shaved right knee joints of Group I were injected with 50 µl of normal saline solution which served as healthy control. Animals from the second group were injected with collagenase type II (from *Clostridium histolyticum*, obtained from Sigma Aldrich, USA).

Collagenase was dissolved in saline and 50 µl (50 units) was injected intra-articularly into the right knee joint. The injection was given twice, on days 1^{st} and 4^{th} of the experiment (Yeh et al., 2008; AL-Saffar et al., 2009). Injections were given using 31-gauge 0.25x8 mm needle.

Table 1: Grouping of animals and treatment specifications for evaluation of selected plants using CIOA model

Group No.	Group	Group code	Treatment
I	Healthy control	HC	Saline
II	Osteoarthritic control	CIOA	Collagenase
III	Positive control	INDO	Collagenase+Indomethacin (3 mg/kg b. wt.)
IV	*S. cordifolia*	SC	Collagenase+Test groups (270 mg/kg b. wt.)
V	*P. longum*	PL	
VI	*Z. officinale*	ZO	
VII	*R. communis*	RC	
VIII	*V. negundo*	VN	
IX	*T. terrestris*	TT	

4.2.1.5 Body weight, Knee diameter, Paw volume and Paw retraction measurement

Changes in body weight and knee diameter were measured on days 0^{th}, 5^{th}, 10^{th}, 15^{th}, 20^{th}, 25^{th} and 30^{th}. Knee diameter was measured using digital vernier caliper (Mitutoyo, Japan). Mean changes in body weight and joint swelling after treatment were calculated. Paw volume and paw retraction was measured once in a week using digital plethysmometer (Orchid Scientifics, India) and tail flick unit (Ugobasile unit, Italy), respectively. Paw latency after treatment and % Inhibition of paw edema with respect to CIOA was calculated using following formula:

Percent inhibition of paw edema = (Vc-Vt)/Vcx100

Where, Vc: Paw Volume of CIOA group, Vt: Paw Volume of test group

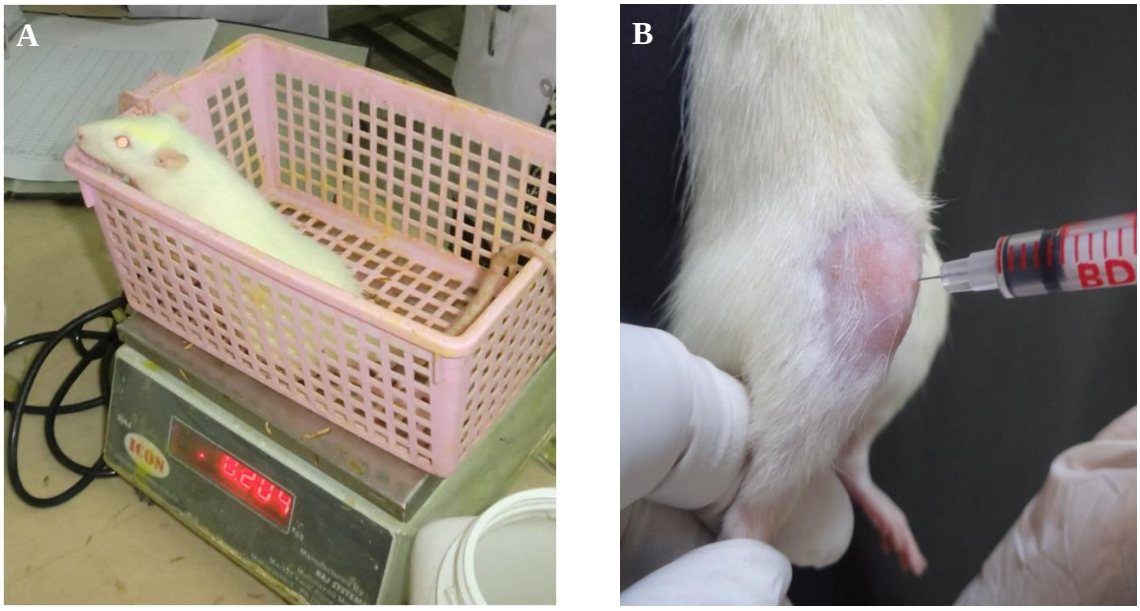

Figure 13: (A) Body weight measurement using digital weighing balance and (B) intra-articular injection to knee joint.

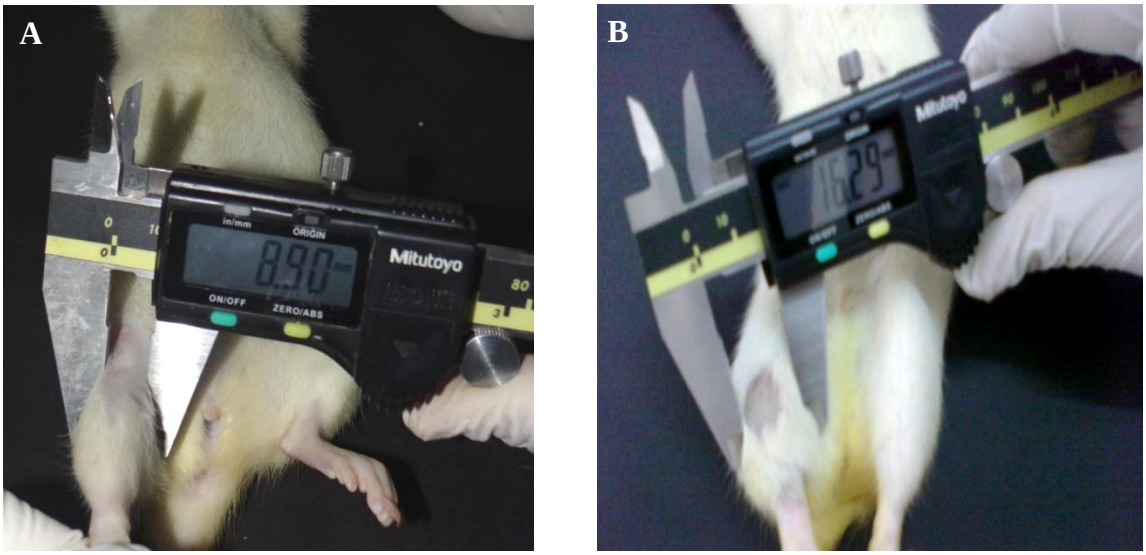

Figure 14: Measurement of knee diameter. (A) Measurement of knee joint diameter before intra-articular injection of collagenase and (B) measurement of knee joint diameter after collagenase injection.

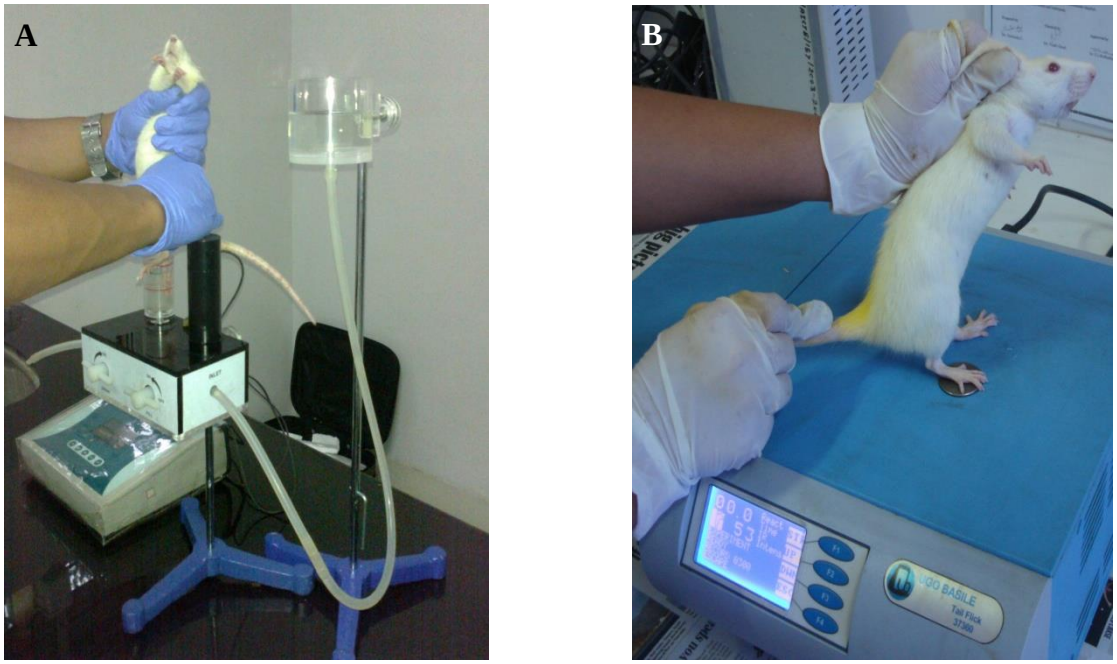

Figure 15: (A) Measurement of paw volume using Plethysmometer and (B) measurement of paw retraction using Tail flick unit

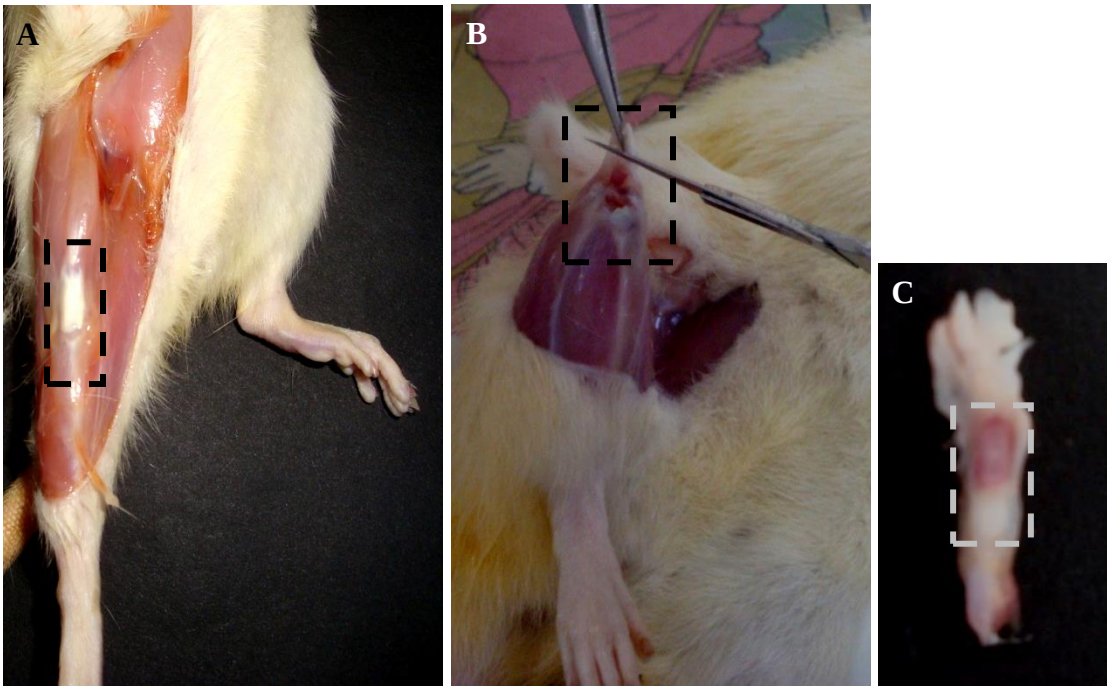

Figure 16: Harvesting knee joint and synovium tissue. (A) Knee joint of the animal with the skin removed; (B) Cut made in the joint for removing synovium with patella. (C) Appearance of the patella with synovium resting on the patellar ligament, free from bone.

4.2.1.6 Glycosaminoglycans (GAG) release

Blood was taken from rats before and after the treatment through retro-orbital vein puncture and serum was separated.

Extracellular matrix of cartilage contains proteoglycans, which consists of a core protein to which glycosaminoglycans (GAGs) chains are covalently attached (Ishiguro et al., 2002). The ability of the cartilage to stand compressive forces is aided by the sulfated GAGs such as chondroitin sulfate (Sutton et al., 2009). GAG release from explants into the surrounding fluid, is a proven marker of cartilage matrix damage. GAG content in serum was measured by 1, 9-dimethyl methylene blue (DMMB) dye binding assay (Hoemann et al., 2002).

- **Principle**

1, 9-dimethyl methylene blue (DMMB) is a metachromatic cationic dye which binds anionic GAG molecules. The degree of metechomasia is directly proportional to GAG present in the reaction mixture which can be measured spectrophotometricaly.

- **Sample preparation**

Blood was centrifuged at 2000 rpm and serum was separated.

- **Reagents**

- 40 μM Glycine (Sigma, USA) in distilled water.
- 40 μM NaCl (Sigma, USA) in distilled water.
- 1X Phosphate buffered saline (PBS):

  10X PBS was prepared by mixing 0.2 gm Potasium dihydrogen phosphate (KH_2PO_4, Merck, India), 8 gm Sodium chloride (NaCl, Merck, India) and 1.14 gm Sodium hydrogen phosphate (Na_2HPO_4) in 100 ml distilled water. 1X PBS was prepared by diluting 10X stock.
- Preparation of DMMB dye:

  DMMB (Sigma Aldrich, USA) stock: 23 mM stock of DMMB was prepared in triple distilled water. It can be used up to 1 week. Stock was stored at room temperature.

  Working stock of DMMB dye: 23 μM DMMB was prepared in 40 μM glycine and 40 μm NaCl. pH of the working dye was adjusted to 3 using 2N HCl. Prepared freshly just before assay.
- Preparation of Chondroitin sulfate:

10 mg/ml Chondroitin sulfate (Sigma Aldrich, USA) stock solution was prepared in 1X PBS. 1 mg/ml working stock was prepared from 10 mg/ml Chondroitin sulfate stock in 1X PBS.

- **Procedure**

Using digestive buffer as diluent, a standard curve was prepared from chondroitin sulphate. Duplicate aliquots of 200 µl of either digestive buffer blanks, standards or samples (1:5 dilution) and 400 µl of 70µM DMMB solution was added into microcentrifuge tubes. Absorbance was read at 540 nm (Biorad, Hercules, CA). GAG was calculated as µg chondroitin sulphate equivalents.

4.2.1.7 Radiological analysis

Before the termination of experiment, the animals were anesthetized using diethyl ether and anterior posterior (AP) X-rays using (GE Medical Systems- DXD 300, 300MA, America) thermal laser AGFA digital photo films (digitizer CR-30, AGFA photo film, Belgium) were taken for the knee joints of the animals to evaluate the cartilage degradation and joint space reduction. The X-ray apparatus was operated at 300 MA, 50 KV, 0.02 second exposure time and a 100 cm tube to film distance for AP projection. X-rays were analysed by Dr. Anant S. Bhagwat, M.D. (Radiology), Bhagwat X-Ray Clinic, Pune, Maharashtra, India.

4.2.1.8 Histopathological analysis

On day 34^{th}, animals were sacrificed. The right knee joints were dissected out and fixed in 10% phosphate buffered formalin (Sigma Aldrich, USA). They were than decalcified, sectioned and finally stained with hematoxyline and eosin (H and E), masson's trichome and safranin-O. Synovium was dissected out and fixed in 10% phosphate buffered formalin for H and E staining. Tissue samples were prepared for light microscopy using standard procedures. Slides were analysed by Dr. Vinayak D. Kulkarni, MD (Pathology), OM Laboratory, Pune, Maharashtra, India.

4.2.1.8.1 Hematoxylin and Eosin staining

- **Principle**

Haematoxylin and eosin stains are used to analyse tissue architecture. The basic dye component, haematoxylin is having high affinity for acidic structures such as nucleic acid, stains the cell nuclei blue/black. Whilst eosin, an acid dye, having high affinity for basic structures, stains cell cytoplasm and most connective tissue fibers in various shades and intensities of pink, orange and red (King and King, 1986).

- **Reagents**

- Xylene.
- Absolute ethanol.
- Iso-propyl alcohol.
- 95% alcohol: 95 ml absolute ethanol was added in 5 ml distilled water.
- 70% alcohol: 70 ml absolute ethanol was added in 30 ml distilled water.
- Harris Hematoxylin:

  Chemicals: Hematoxylin: 2.5 gm, Absolute ethanol: 25 ml, Potassium or ammonium (alum): 50 gm, Distilled water: 500 ml, Mercuric oxide: 1.25 gm, Glacial acetic acid: 20 ml. Stored in amber coloured bottle at room temperature.

  Preparation: In 500 ml distilled water, 50 gm of potassium alum was dissolved by heating and shaking. The solution was allowed to reach at 60^0C. 2.5 gm Hematoxylin dissolved in 25 ml absolute alcohol was added to above solution. Allowed it to boil rapidly and removed from flame when it started boiling. 1.25 gm of mercuric acid was added. Mixed by swirling gently and heating was continued for 3 minutes till solution becomes purple coloured. When it gets cooled, 20 ml Glacial acetic acid and 25 ml Absolute alcohol was added. Stain was then filtered and used.
- Acid alcohol solution (for differentiation): 2 ml concentrated HCL were added in 300 ml Iso-propanol and mixed well.
- 0.2% Ammonia Water Solution (Bluing): 2 ml Ammonium hydroxide (concentrated) were added in 1000 ml distilled water and mixed well.
- Lithium Carbonate Solution (Saturated): 10 gm Lithium carbonate was dissolved in 100 ml distilled water.

- Eosin:

  Preparation of 1% aqueous Eosin: 1 gm Eosin was dissolved in 100 ml distilled water

  1% W/V alcoholic Eosin: 1%, 100 ml aqueous Eosin was dissolved in 780 ml absolute alcohol and 4 ml glacial acetic acid.

➢ **Procedure**

Slides were heated onto the hot plate to melt paraffin. Sections was deparaffinized in 3 changes of xylene, 10 min each and rehydrated in 2 changes of absolute alcohol, 5 minutes each and then changes of 95% alcohol and 70% alcohol, each for 2 minutes. Slides were washed under tap and stained in Harris hematoxylin for 5 minutes. Rinsed under tap water for 1 minute and differentiated in acid alcohol (1 dip) and dipped in 10% Lithium carbonate (1 dip). Slides were kept under running tap water for 10 minutes and stained in Eosin for 30 seconds. Further, dehydrated in two changes of isopropyl alcohol for 1 min each and were cleared in three changes of xylene, 5 minutes each. Slides were then mounted in DPX (Tran et al., 2000).

4.2.1.8.1 Masson's Trichome staining

➢ **Principle**

This method is used for the detection of collagen fibers in tissues. The collagen fibers will be stained blue and the nuclei will be stained black and the background is stained red (Meinen et al., 2007).

➢ **Reagents**

- Absolute ethyl alcohol.
- Zenker’s fluid.
- Xylene.
- 95% Ethyl alcohol: 95 ml ethyl alcohol dissolved in 5 ml distilled water (for 100 ml solution).
- 70% Ethyl alcohol: 70 ml ethyl alcohol dissolved in 30 ml distilled water.
- 2% Sodium thiosulphate: 2gm Sodium thiosulphate was dissolved in 100 ml distilled water.
- Weigert's Iron Hematoxylin Solution:

Stock Solution A: 1 gm Hematoxylin dissolved in 100 ml 95% alcohol.

Stock Solution B: 4 ml, 29% Ferric chloride in water dissolved in 95 ml dstilled water and 1 ml Hydrochloric acid, concentrated.

Weigert's Iron Hematoxylin Working Solution: Equal parts of stock solution A and B were mixed. This working solution is stable for 3 months.

- 1% Acetic Acid Solution: 1 ml Glacial Acetic acid was dissolve in 100 ml distilled water.
- Biebrich Scarlet-Acid Fuchsin Solution: 90 ml, 1% aqueous Biebrich scarlet, 10 ml 1% aqueous Acid fuchsin and 1 ml Glacial Acetic acid were mixed.
- Phosphotungstic Acid: 5 gm phosphotungstic acid was dissolved in 100 ml distilled water.
- Light green: 5 gm light green was dissolved in 250 ml distilled water and 2 ml Glacial Acetic acid.

➢ **Procedure**

Sections were deparaffinized and rehydrated through 100% alcohol, 95% alcohol and 70% alcohol. Slides were placed in Zenker's fluid at 56^0C for 1 hour and washed in distilled water, and bleached in 2% Sodium-thiosulphate. Slides were then stained in Weigert's iron hematoxylin working solution for 10 minutes. Nuclei were differentiated in 1% acid alcohol. Further washed in running tap water for 10 minutes and rinsed with distilled water. Then stained in Biebrich scarlet-acid fuchsin solution for 10-15 minutes and rinsed in distilled water. Light green is used as a counter stain. Slides were differentiated in Phosphotungstic acid for 10-15 minutes or until collagen is not red and again rinsed and stained with 2% light green for 1-3 minutes. Slides were rinsed in distilled water and differentiated in 1% acetic acid solution for 2-5 minutes. Dehydrated very quickly through 95% ethyl alcohol, absolute ethyl alcohol (this step wipes off Biebrich scarlet-acid fuchsin staining) and clear in xylene. Slides were mounted in DPX (Meinen et al., 2007).

4.2.1.8.3 Safranin O staining

- **Principle**

This method is use for the detection of cartilage, mucin, and mast cell granules on formalin-fixed, paraffin-embedded tissue sections, and may be used for frozen sections as well. The cartilage and mucin will be stained orange to red, and the nuclei will be stained black. The background is stained green (Ashraf, 2011).

- **Reagents**

- Weigert's Iron Hematoxylin Solution: Prepared as described Masson's Trichome staining.
- 0.05% Fast Green (FCF) Solution: 0.5 gm Fast green, FCF, C.I. 42053 was dissolved in 1000 ml distilled water.
- 1% Acetic Acid Solution: 1 ml Glacial Acetic acid was dissolved in 100 ml distilled water.
- 0.1% Safranin O Solution: 0.1 gm Safranin O, C.I. 50240 was dissolved in 100 ml distilled water.

- **Procedure**

Slides were deparaffinised and hydrated to distilled water. Stained with Weigert's iron hematoxylin working solution for 10 minutes and washed in running tap water for 10 minutes. Slides then stained with fast green (FCF) solution for 5 minutes and rinsed quickly with 1% acetic acid solution for no more than 10 –15 seconds. Further slides stained with 0.1% safranin O solution for 5 minutes. Dehydrated and cleared with 95% ethyl alcohol, absolute ethyl alcohol, and xylene, using 2 changes each, 2 minutes each and slides were mounted in DPX (Tran et al., 2000).

4.2.1.9 Quantitative real-time reverse transcription-polymerase chain reaction (qPCR) analysis

Animals were killed at the end of the experiment and their synovial tissue was removed. The synovium was flash frozen immediately in liquid nitrogen and stored at -80^0C until further use.

4.2.1.9.1 RNA isolation

Kit specifications

Name: PureLink RNA mini kit.

Make: Invitrogen CA, USA.

Catalog number: 12183-018A.

Storage conditions: Stored at room temperature.

For qPCR analysis, total RNA from isolated knee synovium tissue was extracted using Trizol reagent (Sigma Aldrich, USA).

- **Kit contents**

Lysis buffer, Wash buffer I, Wash buffer II, RNase-Free Water, Spin cartridges (with collection tubes) and Recovery tubes.

- **Reagents**

0.1% Trizol reagent, Absolute ethanol (Chang Shu Yung chemicals, China), 70% ethanol in DEPC (Diethyl pyrocarbonate) (Invitrogen, USA), Wash buffer II: 60 ml absolute ethanol was added to it.

- **Procedure**

Working table area was wiped out using DEPC water. Entire synovium tissue was homogenized in 1 ml trizol, in 1.5 ml eppendorf tubes and kept at room temperature (RT) for 5 minutes. 200 µl of chloroform was added to it and tubes were vigorously shaken immediately for 15 seconds and kept at RT for 3 minutes. Tubes were then centrifuged at 12000xg for 15 minutes at 4^0C. 400 µl supernatant was taken from the solution in fresh eppendorf tubes and one volume of 70% ethanol was added to it. Tubes were then vortexed for 30 seconds (to disperse any visible precipitate that may form after adding alcohol) and 500 µl was transferred to spin cartridge. It was than centrifuged for 30 seconds at 12000xg at RT. Flow-through was discarded. Above step was repeated until entire sample has got processed. 700 µl Wash buffer I was added to spin cartridge and was centrifuged for 30 seconds at 12000xg at RT. Collection tube was than discarded. Spin cartridge was placed in a new collection tube. Further 500 µl Wash buffer II was added to spin cartridge and it was centrifuged for 30 seconds at 12000xg at RT. Flow-through was discarded and this step was again repeated. Further, spin cartridge was centrifuged for 1 minute at 12000xg at RT, to dry the membrane with bound RNA.

Collection tube was discarded and spin cartridge was inserted into recovery tube. 30 µl RNase-free water was added to the centre of the spin cartridge and incubated for 2 minute at RT. The spin cartridge was than centrifuged for 1 minute at 12000xg at RT to elute the RNA. Quality of the isolated RNA was determined using denaturing agarose gel electrophoresis followed by quantification by measuring absorbance at 260 nm and the first strand cDNA was synthesized.

4.2.1.9.2 cDNA synthesis

Kit specifications

Name: High capacity cDNA reverse transcription kit.

Make: Invitrogen CA, USA.

Catalog number: 4368814.

Storage conditions: Stored at -20^{0}C.

- **Starting material**

  2 µg of total RNA was used per 20µl reaction.

- **Kit components**

  10X RT Buffer, 10X RT Random Primers, 25X dNTP Mix (100 mM), MultiScribe™ Reverse Transcriptase, 50 U/µl RNase Inhibitor.

- **Reagents**

  Nuclease-free water.

- **Procedure**

Kit components were allowed to thaw on ice. Volume of components required was calculated as per number of reactions. Kit components required to prepare one reaction of the 2X RT master mix are: 10X RT Buffer: 2.0 µl, 10X RT Random Primers: 2.0 µl, 25X dNTP Mix (100 mM): 0.8 µl, MultiScribe™ Reverse Transcriptase: 1µl, Nuclease-free water: 4.2 µl. Total reaction volume is 10 µl. 2X RT master mix was placed on ice and mixed gently. To prepare cDNA RT reaction, in a PCR tubes-10 µl RNA was added into the prepared 10 µl 2X RT master mix. It was mixed by pipetting up and down two times. Tubes was then briefly centrifuged to spin down the contents and to eliminate any air bubbles. Further these tubes were placed into thermal cycler. Thermal cycler was programmed according to following conditions:

	Step 1	Step 1	Step 1	Step 1
Temperature (^{0}C)	25	37	85	4
Time	10 min	120 min	5 min	∞

Reaction volume was set to 20 µl and reverse transcription run was started. After completion, cDNA was stored at -20^{0}C. The qPCR analysis was performed with the help of a StepOne realtime PCR system (Applied Biosystems, CA, USA).

4.2.1.9.3 Quantitative real-time reverse transcription-polymerase chain reaction (qPCR)

The qPCR analysis was performed with the help of a StepOne realtime PCR system (Applied Biosystems, CA, USA) using TaqMan gene expression assays and TaqMan gene expression master mix.

- **Reagents**

TaqMan gene expression assays (Applied Biosystems, CA, USA. Stored at -20^{0}C), TaqMan gene expression master mix (Applied Biosystems, CA, USA. Stored at -4^{0}C), sterile distilled water.

- **Procedure**

cDNA, master mix and assay were allowed to thaw on ice. They were resuspended by gentle mixing and briefly centrifuged to bring liquid to the bottom of the tube. Number of reactions required for each assay was calculated. Volume required for PCR reaction mix component per 10 µl per single reaction are: TaqMan gene expression assay: 0.5 µl, TaqMan gene expression master mix: 5.0 µl, cDNA template: 2.5 µl and sterile distilled water: 2.0 µl. PCR reaction mix was prepared into a microcentrifuge tube. All the solutions were mixed well and the tube was centrifuged briefly. 7.5 µl of PCR reaction mix was transferred into each well of 48-well reaction plate. Then, 2.5 µl of cDNA (appropriately diluted) was added in duplicate in all the wells except NTC well. After this, 2.5 µl of sterile distilled water was added to the NTC well. Plate was sealed, centrifuged briefly and loaded into the instrument. Plate was run according to cycling conditions: 50°C for 2 min; 95°C for 10 min; and 40 cycles of 95°C for 15 s, 60°C for 1 min. The TaqMan gene expression assays that were used in this study are SOD (Sod1;

Rn00566938_m1), GPx (Gpx1; Rn00577994_g1), CAT (Cat; Rn00560930_m1), Paraoxonase-1 (Pon1) (Pon1; Rn01455909_m1), MMP-3 (Mmp3; Rn00591740_m1), MMP-9 (Mmp9 Rn00579162_m1) and TIMP-1 (Timp1; 1 Rn00587558_m1). The data was analyzed using Data Assist software version 3.0. The data is representative of synovium of at least from three rats. The relative abundance of the RNA was calculated to the amount of β-actin (Actb; Rn00667869_m1) using StepOne software version 2.2.2, DataAssist version 3.0 (Applied Biosystems, CA, USA) and the ΔΔ Ct method (Schmittgen and Livak, 2008).

4.3 To comparatively evaluate anti-osteoarthritic potential of selected gums and formulations using *in vivo* model

In this objective, comparative evaluation of selected three gums and three formulations were carried out using CIOA rat model to prioritize potential gum and formulation. Prioritized gum and formulation were evaluated for their mechanism of action using qPCR studies by studying OA biomarkers.

4.3.1 Collagenase type II-induced osteoarthritis (CIOA) rat model

Institutional animal ethics committee approval for the experimental protocol was obtained before initiation of the study (BVDUMC/CPCSEA/1563/2013-14). Guidelines laid down by the CPCSEA were observed throughout the study for animal handling and experimentation. Rats were obtained from National Institute of Biosciences (Pune, India).

4.3.1.1 Experimental animals

54 female wistar rats weighing between 180-300 gm were used for the experiment. They were housed for two weeks in solid bottomed polypropylene cages for acclimatization before use, maintained under standard conditions and fed standard rat chaw with water *ad libitum*.

4.3.1.2 Drug and dosage

The dose was selected based on the human dose mentioned in the *Ayurvedic* literature and dose was calculated for animals based on the body surface area ratio (Methekar et al., 2012). Powders of gums, *Triphala* and '*Triphala Guggul*' were administered orally at the dose of 270 mg/kg b. wt. p. o. in the form of suspension prepared in water. *Dashamoolarishta* was administered orally at the dose of 0.9 ml/kg b. wt. p. o. Standard drug indomethacin (purchased from local pharmacy, manufactured by Jagsonpal pharmaceuticals Ltd., India) was given at the dose of 3 mg/kg b. wt. p. o. in 0.5% sodium-carboxy methyl cellulose based on previous reports (Rasool and Varalakshmi, 2007).

4.3.1.3 Treatment

Rats were divided into nine groups of 6 animals each.

Table 2: Grouping of animals and treatment specifications for evaluation of selected formulations and gums using CIOA model

Group No.	Group	Group code	Treatment
I	Healthy control	HC	-
II	Osteoarthritic control	CIOA	Collagenase
III	Positive control	INDO	Collagenase+Indomethacin (3 mg/kg b. wt.)
IV	*Triphala*	TC	Collagenase+Test groups
V	'*Triphala Guggul*'	TG	
VI	*Dashamoolarishta*	DA	
VII	*B. ceiba*	BC	
VIII	*B. serrata*	BS	
IX	*A. nilotica*	AN	

4.3.1.4 Induction of OA

OA was induced as described in section 4.2 (4.2.1.4).

4.3.1.5 Body weight, Knee diameter, Paw volume measurement

Body weight, knee diameter and paw volume was measured as described in section 4.2 (4.2.1.5).

4.3.1.6 Glycosaminoglycan (GAG) release

GAG release was measured from serum as described in section 4.2 (4.2.1.6).

4.3.1.7 Radiological analysis

Carried out as per protocol described in section 4.2 (4.2.1.7).

4.3.1.8 Histopathological analysis

On day 34th, animals were sacrificed. The right knee joint were dissected out and fixed in 10% phosphate buffered formalin (Sigma Aldrich, USA). The joints were than decalcified, sectioned and finally stained with hematoxyline and eosin (H and E), Masson's trichome and Safranin-O. Synovium was dissected out and fixed in 10% phosphate buffered formalin for staining. Tissue samples were prepared for light microscopy using standard procedures.

Histopathology studies were performed as described in section 4.2 (4.2.1.8). Slides were analysed by Dr. Bhupendra D. Mohole, MBBS, DNB-Pathology, DCP-Pathology, Sinhagad Pathology Laboratory, Pune, Maharashtra, India.

4.3.1.9 Quantitative real-time reverse transcription-polymerase chain reaction (qPCR) analysis

Carried out as per protocol described in section 4.2 (4.2.1.9). Animals were killed at the end of the experiment and their synovial tissue was removed. The synovium were flash frozen immediately in liquid nitrogen and stored at -80^{0}C until further use. The TaqMan gene expression assays that were used in this study are SOD (Sod1; Rn00566938_m1), GPx (Gpx1; Rn00577994_g1), CAT (Cat; Rn00560930_m1), Paraoxonase-1 (Pon1) (Pon1; Rn01455909_m1), MMP-3 (Mmp3; Rn00591740_m1) and TIMP-1 (Timp1; 1 Rn00587558_m1). The data was analyzed using Data Assist software version 3.0. The data is representative of synovium from at least three rats. The relative

abundance of the RNA was calculated to the amount of β-actin (Actb; Rn00667869_m1) using StepOne software version 2.2.2, DataAssist version 3.0 (Applied Biosystems, CA, USA) and the ΔΔ Ct method (Schmittgen and Livak, 2008).

4.4 To develop and comparatively evaluate anti-osteoarthritic potential of selected new combination formulations using *in vivo* model

Development of new combination formulation

Based on comparative evaluation of individual plants, *S. cordifolia* (S), *P. longum* (P) and *Z. officinale* (Z) were prioritized as they exhibits highest ant-osteoarthritic potential. Different combinations of these plants were prepared according to the mathematical formula ${}^{n}C_{r}$.

$${}^{n}C_{r} = {}^{3}C_{1} + {}^{3}C_{2} + {}^{3}C_{3}$$
$$= 3 + 3 + 1 = 7$$

Where, n= Number of selected plants, r= Number of plants taken at a time.

Possible combinations of five selected plants:

${}^{3}C_{1}$: S, P and Z

${}^{3}C_{2}$: SP, SZ and PZ

${}^{3}C_{3}$: SPZ

Where, S= *S. cordifolia*, P= *P. longum*, Z= *Z. officinale.*

Thus, four different new combination formulations were prepared from these prioritized plants and were comparatively evaluated using CIOA model.

Table 3: The composition of new combination formulations

Sr. No.	Formulation code	Composition	Combination
1	SP	Powders of *S. cordifolia* roots and *P. longum* inflorescence	1:1
2	PZ	Powders of *P. longum* inflorescence and *Z. officinale* rhizomes	1:1
3	SZ	Powders of *S. cordifolia* roots and *Z. officinale* rhizomes	1:1
4	SPZ	Powders of *S. cordifolia* roots, *P. longum*, inflorescence and *Z. officinale* rhizomes	1:1:1

Prioritized formulation was further evaluated for its mechanism of action using qPCR studies by studying OA biomarkers. Further acute and sub-acute toxicity studies of prioritized new formulation were carried out.

4.4.1 Collagenase type II-induced osteoarthritis (CIOA) rat model

Institutional animal ethics committee approval for the experimental protocol was obtained before initiation of the study (BVDUMC/CPCSEA/2679/2013-14). Guidelines laid down by the CPCSEA were observed throughout the study for animal handling and experimentation. Rats were obtained from National Institute of Biosciences (Pune, India).

4.4.1.1 Experimental animals

90 female wistar rats weighing between 180-300 gm were used for the experiment. They were housed for two weeks in solid bottomed polypropylene cages for acclimatization before use, maintained under standard conditions and fed standard rat chaw with water *ad libitum*.

4.4.1.2 Drug and dosage

All formulations were administered to rats at lower dose (135 mg/kg b. wt. p. o.), middle dose (270 mg/Kg b. wt. p. o.) and higher dose (540 mg/Kg b. wt. p. o.). As per suggestion given by ethical committee, standard drug diclofenac (Novartis dispersible diclofenac tablets-voveran D, manufactured by Medreich Limited, India) was used and given at the dose of 10 mg/kg b. wt. in 0.5% sodium-carboxy methyl cellulose. The formulation doses were selected based on the human dose mentioned in the *Ayurvedic* literature and was calculated for animal use based on the body surface area ratio. Treatment was given as a suspension of doses in water, once per day orally through feeding needles from day 14^{th} to 34^{th}.

4.4.1.3 Treatment

Rats were divided into fifteen groups of 6 animals each.

4.4.1.4 Induction of OA

OA was induced as described in section 4.2 (4.2.1.4).

4.4.1.5 Body weight, Knee diameter and Paw volume measurement

Body weight, knee diameter and paw volume was measured as described in section 4.2 (4.2.1.5).

Table 4: Grouping of animals and treatment specifications for selected new combination formulations using CIOA model

Group No.	Group	Group code	Treatment
I	Healthy control	HC	Saline
II	Osteoarthritic control	CIOA	Collagenase
III	Positive control	DICLO	Collagenase+Diclofenac (10 mg/kg b. wt.)
IV	*S. cordifolia+P. longum*	SPL	Collagenase+Test drug (135 mg/kg b. wt.)
V		SPM	Collagenase+Test drug (270 mg/kg b. wt.)
VI		SPH	Collagenase+Test drug (540 mg/kg b. wt.)
VII	*P. longum+Z. officinale*	PZL	Collagenase+Test drug (135 mg/kg b. wt.)
VIII		PZM	Collagenase+Test drug (270 mg/kg b. wt.)
IX		PZH	Collagenase+Test drug (540 mg/kg b. wt.)
X	*S. cordifolia+Z. officinale*	SZL	Collagenase+Test drug (135 mg/kg b. wt.)
XI		SZM	Collagenase+Test drug (270 mg/kg b. wt.)
XII		SZH	Collagenase+Test drug (540 mg/kg b. wt.)
XIII	*S. cordifolia+P. longum+Z. officinale*	SPZL	Collagenase+Test drug (135 mg/kg b. wt.)
XIV		SPZM	Collagenase+Test drug (270 mg/kg b. wt.)
XV		SPZH	Collagenase+Test drug (540 mg/kg b. wt.)

4.4.1.6 Quantitative determination of C-reactive protein (CRP)

Blood was taken from rats before and after the treatment through retro-orbital vein puncture and serum was separated. CRP is an acute-phase protein present in normal serum, which increases significantly after most forms of tissue injuries, bacterial and virus infections, inflammation and malignant neoplasia. CRP was quantitatively determined using kit,

Kit specifications:

Name: CRP turbilatex

Make: Spinreact

Catalog number: TLIS01

Storage conditions: Stored at 2-8^{0}C.

- **Test principle**

CRP is quantitatively determined, before and after treatment from serum using CRP-Turbilatex turbidometric test. Latex particles coated with specific anti-CRP are agglutinated when mixed with samples containing CRP. The agglutination causes an absorbance change, dependent upon the CRP contents of the samples that can be quantified by comparison from a calibrator of unknown CRP concentration.

- **Sample preparation**

Samples were centrifuged and serum was isolated for assay.

- **Reagents**

- **Diluent:** Tris buffer 20 mmol/litre, pH: 8.2. Preservative.
- **Latex:** Latex particles coated with IgG anti-human CRP, pH: 7.3. Preservative.
- **CRP Calibrator:** Reconstituted with 1.0 ml of distilled water. Mixed gently and incubated for 10 minutes at room temperature before use.

- **Procedure**

Working reagents were allowed to reach at room temperature and the photometer (cuvette holder) to 37ºC. Assay conditions: Wavelength: 540 nm (530-550) Temperature: 37ºC. Instrument was adjusted to zero with distilled water. 1.0 ml working reagent and 5 µl calibrator or sample was pipette into cuvette. Mixed and absorbance was read immediately (A1) and after 2 minutes (A2) of the sample addition.

CRP (mg/l): $[(A2\text{-}A1)_{sample}/(A2\text{-}A1)_{calibrator}]$xCalibrator concentration.

4.4.1.7 Glycosaminoglycans (GAG) release

GAG release was measured from serum as described in section 4.2 (4.2.1.6).

4.4.1.8 Determination of Alkaline phosphatase (ALP)

Blood was taken from rats before and after the treatment through retro-orbital vein puncture and serum was separated. ALP found in plasma membranes of osteoblastic cells, have role in bone mineralization (Padmini et al., 2004). It's high level in blood may indicate bone, liver or bile duct disease (Sodicoff 1995, Alam et al., 2006). ALP was determined using kit,

Kit specifications

Name: Alkaline Phosphatase Kit (Mod. Kind & King's Method).

Make: Coral Clinical Systems, Goa, India.

Catalog Number: ALP 010 / ALP 011.

- **Principle**

ALP at an alkaline pH hydrolyses di Sodium Phenylphosphate to form phenol. The Phenol formed reacts with 4-Aminoantipyrine in the presence of Potassium Ferricyanide ($K_3Fe(CN)_6$), an oxidising agent, to form a red coloured complex. The intensity of the colour formed is directly proportional to the activity of ALP present in the sample.

- **Kit components**

L1: Buffer reagent, L2: Substrate reagent, L3: Colour reagent, S: Phenol standard (10 mg/dl).

- **Procedure**

All the test tubes were marked properly as blank (B), standard (S), control (C), and test (T). Distilled water (1.05 ml in B and 1.0 ml in S, C and T) was added in the tubes. L1 Reagent (1 ml) and L2 Reagent (0.10 ml) were added in all the tubes. The contents were mixed well and incubated at 37°C for 3 min. Serum (0.05 ml) was added in test (T) and Phenol standard (0.05 ml) was added in standard (S). Contents of the tubes were mixed well and incubated at 37°C for 15 min. L3 Reagent (1 ml) was added in all the tubes. Serum (0.05 ml) was added in control (C). Contents of the tubes were mixed well and absorbance was read at 510 nm against distilled water as blank. Serum alkaline phosphatase activity is expressed as KA units.

Total ALP activity (K.A. Units)**:** (Abs.T-Abs.C/ Abs.S-Abs.B)x10.

4.4.1.9 Radiological analysis

Carried out as per protocol described in section 4.2 (4.2.1.7).

4.4.1.10 Histopathological analysis

On day 34th, animals were sacrificed. The right knee joint were dissected out and fixed in 10% phosphate buffered formalin (Sigma Aldrich, USA) and were decalcified, sectioned and finally stained with hematoxyline and eosin (H and E),

Masson's trichome and Safranin-O. Synovium was dissected out and fixed in 10% phosphate buffered formalin for staining. Tissue samples were prepared for light microscopy using standard procedures.

Histopathology studies were performed as described in section 4.2 (4.2.1.8). Slides were analysed by Dr. Bhupendra D. Mohole, MBBS, DNB-Pathology, DCP-Pathology, Sinhagad Pathology Laboratory, Pune, Maharashtra, India.

4.4.1.11 Quantitative real-time reverse transcription-polymerase chain reaction (qPCR) analysis

The qPCR analysis was carried out as per protocol described in section 4.2 (4.2.1.9). Animals were killed at the end of the experiment and their synovial tissue was removed. The synovium were flash frozen immediately in liquid nitrogen and stored at -80^0C until further use. The TaqMan gene expression assays that were used in this study are SOD (Sod1; Rn00566938_m1), GPx (Gpx1; Rn00577994_g1), CAT (Cat; Rn00560930_m1), MMP-3 (Mmp3; Rn00591740_m1), MMP-9 (Mmp9 Rn00579162_m1) and TIMP-1 (Timp1; 1 Rn00587558_m1). The data was analyzed using Data Assist software version 3.0. The data is representative of synovium at least from three rats. The relative abundance of the RNA was calculated to the amount of β-actin (Actb; Rn00667869_m1) using StepOne software version 2.2.2, DataAssist version 3.0 (Applied Biosystems, CA, USA) and the ΔΔ Ct method (Schmittgen and Livak, 2008).

4.4.2 Toxicity studies of prioritized formulation

The safety profile of prioritized developed formulation viz. SZ was further evaluated by investigating acute and sub-acute toxicities in rodents. Institutional animal ethics committee approval for the experimental protocol was obtained before initiation of the study (BVDUMC/CPCSEA/2679/2013-14). Guidelines laid down by the CPCSEA were followed throughout the study for animal handling and experimentation. Rats were obtained from National Institute of Biosciences (Pune, India).

4.4.2.1 Acute toxicity test

The acute toxicity study of SZ was carried out according to the Organization for Economic Cooperation and Development (OECD) guideline 420 (OECD, 2001).

4.4.2.1.1 Experimental animals

12 Female wistar rats (average weight 80-100 g) were selected for this study. They were housed for two weeks in solid bottomed polypropylene cages for acclimatization before use, maintained under standard conditions and fed standard rat chaw with water *ad libitum*.

4.4.2.1.2 Experimental protocol

Animals were randomly divided into 2 groups comprising six animals per group. Group 1 served as control and Group 2 served with formulation SZ. SZ was administered orally at a single dose of 2000 mg/kg. Animals were observed individually after dosing at least once during the first 30 min, periodically during the first 24 hr, with special attention given during the first 4 hr and daily thereafter, for a total of 14 days. All observations were systematically recorded, with individual records being maintained for each animal. Animals were observed for toxicity signs like tremors, convulsions, salivation, diarrhoea, lethargy, sleep and coma. Along with this, individual weights of animals were determined shortly before the test formulation was administered and at least weekly thereafter. Body weights of the animals were recorded on day 0 and at an interval of 5 days till the end of the experiment. At the end of the experiment, all animals were sacrificed by cervical decapitation under light ether anesthesia for gross necropsy findings.

4.4.2.2 Sub-acute toxicity test (Repeated dose 28 day oral toxicity study)

Sub-Acute toxicity study was carried out according to the Organization for Economic Cooperation and Development (OECD) guideline 407 (OECD, 2008).

4.4.2.2.1 Experimental animals

Male and female wistar rats (average weight 80-100 gm) were used for this study. They were housed for two weeks in solid bottomed polypropylene cages for acclimatization before use, maintained under standard conditions and fed standard rat chaw with water *ad libitum*.

4.4.2.2.2 Experimental protocol

The animals were randomly divided into 6 groups comprising 5 males and 5 females (total of 10 animals) rats per group. Group 1 served as Control; group 2 was served as reversal control group, while SZ group kept as test formulation group with different doses (Table 5). The animals were given dose of SZ daily for 28 days. Three doses were selected according to acute toxicity study results. The additional Reversal groups of the control and the high dose group of SZ were kept for 14-28 days post-treatment to observe the reversibility, persistence, or delayed occurrence of toxic effects of the test compound.

Table 5: Grouping of animals and test specifications for subacute toxicity study

Group No.	Test groups	Treatment specification	Period of study
1.	C	Control	28 days
2.	C-REV	Control-REV	42 days
3.	SZ-L	SZ (135 mg/kg b. wt.)	28 days
4.	SZ-M	SZ (270 mg/kg b. wt.)	28 days
5.	SZ-H	SZ (540 mg/kg b. wt.)	28 days
6.	SZ-REV	SZ (540 mg/kg b. wt.)	42 days

Note: n=10; 5 male wistar rats and 5 female wistar rats per group. L: Lower dose, M: Middle dose, H: Higher dose, REV: Reversal group.

4.4.2.2.3 Assessment of body weight and food consumption

Changes in body weight were measured at specific time intervals and food consumption was evaluated daily for all rat groups.

4.4.2.2.4 Hematological and biochemical analysis

At the end of the experiment, blood samples were collected from the orbital sinus for biochemical and hematological analysis. The hematological parameters included the Haemoglobin, Red blood cell (RBC) count, Hematocrit contents, Mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), white blood cell (WBC) count, Neutrophils, Lymphocytes, Eosinophils, Monocytes and Platelet count. The biochemical parameters were studied in serum obtained after centrifugation of blood at 2000 RPM for 15 min. The enzymes evaluated include Alkaline phosphatase (ALP), serum glutamic-oxaloacetic transaminase (SGOT), serum glutamic-pyruvic transaminase (SGPT), Direct Bilirubin, Total bilirubin, Uric acid, and Urea, which were assessed using the commercial kits (Coral Clinical Systems, Goa, India).

A) Alkaline Phosphatase

Alkaline Phosphatase (ALP) is an enzyme of the Hydrolase class of enzymes and acts in an alkaline medium. It is found in high concentrations in liver, biliary tract epithelium and in the bones. Normal levels are age dependent and increase during bone development. Increased levels are associated mainly with liver and bone diseases. Moderate increases are seen in Hodgkins diseases and congestive heart failure.

ALP was determined as described in section 4.4 (4.4.1.8).

B) Serum glutamic-oxaloacetic transaminase (SGOT)

Kit specifications

Name: SGOT (ASAT) Kit (Reitman & Frankel's method)

Make: Coral Clinical Systems

Catalog Number: GOT 010

SGOT is an enzyme found mainly in heart muscle, liver cells, skeletal muscle and kidneys. Injury to these tissues results in the release of the enzyme in blood stream. Elevated levels are found in myocardial infarction, Cardiac operations, Hepatitis, Cirrhosis, acute pancreatitis, acute renal diseases, and primary muscle diseases. Decreased levels may be found in Pregnancy, Beri Beri and Diabetic ketoacidosis.

- **Principle**

SGOT converts L-Aspartate and α Ketoglutarate to Oxaloacetate and Glutamate. The Oxaloacetate formed reacts with 2, 4, Dinitrophenyl hydrazine to produce a hydrazone derivative, which in an alkaline medium produces a brown coloured complex whose intensity is measured. The reaction does not obey Beer's law and hence a calibration curve was plotted using a Pyruvate standard. The activity of SGOT (ASAT) was read off this calibration curve.

- **Reagents**

L1: Substrate Reagent

L2: DNPH Reagent

L3: NaOH Reagent (4N)

S: Pyruvate Standard (2 mM)

Note: 4N NaOH reagent diluted 1:10 with distilled water before use (Working NaOH reagent).

- **Calibration curve**

Test tubes were labeled as 1, 2, 3, 4 and 5. Table 6 represents the protocol for the calibration curve.

Table 6: Protocol for calibration curve for SGOT

Addition sequence	**1**	**2**	**3**	**4**	**5**
Enzyme activity (U/ml)	0	24	61	114	190
	(ml)	(ml)	(ml)	(ml)	(ml)
L1	0.50	0.45	0.40	0.35	0.30
S	--	0.05	0.10	0.15	0.20
Distilled water	0.10	0.10	0.10	0.10	0.10
L2	0.50	0.50	0.50	0.50	0.50
Mix well and allow to stand at R.T. for 20 minutes					
L3 (Working reagent)	5.00	5.00	5.00	5.00	5.00

The solutions were mixed well and allowed to stand at R.T. for 10 min. Absorbance of the tubes 2-5 against tube 1 (Blank) at 505 nm was measured. Graph of the absorbances of tubes 2-5 on the 'Y' axis versus the corresponding enzyme activity on the 'X' axis was plotted.

- **Assay Protocol**

Assay was performed as per Table 7. Test tubes were labeled as Blank (B) and Test (T).

Table 7: Assay protocol for SGOT

Addition sequence	B (ml)	T (ml)
L1	0.50	0.50
Incubate at 37°C for 3 minutes		
Sample	--	0.10
Incubate at 37°C for 60 minutes		
L2	0.50	0.50
Mix well and allow to stand at R.T. for 20 minutes		
Distilled water	0.10	--
L3 (Working reagent)	5.00	5.00

Solutions were mixed well and allowed to stand at R.T. for 10 min. Absorbance of the Test (T) against Blank (B) at 505 nm was measured and read the activity of the test samples from the calibration curve graph.

C) Serum glutamic-pyruvic transaminase (SGPT)

Kit specifications

Name: SGPT (ALAT) Kit (Reitman & Frankel's method)

Make: Coral Clinical Systems

Catalog Number: GPT 010 / GPT 011

SGPT is found in a variety of tissues but is mainly found in liver. Increased levels are found in hepatitis, cirrhosis, obstructive jaundice and other hepatic diseases. Slight elevation of the enzymes is also seen in myocardial infarction.

- **Principle**

SGPT converts L-Alanine and α Ketoglutarate to Pyruvate and Glutamate. The Pyruvate formed reacts with 2, 4, Dinitrophenyl hydrazone, which in an alkaline medium produces a brown coloured complex whose intensity is measured. The reaction does not obey Beer's law and hence a calibration curve is plotted using a Pyruvate standard. The activity of SGPT (ALAT) is read off this calibration curve.

- **Reagents**

L1: Substrate Reagent

L2: DNPH Reagent

L3: NaOH Reagent (4N)

S: Pyruvate Standard (2 mM)

Note: 4N NaOH reagent diluted 1:10 with distilled water before use (Working NaOH reagent).

- **Calibration curve:**

Test tubes were labeled as 1, 2, 3, 4 and 5. Table 8 represents the protocol for the calibration curve.

Table 8: Protocol for calibration curve for SGPT

Addition sequence	**1**	**2**	**3**	**4**	**5**
Enzyme activity (U/ml)	0	24	61	114	190
	(ml)	(ml)	(ml)	(ml)	(ml)
L1	0.50	0.45	0.40	0.35	0.30
S	--	0.05	0.10	0.15	0.20
Distilled water	0.10	0.10	0.10	0.10	0.10
L2	0.50	0.50	0.50	0.50	0.50
Mix well and allow to stand at R.T. for 20 minutes					
L3 (Working reagent)	5.00	5.00	5.00	5.00	5.00

Mixed well and allowed to stand at R.T. for 10 min. Absorbance of the tubes 2-5 against tube 1 (Blank) at 505 nm was measured. Graph of the absorbance's of tubes 2-5 on the 'Y' axis versus the corresponding Enzyme activity on the 'X' axis was plotted.

- **Assay Protocol**

Assay was performed as per Table 9. Test tubes were labeled as Blank (B) and Test (T).

Table 9: Assay protocol for SGPT

Addition sequence	B (ml)	T (ml)
L1	0.50	0.50
Incubate at 37°C for 3 minutes		
Sample	--	0.10
Incubate at 37°C for 30 minutes		
L2	0.50	0.50
Mix well and allow to stand at R.T. for 20 minutes		
Distilled water	0.10	--
L3 (Working reagent)	5.00	5.00

Solutions were mixed well and allowed to stand at R.T. for 10 min. Absorbance of the Test (T) against Blank (B) at 505 nm was measured and read the activity of the test samples from the calibration curve graph.

D) Bilirubin

Kit specifications

Name: Bilirubin Kit (Mod. Jendrassik & Grof's Method)

Make: Coral Clinical Systems

Catalog Number: BIL 010 / BIL 011 / BIL 012

Bilirubin is mainly formed from the heme portion of aged or damaged RBC'S. It then combines with albumin to form a complex, which is not water-soluble. This is referred to as indirect or unconjugated Bilirubin. In the liver this Bilirubin complex is combined with glucuronic acid into a water-soluble conjugate. This is referred to as conjugated or direct Bilirubin. Elevated levels of bilirubin are found in liver diseases (Hepatitis, cirrhosis), excessive haemolysis / destruction of RBC (hemolytic jaundice)

obstruction of the biliary tract (obstructive jaundice) and in drug induced reactions. The differentiation between the direct and indirect bilirubin is important in diagnosing the cause of hyperbilirubinemia.

- **Principle**

Bilirubin reacts with diazotised sulphanilic acid to form a coloured azobilirubin compound. The unconjugated bilirubin couples with the sulphanilic acid in the presence of a caffein- benzoate accelerator. The intensity of the colour formed is directly proportional to the amount of bilirubin present in the sample.

- **Reagents**

L1: Direct Bilirubin Reagent

L2: Direct Nitrite Reagent

L1: Total Bilirubin Reagent

L2: Total Nitrite Reagent

S: Artificial Standard (10 mg/dl)

- **Direct Bilirubin Assay Protocol**

Assay was performed as per Table 10. Test tubes were labeled as Blank (B) and Test (T).

Table 10: Assay protocol for Direct Bilirubin

Addition sequence	B (ml)	T (ml)
L1 (Direct Bilirubin Reagent)	1.00	1.00
L2 (Direct Nitrite Reagent)	--	0.05
Sample	0.10	0.10

The solutions were mixed well and incubated at R.T. for exactly 5 min. Absorbance of the Test (A_T) against respective Blanks (A_B) at 546 nm was measured.

- **Total Bilirubin Assay Protocol**

Assay was performed as per Table 11. Test tubes were labeled as Blank (B) and Test (T).

Table 11: Assay protocol for Total Bilirubin

Addition sequence	B (ml)	T (ml)
L1 (Total Bilirubin Reagent)	1.00	1.00
L2 (Total Nitrite Reagent)	--	0.05
Sample	0.10	0.10

Mixed well and incubated at R.T. for exactly 10 min. Absorbance of the Test (A_T) against respective Blanks (A_B) at 546 nm was measured.

Total or Direct Bilirubi in mg/dl = $A_T \times 13$

E) Uric acid

Kit specifications

Name: Uric Acid Kit (Uricase/PAP method)

Make: Coral Clinical Systems

Catalog Number: UAC 010 / UAC 011 / UAC 012 / UAC 013 / UAC 014

Uric acid is the end product of the Purine metabolism. Uric acid is excreted to a large degree by the kidneys and to smaller degree in the intestinal tract by microbial degradation. Increased levels are found in Gout, arthritis, impaired renal functions, and starvation. Decreased levels are found in Wilson's disease, Fanconis syndrome and yellow atrophy of the liver.

- **Principle**

Uricase converts uric acid to allantoin and hydrogen peroxide. The hydrogen peroxide formed further reacts with a phenolic compound and 4 aminoantipyrine by the catalytic action of peroxidase to form a red coloured quinoneimine dye complex. Intensity of the colour formed is directly proportional to the amount of uric acid present in the sample.

- **Reagents**

L1: Buffer Reagent

L2: Enzyme Reagent

S: Uric Acid Standard (8 mg/dl)

- **Reagent preparation**

  Working reagent: Mix together 4 parts of L1 (Buffer Reagent) and 1 part of L2 (Enzyme Reagent).

- **Assay Protocol**

Assay was performed as per Table 12. Test tubes were labeled as Blank (B), Standard (S) and Test (T).

Table 12: Assay protocol for Uric acid

Addition sequence	B (ml)	S (ml)	T (ml)
Working Reagent	1.00	1.00	1.00
Distilled water	0.02	--	--
S	--	0.02	--
Sample	--	--	0.02

All the solutions were mixed well and incubated at 37°C for 5 min. Absorbance of the Standard (A_S) and Test Sample (A_T) against Blank (A_B) within 30 min at 520 nm was measured. $\textbf{Uric Acid in mg/dl} = \frac{A_T}{A_S} \times 8$

F) Urea

Kit specifications

Name: Urea Kit (Mod. Berthelot method)

Make: Coral Clinical Systems

Catalog Number: URE 020 / URE 021 / URE 022

Urea is the end product of the protein metabolism. It is synthesized in the liver from the ammonia produced by the catabolism of amino acids. It is transported by the blood to the kidneys from where it is excreted. Increased levels are found in renal diseases, urinary obstructions, shock, congestive heart failure and burns. Decreased levels are found in liver failure and pregnancy.

- **Principle**

Urease hydrolyzes urea to ammonia and CO .The ammonia formed further reacts with a phenolic chromogen and hypochlorite to form a green coloured complex. Intensity of the colour formed is directly proportional to the amount of urea present in the sample.

- **Reagents**

L1: Buffer Reagent

L2: Enzyme Reagent

L3: Chromogen Reagent

S: Urea Standard (40 mg/dl)

- **Assay Protocol**

Assay was performed as per Table 13. Test tubes were labeled as Blank (B), Standard (S) and Test (T).

4.4.2.2.5 Organ toxicity

After necropsy, the gross examinations of target vital organs of all animals were done to check any significant changes in weight, texture and shape. The weights of major organs viz. Brain, Liver, Kidney, Adrenals, Heart, Thymus, Spleen, Testis, Uterus, Epididymis, Ovaries and Lungs were recorded.

Table 13: Assay protocol for Urea

Addition sequence	B (ml)	S (ml)	T (ml)
L1	1.00	1.00	1.00
L2	0.10	0.10	0.10
Distilled water	0.01	--	--
S	--	0.01	--
Sample	--	--	0.01
Mix well and incubate 37°C for 5 minutes			
L3	0.20	0.20	0.20

The solutions were mixed well and incubated at 37°C for 5 min. Absorbance of the Standard (A_S) and Test Sample (A_T) against Blank (A_B) within 60 min at 570 nm was measured. $\textbf{Urea in mg/dl} = \frac{A_T}{A_S} \times 40$

4.4.2.2.6 Histopathological examinations

Major organs like liver, kidney, brain and lungs were removed from each rat after dissection. All these organs were washed once with 1X PBS and immediately fixed in 10% formalin. The formalin fixed organs were dehydrated with increasing concentrations of ethanol and were further embedded in paraffin. The 4 µm sections of each organ were deposited on a slide and later stained with hematoxylin and eosin (protocol: 2.1.8.1). Slides were observed microscopically and photographed.

4.5 To evaluate anti-arthritic effects of new combination formulation using HIG-82, rabbit synoviocytes

As the formulation found to be safe in toxicity studies, we have then elucidated its mechanism of action in inflammation induced synoviocytes cells. Tissue culture grade media, lipopolysaccahride, enzymes and chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Analytical grade chemicals were purchased from Qualigens. Cell culture plasticware were purchased from BD Biosciences, CA, USA and Axygen Scientific Inc, CA, USA.

4.5.1 Extract preparation

Water extract of SZ (7.5%) was prepared and it was incubated overnight at 37°C at 110 rpm. SZ extract was filtered using filter paper and centrifuged at 5000 rpm for 10 minutes. Extract supernatant was filtered through 0.4 micron sterile filters and yield was calculated. Based on yield, stock of 1 mg/ml was prepared and used further for assay using different concentrations.

4.5.2 Rabbit Synoviocytes sarcoma (HIG-82) cell line

The rabbit synoviocyte sarcoma cell line, HIG-82, used in the study was obtained from National Centre for Cell Science (NCCS), Pune, India.

4.5.1.2 Maintenance of HIG-82 cell line

HIG-82 cell line was routinely cultured in T25 culture flask and grown in media supplemented with 5 ml of Nutrient mixture Hams F-12 supplemented with 10% FBS, 2 mM L-glutamine and 1% penicillin–streptomycin. Three or four days later the cultures were given a change of growth medium. They normally became confluent in five to six days later.

Confluent cultures were trypsinized using following protocol,

The growth medium was warmed at 37ºC incubator for at least 30 min prior to trypsinization. Trypsin EDTA (1 ml) was added to cover the adherent cells in culture flask and incubated for 5 min at RT or in a 37ºC incubator, checked for cell detachment under a microscope. If cells have not detached, flask was gently taped to dislodge the cells, or let the cells incubate longer, checking them every minute under a microscope. When all the cells had detached, serum-containing media was added to a final volume of 5 ml (Note: the media must contain serum to inactivate the trypsin). The cells were gently pipetted up and down to mix, and then cell suspension was transferred to a centrifuge tube. The cells were centrifuged at 2000 rpm for 5 min to pellet. Media was aspirated and cell pellet was washed with 5 ml of cold 1X PBS. The cells were spun at 2000 rpm for 5 min to pellet. Cell pellet was resuspended in 5 ml of growth media using a 1:2 or 1:4 split ratio. This process was repeated until cultures were no longer able to achieve confluence.

4.5.2.2 Cell counting using hemocytometer

Cell counting was performed according to Neubauer improved method. The glass hemocytometer and its cover slip was cleaned using 70% ethyl alcohol (ethanol). A small aliquot (10 µl) of resuspended cells was taken in separate eppendorf tube and 400 µl of 0.4% Trypan Blue was added and mixed gently. Trypan Blue-treated cell suspension (10 µl) was taken on the hemocytometer. Both chambers were filled very gently underneath the coverslip, allowing the cell suspension drawn out by capillary action. Using an inverted microscope, hemocytometer grid lines was focused with a 10X objective. The live and unstained cells were counted using a hand tally counter (live cells do not take up Trypan Blue) in one set of 16 squares. Further, hemocytometer was moved to the next set of 16 corner squares and counting was carried out until all 4 sets of 16

corners were counted. The average cell count from each of the sets of 16 corner squares was noted. Further, multiplied by 10,000 (10^4) and by 5 to correct for the 1:5 dilution from the Trypan Blue addition. The final value was the number of viable cells/ml in the original cell suspension.

4.5.3 Cell toxicity using MTT assay

- **Principle**

This is a colorimetric assay that measures reduction of yellow 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) by active mitochondrial succinate dehydrogenase. The MTT enters the cells and passes into the mitochondria where it is reduced to an insoluble, coloured (dark purple) formazan product. These formazan crystals were then solubilised with an organic solvent, isopropanol and the released, solubilised formazan reagent is measured spectrophotometrically. Since reduction of MTT can only occur in metabolically active cells, it is taken as a measure of viability of the cells (Gerlier and Thomasset, 1986).

- **Reagents**

- Preparation of MTT (5 mg/ml):

  100 mg of MTT powder was dissolved in 20 ml of 1X PBS. Solution was filtered through syringe filter and protected from light. The stock solution was stored at 2-8°C till further use. Main stock was diluted in 1:6 ratio, 1 part of 5 mg/ml MTT and 5 parts of 10% supplemented FBS in 1X PBS and stored at 2-8ºC for 1 week.

- 20% Sodium dodecyl sulfate (SDS) in 50% Dimethylformamide (DMF):

  To 5g SDS, 12.5 ml of DMF was added, solution was heated at 60ºc in water bath, once the SDS-DMF solution was dissolved, 12.5 ml distilled water was added.

- **Procedure**

MTT assay was performed as described in Gerlier and Thomasset (1986). Briefly, the synoviocytes was seeded at a density of $1x10^5$ cells/ml density in 96-well plates. An untreated group was kept as a negative control. SZ extract was added in linear concentrations: 0, 10, 20, 40, 80, 160 and 320 µg/ml in triplicates and this step was repeated for three times. MTT solution (5 mg/ml) was added to each well and the cells were grown for another 4 hr at 37°C in 5% CO_2 incubator. Formazan crystals were

dissolved by addition of 90 µl of sodium dodecyl sulphate (SDS)-dimethyl formamide (DMF) (20% SDS in 50% DMF). After 15 min, amount of coloured formazan derivative was determined by measuring optical density (OD) at 570 nm using the ELISA microplate reader (Biorad, Hercules, CA).

The percent viability: [OD of treated cells/OD of control cells] ×100

4.5.4 LPS-stimulated rabbit synoviocytes sarcoma model

- **Reagents**
  - **Reconstitution of LPS (Lipopolysaccharide)**

    Entire vial of 5 mg LPS was dissolved in sterile 5 ml 1X PBS (stock 1 mg /ml), mixed well.
  - **Preparation of 1X PBS**

    To make 10X buffer, weighed 1 g Potassium chloride (KCL), 1 g mono potassium phosphate (KH_2PO_4), 40 g Sodium Chloride (NaCl), 5.75 g disodium phosphate (Na_2HPO_4) and dissolved in 500 ml triple distilled water, mixed well. It was Autoclaved. 1X PBS was prepared by diluting it 1:10 in triple distilled water.
- **Procedure**

To mimic synovial inflammation in OA, HIG-82 cell line was stimulated with different concentrations of LPS (1, 5, 10, 20 µg/ml) for 24, 48 and 72 h using MTT dye (Ray and Ray, 1998). The condition media was evaluated for cellular inflammation measured in the form of NO released. Briefly, the synoviocytes was seeded at a density of 1×10^5 cells/ml in 96-well plates and incubated for 24 h. Next day, removed existing media (0.2 ml) and cells were treated with 10 µg/ml LPS in triplicate and grown overnight at 37°C in 5% CO_2 incubator. An untreated group was kept as a negative control. LPS treated cells kept as positive control and remaining LPS treated cells were treated with SZ extract at 20, 80 and 160 µg/ml concentration in triplicates and incubated for another 72 h. After 72 hr incubation, supernatant was collected for NO assay as an inflammatory marker. Once the NO levels were induced, the cells were further treated with respective SZ concentrations for another 24 hr. After incubation, the cells and supernatant were harvested for further biochemical analysis.

4.5.4.1 Determination of Nitric Oxide (NO) levels

Nitric oxide (NO), which is synthesized by nitric oxide synthase (NOS), is a short-lived free radical and an intercellular messenger produced by a variety of mammalian cells. The inducible forms of NOS are the most important pro-inflammatory enzymes responsible for increasing the levels of NO. Three isoforms of NOS have been identified. Expression of iNOS (inducible NOS), catalyses the formation of large amount of NO which plays a key role in the pathogenesis of a variety of inflammatory diseases. Therefore, the level of NO induced by iNOS provides an indicator to evaluate inflammatory processes (Yang et al., 2009). NO levels were measured using Griess reaction (Griess, 1879).

- **Principle**

NO levels were measured by colorimetric assay that allows determination of organic nitrite compounds, in this case NO released by the cells in the surrounding media. The nitrite compounds react with the reagent sulphanilic acid to form diazonium salt. When the reagent naphthylenediamine dihydrochloride (NEDD) is added, pink color develops which can be estimated photometrically and compared with the control of the samples. The amount of dissolved NO per ml of sample can be determined from a standard of sodium nitrate.

- **Reagents**

- Preparation of 5% ortho-phosphoric acid:

  28.409 ml of 88% ortho phosphoric acid stock was taken and volume was made up to 500 ml with distilled water.

- Griess Reagent A (1% sulphanilamide or sulphanilic acid):

  The reagent was prepared by adding 0.25 g of sulphanilamide or sulphanilic acid in 25 ml of 5% phosphoric acid solution (1.25 ml of phosphoric acid diluted to 25 ml with deionized water). The solution was than stored at cool temperature (not freezing) and protected from light.

- Griess Reagent B (0.1% N-1-Naphthylethylenediamine dihydrochloride):

  The reagent was prepared by dissolving 0.025 gm of N-1-Naphthyl- ethylenediamine dihydrochloride in 25 ml deionized water, and stored at cool temperature (not freezing) and protected from light.

- **Procedure**

100 µl of conditioned media was appropriately diluted using 1X PBS in 1.5 ml eppendorf tubes. To this solution 1% SA (50 µl) and 0.1% NEDD (50 µl) prepared in 5% phosphoric acid was added in 1:1 ratio. After incubation for 10 min at RT, 26 µl of solution was dispensed from each tube in 96-well U bottom plate. Absorbance was measured at 540 nm in a plate reader (Biorad, Hercules, CA). Further calculations were done from a standard curve made with linear concentrations of sodium nitrate.

- **Calculation of results**

A standard curve was created by plotting the mean absorbance for each standard verses the concentration and draw a best fit curve through the points on the graph.

4.5.4.2 Determination of Superoxide Dismutase (SOD) activity

Kit specifications

Name: Superoxide Dismutase (SOD) activity assay kit

Make: Biovision research products, USA

Catalog Number: K335-100

Storage condition: Stored at 4°C

- **Principle**

SOD is one of the most important anti-oxidative enzymes. It catalyzes the dismutation of the superoxide anion into hydrogen peroxide and molecular oxygen. The sensitive SOD assay kit utilizes WST-1 that produces a water-soluble formazan dye upon reduction with superoxide anion. The rate of the reduction with a superoxide anion is linearly related to the xanthine oxidase (XO) activity, and is inhibited by SOD. Therefore, the inhibition activity of SOD can be determined by a colorimetric method.

- **Kit contents**

WST solution, SOD enzyme solution, SOD assay buffer and SOD dilution buffer

- **Sample Preparation**

Homogenized 10^6 cells in ice cold 0.1M Tris/HCl (Sigma), pH 7.4 containing 0.5% Triton X-100 (Sigma), 5mM β –ME (2-Mercaptoethanol, Sigma) and 0.1mg/ml PMSF (Phenylmethanesulfonyl fluride, Sigma). The mixture was than centrifuged at 14000xg

for 5 minutes at 4°C. Cell debris was discarded and the supernatant was used for the assay. The supernatant contains total SOD activity from cytosolic and mitochondria.

- **Reagents**

- WST working solution: Diluted 1 ml of WST solution with 19 ml of Assay Buffer Solution. The diluted solution is stable for up to 2 months at 4°C.
- Enzyme working solution: The enzyme solution was centrifuged for 5 seconds. Mixed well by pipeting (The step is necessary, as the enzyme has two layers and must be mixed well before dilution). The 15 µl solution was diluted with 2.5 ml of dilution buffer. The diluted enzyme solution is stable for up to 3 weeks at 4°C.

- **Procedure**

20 µl of Sample solution was added to each sample and in blank 2 well of the 96-well plate and 20 µl H_2O were added to each blank 1 and blank 3 well. Further, 200 µl of the WST working solution was added to each well and 20 µl of dilution buffer were added to each Blank 2 and Blank 3 well. 20 µl of Enzyme working solution was then added to each sample and blank 1 well, mixing thoroughly. Then the plate was incubated at 37°C for 20 minutes. After incubation, absorbance was read at 450 nm using a microplate reader. SOD activity was calculated using following formula.

SOD activity (Inhibition rate %)**:** $(A_{blank1}-A_{blank3})-(A_{sample}-A_{blank2})/(A_{blank1}-A_{blank3})x100$

4.5.4.3 Determination of Catalase activity

Kit specifications

Name: Catalase activity colorimetric/fluorometric assay kit

Make: Biovision research products, USA

Catalog Number: K773-100

Storage condition: Stored at 4°C

- **Principle**

Catalase (EC 1.11.1.6) is a ubiquitous antioxidant enzyme that is present in nearly all living organisms. It catalyzes the decomposition of hydrogen peroxide (H_20_2) to water and oxygen. In the assay, catalase first reacts with H_2O_2 to produce water and oxygen, the unconverted H_2O_2 reacts with OxiRed™ probe to produce a product, which can be

measured at 570 nm Catalase activity is reversely proportional to the signal. The kit can detect 1 µu or less of catalase activity in samples.

- **Kit contents**

Catalase assay buffer, OxiRed Probe (in DMSO), HRP (lyophilized), H_2O_2 (0.88M), Stop solution and Catalase positive control.

- **Sample Preparation**

Cells: Homogenized, 10^6 cells in 0.2 ml ice cold assay buffer. It was centrifuged at 10,000xg for 15 min at 4°C. Cell debris was discarded and the supernatant was used for the assay.

- **Reagents**

- OxiRed™ Probe: Briefly warmed to completely melt the DMSO solution. Stored at 4°C, protected from light and should be used within two months.
- HRP: Dissolved with 220 µl assay buffer. Stored at 4°C and should be used within two months.

  Note: Keep samples, HRP and Catalase on ice while in use.
- Developer mix: It should be prepared according to number of assays to be performed by mixing, 46 µl assay buffer, 2 µl OxiRed™ probe and 2 µl HRP solution

- **Procedure**

H_2O_2 Standard Curve

5 µl of 0.88M H_2O_2 was diluted to 215 µl dH_2O to generate 20 mM H_2O_2. Than took 50 µl of the 20 mM H_2O_2 solution was taken and further diluted to 0.95 ml dH_2O to generate 1 mM H_2O_2 solution. Further 0, 2, 4, 6, 8, 10 µl of 1 mM H_2O_2 solution were added to the 96-well plate to generate 0, 2, 4, 6, 8, 10 nmol/well H_2O_2 standard. Final volume was adjusted to 90 µl with assay buffer. 10 µl Stop Solution was added into each well. After this 50 µl of the developer mix was added to each test samples, controls, and standards. All the solutions were mixed well and incubated at 25°C for 10 min. The absorbance was read at 570 nm using a microplate reader.

Note: Diluted H_2O_2 is unstable, prepare fresh dilution each time.

Sample

50 µl of samples and sample high control (HC) were added into each well, and total volume was adjusted to 78 µl with assay buffer. Than 10 µl of stop Solution was added

into the sample HC, mixed and incubated at 25°C for 5 min to completely inhibit the catalase activity. 12 µl of fresh 1 mM H_2O_2 was than added into each well of both samples and sample HC to start the reaction and incubated at 25°C for 40 min. Than 10 µl stop solution was added into each sample well to stop the reaction. 50 µl of the developer mix was added to each test samples, controls, and standards. Mixed well and incubated at 25°C for 10 min. Absorbance was read at 570 nm using a microplate reader.

Catalase Activity (nmol/min/ml)**:** [B/(40xV)]xsample dilution factor

Where, B: Decomposed H_2O_2 amount from H_2O_2 Standard Curve (in nmol).

V: Pretreated sample volume added into the reaction well (in ml).

40: The reaction time 40 min.

$\Delta A = A_{HC}-A_{sample}$, where A_{HC}: Reading of sample High Control and A_{sample}: Reading of sample in 40 min.

H_2O_2 standard curve was plotted. ΔA was applied to the H_2O_2 standard curve to get B nmol of H_2O_2 decomposed by catalase in 40 min reaction.

4.5.4.4 Estimation of matrixmetalloproteinase-3 (MMP-3) levels

Kit specifications

Name: Rabbit stromelysin-1, SL-1 ELISA kit

Make: EIAab, China

Catalog Number: E0101Rb

Storage condition: Stored at -20°C

- **Test principle**

The microtiter plate provided in this kit has been pre-coated with an antibody specific to SL-1. Standards or samples are then added to the appropriate microtiter plate wells containing a biotin-conjugated polyclonal antibody preparation specific for SL-1 and Avidin conjugated to Horseradish Peroxidase (HRP) and incubated. Then a TMB substrate solution is added to each well. Only those wells that contain SL-1, biotin-conjugated antibody and enzyme-conjugated Avidin will exhibit a change in color. The enzyme-substrate reaction is terminated by the addition of a sulphuric acid solution and the color change is measured spectrophotometrically at a wavelength of 450 nm ± 2 nm.

The concentration of SL-1 in the samples is then determined by comparing the O.D. of the samples to the standard curve.

- **Kit components**

Assay plate, Standard, Sample Diluent, Assay Diluent A, Assay Diluent B, Detection Reagent A, Detection Reagent B, Wash Buffer (25 x concentrate), Substrate, Stop Solution and Plate sealer for 96 wells.

- **Storage of the kits**

The Assay Plate, Standard, Detection Reagent A and Detection Reagent B were stored at -20℃ upon being received. After receiving the kit, Substrate was stored at 4℃. Other reagents are kept according to the labels on vials. But for long term storage, the whole kit was kept at -20℃.

- **Sample preparation**

$3x10^4$ cells were centrifuged and particulates were removed. Supernatant was used for the assay.

- **Reagents**

- Wash buffer - Warmed to room temperature and mixed gently until the crystals dissolved completely. 30 ml of wash buffer Concentrate was diluted with deionized water to prepare 750 ml of wash buffer.
- Standard: Standard was reconstituted with 1.0 ml of sample diluent. This reconstitution produced a stock solution of 50.0 ng/ml. The standard solution was allowed to sit for a minimum of 15 min with gentle agitation prior to making serial dilutions. The undiluted standard serves as the high standard (50.0 ng/ml). Serial dilutions of undiluted standard were made using sample diluents, to prepare 25, 12.5, 6.25, 3.12, 1.56 and 0.78 ng/ml concentrations of standard. The sample diluent serves as the zero standard (0 ng/ml).

- **Procedure**

All reagents were allowed to reach room temperature. All the reagents were mixed thoroughly by gently swirling before pipetting (Avoid foaming). Appropriate number of strips was taken and extra strips were removed from microtiter plate. Removed strips were resealed and stored at 4℃ until the kits expiry date. 100 µl of Standard, Blank, or Sample was added per well. Plate was covered with sealer and incubated for 2 hours at

37℃. Solutions of each well was taken out then without washing the plate, 100 µl of Detection Reagent A working solution was added to each well. Plate was covered with sealer and incubated for 1 hour at 37^0C. Each well was aspirate and washed, repeating the process three times for a total of three washes. Wells were washed by filling each well with Wash Buffer (approximately 400 µl) with multi-channel pipette. Complete removal of liquid at each step is essential for good performance. Last wash was aspirated and any remaining Wash Buffer was removed by decanting. The plate was inverted and blotted against clean paper towels. 100 µl of Detection Reagent B working solution was added to each well. Plate was covered with a new plate sealer and Incubated for 1 hour at 37^0C. Three washes were repeated as described above. 90 µl of Substrate Solution was added to each well. Plate was covered with a new Plate sealer and incubated within 15-30 minutes at 37℃. The plate was kept protect from light. Than 50 µl of Stop Solution was added to each well. If color change does not appear uniform, gently tap the plate to ensure thorough mixing. The optical density of each well was determined at once, using a microplate reader set to 450 nm.

- **Calculation of results**

Average was taken of the duplicate readings for each standard, control, and sample. The average zero standard optical density was subtracted. A standard curve was created by plotting the mean absorbance for each standard verses the concentration and draw a best fit curve through the points on the graph.

4.5.4.5 Estimation of Matrix metalloproteinase-9 (MMP-9) levels

Kit specifications

Name: Rabbit Matrix metalloproteinase-9, MMP-9 ELISA Kit

Make: EIAab, China

Catalog Number: E0553Rb

Storage condition: Stored at -20°C

- **Test principle**

The microtiter plate provided in this kit has been pre-coated with an antibody specific to MMP-9. Standards or samples are then added to the appropriate microtiter plate wells containing biotin-conjugated polyclonal antibody preparation specific for MMP-9 and

Avidin conjugated to Horseradish Peroxidase (HRP) is added to each microplate well and incubated. Then a TMB substrate solution is added to each well. Only those wells that contain MMP-9, biotin-conjugated antibody and enzyme-conjugated Avidin will exhibit a change in color. The enzyme-substrate reaction is terminated by the addition of a sulphuric acid solution and the color change is measured spectrophotometrically at a wavelength of 450 nm ± 2 nm. The concentration of MMP-9 in the samples is then determined by comparing the O.D. of the samples to the standard curve.

- **Kit components**

Assay plate, Standard, Sample Diluent, Assay Diluent A, Assay Diluent B, Detection Reagent A, Detection Reagent B, Wash Buffer (25 x concentrate), Substrate, Stop Solution and Plate sealer for 96 wells.

- **Storage of the kits**

The Assay Plate, Standard, Detection Reagent A and Detection Reagent B were stored at -20℃ upon being received. After receiving the kit, Substrate was stored at 4℃. Other reagents were kept according to the labels on vials. For long term storage, whole kit was kept at -20℃.

- **Sample preparation**

$3x10^4$ cells were centrifuged and particulates were removed. Supernatant was used for the assay.

- **Reagents**

- Wash buffer: Warmed to room temperature and mixed gently until the crystals have completely dissolved. 30 ml of wash buffer Concentrate was diluted into deionized water to prepare 750 ml of wash buffer.
- Standard: Standard was reconstituted with 1.0 ml of sample diluent. This reconstitution produces a stock solution of 20.0 ng/ml concentration. Standard solution was allowed to sit for a minimum of 15 minutes with gentle agitation prior to making serial dilutions. The undiluted standard served as the high standard (20.0 ng/ml). Serial dilutions of undiluted standard were made using sample diluents, to prepare 10, 5, 2.5, 1.25, 0.625 and 0.312 ng/ml concentrations of standard. The sample diluent served as the zero standard (0 ng/ml).

- **Procedure**

All reagents were allowed to reach room temperature. All the reagents were mixed thoroughly by gently swirling before pipetting (Avoid foaming). Appropriate numbers of strips were taken and extra strips were removed from microtiter plate. Removed strips were resealed and stored at 4℃ until the kits expiry date. 100 µl of Standard, Blank, or Sample was added per well. Plate was covered with sealer and incubated for 2 hours at 37℃. Liquid from each well was removed without washing the plate, 100 µl of working solution of Detection Reagent A was added to each well. Plate was covered with sealer and incubated for 1 hour at 37^0C. Each well was aspirate and washed, repeating the process three times for a total of three washes. Wells was than washed again by filling each well with Wash Buffer (approximately 400 µl) using multi-channel pipette. Complete removal of liquid at each step is essential for good performance. Last wash was aspirated and any remaining Wash Buffer was removed by decanting. The plate was inverted and blotted against clean paper towels. 100 µl working solution of Detection Reagent B was added to each well. Plate was covered with a new plate sealer and Incubated for 1 hour at 37^0C. Three washes were repeated as described above. 90 µl of Substrate Solution was added to each well. Plate was covered with a new Plate sealer and incubated within 15-30 minutes at 37℃ and was protected from light. 50 µl of Stop Solution was added to each well. If color change did not appear uniform, the plate was gently tapped to ensure thorough mixing. The optical density of each well was determined at once, using a microplate reader set to 450 nm.

- **Calculation of results**

Averaged the duplicate readings for each standard, control and sample. The average zero standard optical density was subtracted. A standard curve was created by plotting the mean absorbance for each standard verses the concentration and draw a best fit curve through the points on the graph.

4.6 Statistical analysis

Statistical analysis was carried out by using GraphPad Prism software (SanDiego, CA, USA). The data were expressed as mean ± SD or SEM. $p<0.05$ were considered significant.

Chapter 5
Results

5.1 To collect and process plant material

Herbs and formulations which are traditionally used to treat OA, were listed out.

5.1.1 List of commonly used traditional herbs and formulations for the management of OA

Individual plants

1) *Acacia nilotica* ssp. *indica* (Bth) Brenan (*Babula*, Family: Leguminosae)
2) *Asparagus racemosus* Willd. (*Shatavari*, Family: Liliaceae)
3) *Bombax ceiba* L. (*Shalmali*, Family: Bombacaceae)
4) *Boswellia serrata* Roxb. (*Shallaki*, Family: Burseraceae)
5) *Cammiphora mukul* (Hook. Ex Stocks) Engl. (*Guggula*, Family: Burseraceae)
6) *Emblica officinale* Gaertn. (*Amalaki*, Family: Euphorbiaceae)
7) *Lac* (*Laksha*)
8) *Piper longum* L. (*Pippali*, Family: Piperaceae)
9) *Ricinus communis* L. (*Eranda*, Family: Euphorbiaceae)
10) *Sida cordifolia* L. (*Bala*, Family: Malvaceae)
11) *Symplocos racemosa* Roxb. (*Lodhra*, Family: Symplocaceae)
12) *Tribulus terrestris* L. (*Gokshura*, Family: Zygophyllaceae)
13) *Vitex negundo* var. *incisa* (Lam.) C.B.CI. (*Nirgundi*, Family: Verbenaceae)
14) *Withania somnifera* (L.) Dunal (*Ashwagandha*, Family: Solanaceae)
15) *Zingiber officinale* Rosc. (*Sunthi*, Family: Zingiberaceae)

Formulations

1) *Dashamool churna* (Ten plants in the form of *Churna*)
2) *Dashamoolarishta* (Ten plants in the form of *Arishta*)
3) '*Laksha Guggul*' (Mixure of six plants)
4) *Triphala* (Mixure of three plants)
5) '*Triphala Guggul*' (Main ingredients are *Triphala* and *Guggul*)

Based on review of literature, nine herbs and three formulations were selected for the study.

Total eight plants were identified, collected and authenticated at herbarium of MPCC (Table 14). Rhizomes of *Z. officinale* were purchased from local market. All plants were authenticated at Ayurved College, Bharati Vidyapeeth Deemed University, Pune. Powders of all plant material were stored in zip lock bags, at room temperature. For experimentation, all powders were sieved through mesh no. 120 for further use.

Table 14: List of selected plants and plant parts

No.	Name	Part used	Herb No.
1	*Acacia nilotica* ssp. i*ndica* (Bth) Brenan	Gum	MPCC3465
2	*Bombax ceiba* L.	Gum	MPCC2594
3	*Boswellia serrata* Roxb.	Gum	MPCC3508
4	*Piper longum* L.	Inflorescence	MPCC2330
5	*Ricinus communis* L.	Leaves	MPCC2906
6	*Sida cordifolia* L.	Roots	MPCC2537
7	*Tribulus terrestris* L.	Fruits	MPCC1097
8	*Vitex negundo* var. *incisa* (Lam.) C.B.CI., Dunal	Leaves	MPCC1145
9	*Zingiber officinale* Rosc.	Rhizomes	-

Selected formulations: *Dashamoolarishta*, *Triphala* and '*Triphala Guggul*'.

5.2 To comparatively evaluate anti-osteoarthritic potential of selected herbs using *in vivo* model

5.2.1 Effect of herbs on body weight

It is known that redness and swelling of the joints and, body weight loss usually appears at the onset of arthritis (Bansod et al., 2011); a change in body weight was measured as one of the parameters to assess the course of the disease and stress on the animals. On administering two collagenase injections (on day 1st and 4th) the body weight started decreasing slowly after day 5th. Increase in body weight was once again observed after the commencement of treatment i.e. day 15th onwards, however CIOA group demonstrated body weight loss (Fig. 17). The loss of body weight was observed during the arthritic condition (CIOA) as well as in INDO group. After treatment with

indomethacin, body weight was significantly ($p<0.05$) decreased compared to HC. Whereas, groups treated with herbs significantly increased the body weight. VN (25 ± 12 gm) ($p<0.01$), PL (24 ± 11 gm) ($p<0.05$), RC (23 ± 18 gm) ($p<0.05$) and TT (21 ± 10 gm) ($p<0.05$) groups revealed significant increase in body weight compared to CIOA (5 ± 4.7 gm) and INDO (4 ± 3.7 gm); however SC (10 ± 4.4 gm) and ZO (17 ± 4.8 gm) have not shown any significant increase in body weight (Fig. 17).

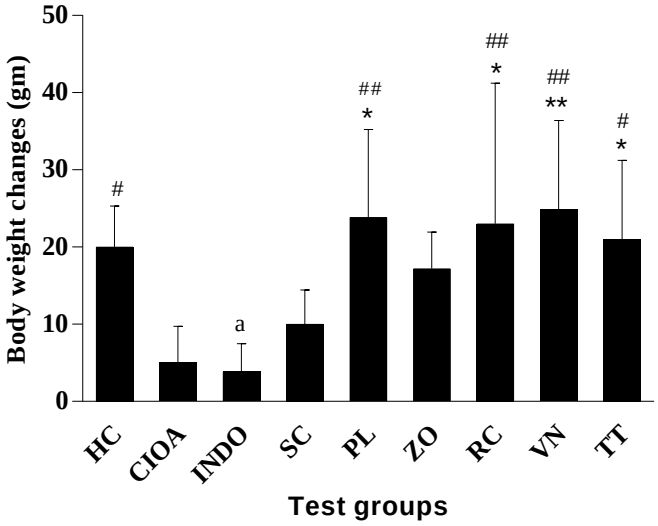

Figure 17: Effect of herbs on change in body weight.

Values are expressed as Mean ± SD; n =6. $^{*}p<0.05$; $^{**}p<0.01$ compared to CIOA. $^{a}p<0.05$ compared to HC. $^{\#}p<0.05$; $^{\#\#}p<0.01$ compared to INDO. Data were analyzed by One-Way ANOVA followed by the Bonferroni's multiple comparison test. HC: Healthy control, CIOA: Osteoarthritic control, INDO: Positive control, SC: *S. cordifolia*, PL: *P. longum*, ZO: *Z. officinale*, RC: *R. communis*, VN: *V. negundo*, TT: *T. terrestris*.

5.2.2 Effect of herbs on knee diameter

CIOA group demonstrated a significant ($p<0.0001$) increase in joint diameter, compared to INDO (Fig. 18). Both INDO and herb treated group substantially attenuated the increase in joint diameter observed in CIOA group animals. After injecting collagenase, visible clinical sign developed i.e. progressive and highly significant knee swelling from day 2nd and after the second injection, on day 4th and maximum swelling was developed on day 5th. In CIOA group, knee diameter (0.79 ± 0.082 mm) did not

reduced until day 34th but with treatment of INDO (0.22 ± 0.16 mm) ($p<0.0001$) and herbs, knee swelling was reduced significantly. On 34th day, CIOA and ZO (1.09 ± 0.16 mm) showed highest knee swelling as compared to other test groups. Interestingly, treatment with SC (0.17 ± 0.074 mm) ($p<0.0001$), PL (0.04 ± 0.016 mm) ($p<0.0001$), ZO (0.62 ± 0.047 mm) ($p<0.05$), RC (0.3 ± 0.191 mm) ($p<0.0001$), VN (0.13 ± 0.031 mm) ($p<0.0001$) and TT (0.38 ± 0.023 mm) ($p<0.0001$) have significantly reduced knee swelling compared to CIOA (Fig. 18). Comparatively PL exhibited highest activity.

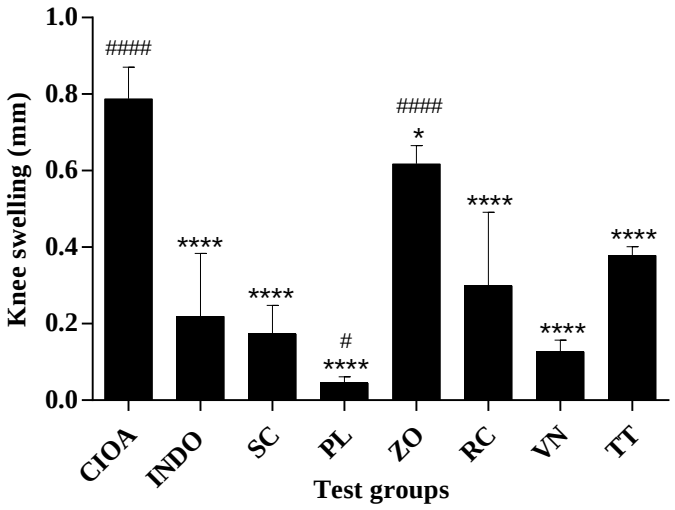

Figure 18: Effect of herbs on knee swelling.

Values are expressed as Mean ± SD; n =6. *p<0.05; ****p<0.0001 compared to CIOA. #p<0.05; ####p<0.0001 compared to INDO. Data were analysed by One-Way ANOVA followed by the Bonferroni's multiple comparison test. CIOA: Osteoarthritic control, INDO: Positive control, SC: *S. cordifolia*, PL: *P. longum*, ZO: *Z. officinale*, RC: *R. communis*, VN: *V. negundo*, TT: *T. terrestris*.

5.2.3 Effect of herbs on paw volume

Change in paw volume has been found to be associated with an increase in granulocyte and monocytes (Kabra et al., 2011). INDO produced significant inhibition in the rat paw edema (24.65 %) (Table 15). Groups SC, PL, ZO, RC, VN and TT produced 24.05 %, 23.69 %, 19.55 %, 23.98 %, 24.85 % and 23.71 % inhibition of rat paw edema, respectively after 28 days, as compared with CIOA (Table 15), which shows anti-

inflammatory effect of herbs and, comparatively SC group produced highest inhibition in the rat paw edema.

5.2.4 Effect of herbs on paw retraction

ZO group showed significant decrease in paw latency compared to CIOA group. Paw latencies between other groups were not significant at any time points (Fig. 19).

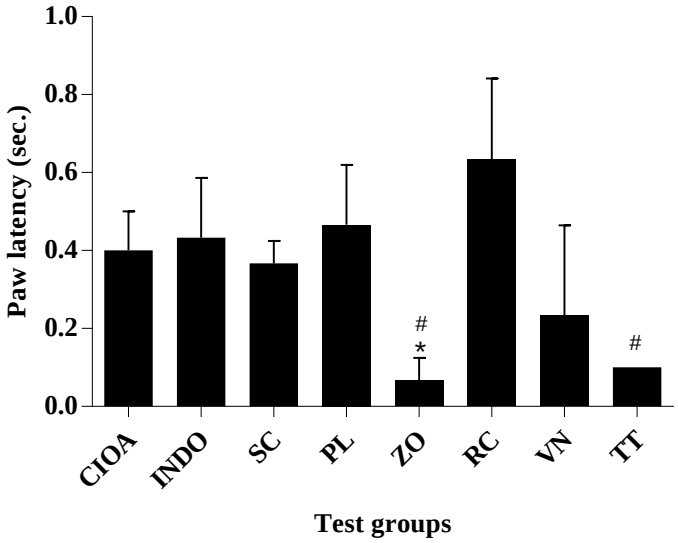

Figure 19: Effect of herbs on paw retraction.

Values are presented in Mean ± SD; n=6. $^{*}p<0.05$ compared to CIOA. $^{\#}p<0.05$ compared to INDO. Data were analyzed by One-Way ANOVA followed by Dunnett's multiple comparison test. CIOA: Osteoarthritic control, INDO: Positive control, SC: *S. cordifolia*, PL: *P. longum*, ZO: *Z. officinale*, RC: *R. communis*, VN: *V. negundo*, TT: *T. terrestris*.

Table 15: Effect of herbs on paw volume.

Treatment	Paw oedema volume (ml)					Percent inhibition of paw oedema		Mean % inhibition (21^{st}+28^{th})
	0^{th} Day	7^{th} Day	14^{th} Day	21^{st} Day	28^{th} Day	21^{st} Day	28^{th} Day	
CIOA	2.47±0.38	3.23±0.92	3.38±0.26	3.45±0.18	3.67±0.82	-	-	-
INDO	2.30±0.02	3.00±0.07	3.12±0.21	2.66±0.45**	2.67±0.39****	23.34±9.1	25.96±11	24.65
SC	2.19±0.44	2.91±0.23	2.90±0.46	2.74±0.14**	2.60±0.36****	20.38±6.4	27.72±11	24.05
PL	2.24±0.51	2.95±0.05	3.01±0.11	2.84±0.46*	2.55±0.48****	17.43±14	29.96±5.3	23.69
ZO	2.30±0.11	2.99±0.04	2.97±0.12	2.93±0.32	2.68±0.21****	14.51±12	24.6±14	19.55
RC	2.22±0.21	2.88±0.39	2.87±0.13	2.73±0.28**	2.61±0.34****	20.79±6.7	27.17±12	23.98
VN	2.01±0.22	2.70±0.01	2.67±0.19**	2.55±0.21***	2.76±0.52****	25.89±5.3	23.81±9.7	24.85
TT	2.02±0.40	2.91±0.12	2.87±0.17	2.65±0.21**	2.73±0.57****	22.98±5.3	24.44±13	23.71

Note: Values are expressed as Mean ± SD; n =6. $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$; $^{****}p<0.0001$ compared to CIOA. Data were analysed by Two-Way ANOVA followed by the Bonferroni's multiple comparison test. CIOA: Osteoarthritic control, INDO: Positive control, SC: *S. cordifolia*, PL: *P. longum*, ZO: *Z. officinale*, RC: *R. communis*, VN: *V. negundo*, TT: *T. terrestris*.

5.2.5 Effect of herbs on GAG

CIOA (481 ± 48.6 µg/ml) group showed significant ($p<0.01$) increase in GAG levels in serum, compared to HC (87.7 ± 50.5 µg/ml) (Fig. 20). INDO significantly elevated GAG release (1390 ± 156.5 µg/ml) ($p<0.0001$), suggesting INDO does not have preventive role for GAG release in serum (Fig. 20). Groups with herbs treatment significantly decreased serum GAG levels (Fig. 20).

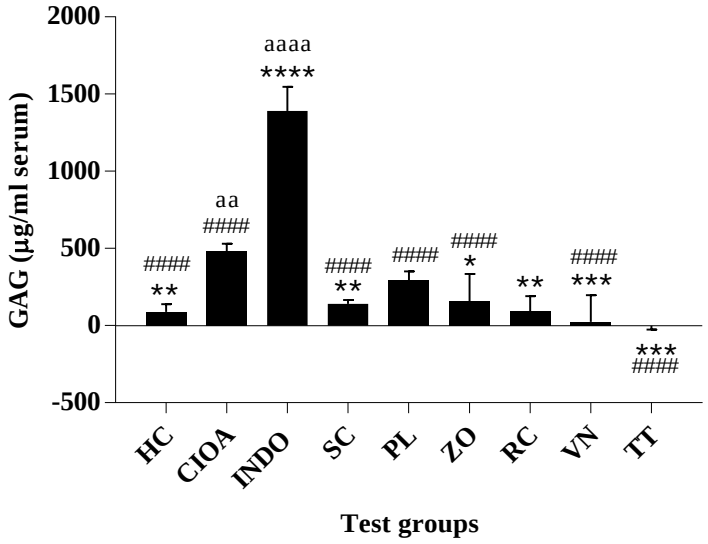

Figure 20: Effect of herbs on GAG release in the serum.

Values are expressed as Mean ± SD; n =6. $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$; $^{****}p<0.0001$ compared to CIOA. $^{\#\#\#\#}p<0.0001$ compared to INDO. $^{aa}p<0.01$; $^{aaaa}p<0.0001$ compared to HC. Data were analysed by One-Way ANOVA followed by Bonferroni's multiple comparison test. HC: Healthy control, CIOA: Osteoarthritic control, INDO: Positive control, SC: *S. cordifolia*, PL: *P. longum*, ZO: *Z. officinale*, RC: *R. communis*, VN: *V. negundo*, TT: *T. terrestris*.

5.2.6 Radiological analysis

Radiographic images of the joints were compared between the right knee (induced) and left knee, for erosion of articular cartilage, reduced joint space and osteophyte formation. CIOA group showed marked erosion of articular cartilage on induced knee, reduction in joint space due to loss of articular cartilage and small osteophyte formation (Fig. 21B). In HC group, joint appeared normal with smooth

articular surface. Knee and patello-femoral joints appeared normal without obvious abnormalities in surrounding tissue (Fig. 21A). INDO treated group revealed osteophyte formation with minimal to mild changes of OA (Fig. 21C). SC group also showed osteophyte formation along with mild changes of OA (Fig. 21D). PL group rats revealed minimal loss of articular cartilage but overall indicated normal architecture of the joint (Fig. 21E). ZO group showed reduction in joint space due to gross loss of articular cartilage (Fig. 21F). RC group exhibited moderate to gross changes of OA with joint space reduction and detached osteophytes (Fig. 21G). VN group showed minimal changes with osteophyte formation (Fig. 21H), while TT group indicated reduction in joint space (Fig. 21I).

5.2.7 Histopathological analysis

Histology of synovium showed 1-2 cell thick lining of the synovium in HC group without sub-epithelial inflammatory cellular infiltration (Fig. 22-A1). In CIOA group, lining cells showed mild increase in layer and stroma indicated mild mononuclear infiltrate (Fig. 22-A2). Effect of herbs on synovium has explained in the table (Table 16 and 17). All these changes in the synovium were most severe in VN treated group while RC group prevented synovium hypertrophy and mononuclear infiltration.

In histology of the articular cartilage in HC group, the cartilage was not degenerated and articular surface was smooth (Fig.22-B1). The matrix was densely stained red with Safranin O and chondrocyte pathology was within normal limits (Fig. 22-D1, 22-C1). In the CIOA group, the cartilage revealed irregular surface with fibrillation and clefting (Fig. 22-B2), with chondrocyte degeneration and evidences of cloning and focal hypercellularity (Fig. 22-C2). Tide mark has shown infiltration by blood vessels, PGs decreased integrity in the upper zone (Fig. 22-D2). Effect of herbs on cartilage has summarized in the table (Table 16 and 17).

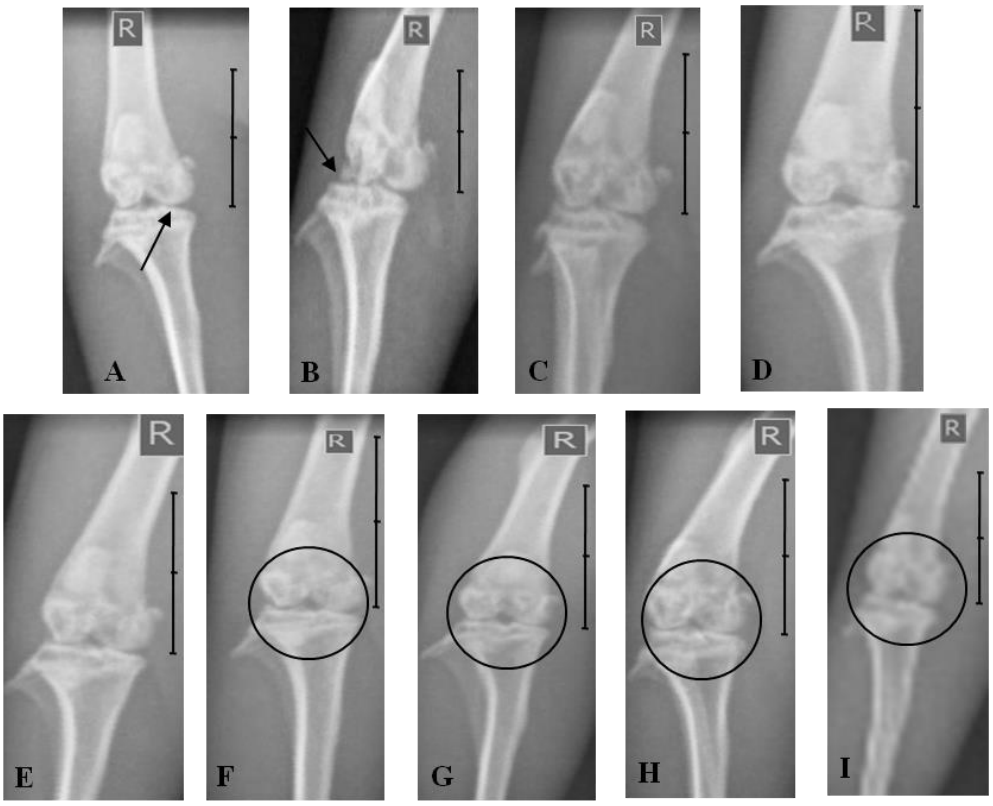

Figure 21: Radiography images of knee joint.

Right knee joint radiographs (in the Anterior Posterior view) of control and experimental animals. All radiographs were taken keeping object to x-ray source distance constant (100 cm) and scale is indicating extent of zoom (1 unit is 0.5 cm). (A) Saline injected groups showed smooth articular surface (arrow) while in (B) CIOA group, erosion of articular surface with joint space reduction and osteophyte can be seen (arrow); Comparatively (C) INDO group revealed minimal changes. (D) SC and (E) PL treated group showed only marginal loss of articular cartilage while (F) ZO; (G) RC; (H) VN and (I) TT treated group exhibited gross reduction in cartilage (circle).

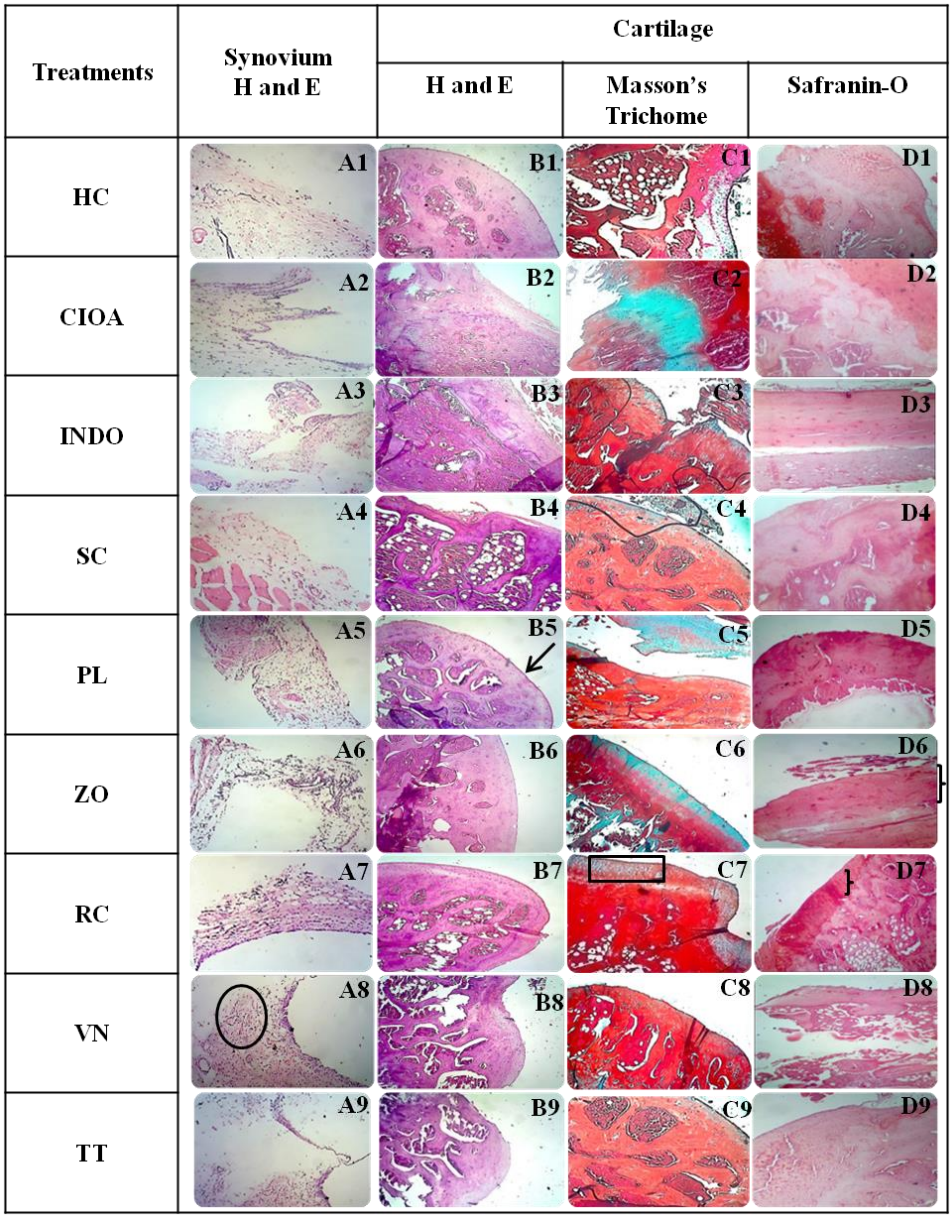

Figure 22: Histopathology images of synovial membrane and cartilage.

First column (A1-A9) showing photographs of synovium stained with H and E and further are photographs of cartilage stained with H and E (B1-B9), Masson's trichome (C1-C9) and safranin-O (D1-D9) respectively. Circle represent synovial cells, arrow represent articular cartilage surface, rectangle represent chondrocyte and right brace represent proteoglycan staining density. HC: Healthy control, CIOA: Osteoarthritic control, INDO: Positive control, SC: *S. cordifolia*, PL: *P. longum*, ZO: *Z. officinale*, RC: *R. communis*, VN: *V. negundo*, TT: *T. terrestris*, H and E: hematoxyline and eosin.

Table 16: Histopathology grading.

I: Microscopic scoring of synovial pathology		
Gross characteristics		**Score**
A	Lining cell layer: 1-2 layers thick Stroma: No cell infiltration	0
B	Lining cell layer: Mild increase Stroma: Mild cell infiltration	1
C	Lining cell layer: Mild hypertrophy Stroma: Mild cell infiltration with congested vessels	2
D	Lining cell layer: Mild to moderate hypertrophy Stroma: moderate cell infiltration with congested vessels	3
II: Grading of cartilage pathology		
Severity of cartilage structure characteristics		**Score**
A	Smooth, intact	0
B	Smooth with minimal clefting	1
C	Mild irregularity with minimal clefting	2
D	Mild irregularity with minimal clefting and fibrillation	3
E	Irregularity with fibrillation and clefting	4
III: Grading of tidemark		
Tide mark integrity		**Score**
A	Intact	0
B	Crossed by blood vessels	1
IV: Grading of chondrocyte pathology		
Severity of chondrocyte characteristics		**Score**
A	Chondroid matrix within normal limits	0
B	Degeneration, diffuse hypercellularity with focal cloning	1
C	Degeneration, diffuse hypercellularity with focal cloning, Dystrophic calcification	2
V: Grading of proteoglycan/collagen integrity		
Severity of proteoglycan/collagen pathology characteristics		**Score**
A	Normal	0
B	Decreased content in upper zone	1
C	Decreased content up to mild zone	2

Table 17: Histopathology scoring.

No.	Parameters	HC	CIOA	INDO	SC	PL	ZO	RC	VN	TT
I	Synovial pathology	0	2	2	0	1	2	0	2	2
II	Cartilage pathology	0	4	3	1	1	1	2	2	2
III	Tide mark integrity	0	1	1	1	1	1	0	1	1
IV	Chondrocyte pathology	0	2	2	1	1	0	0	1	1
V	PG/collagen pathology	0	1	1	1	1	1	0	1	1
Impression		0	9-10	8-10	6-7	6-7	5	4	7-8	7-8

HC: Healthy control, CIOA: Osteoarthritic control, INDO: Positive control, SC: *S. cordifolia*, PL: *P. longum*, ZO: *Z. officinale*, RC: *R. communis*, VN: *V. negundo*, TT: *T. terrestris*.

5.2.8 qPCR analysis

Osteoarthritic control rats showed significant down-regulation (~5.2-fold) of SOD (p=0.0019) in the knee joint synovium by compared to control rats (Fig. 23A). On the contrary, osteoarthritic rats receiving *S. cordifolia* revealed significant up-regulation (~3.3-fold) in the synovial expression of SOD (p=0.0442) (Fig. 23A). Rats receiving *P. longum* and indomethacin also showed up-regulation in SOD expression by ~3.2-fold (p=0.0538) and ~1.7-fold (p=0.4825), respectively (Fig. 23A). Down-regulation of synovial expression of GPx gene by ~2.6-fold (p=0.8206) was observed in osteoarthritic control rats, when compared to control (Fig. 23B). On the contrary, GPx expression was elevated by ~1.7-fold (p=0.9214), ~1.8-fold (p=0.9127) and ~19.4-fold (p=0.0167) in osteoarthritic rats receiving *S. cordifolia*, *P. longum* and indomethacin respectively (Fig. 23B). Compared to control rats, osteoarthritic rats also down-regulated synovial expression of CAT by ~6.8-fold (p=0.6134) (Fig. 23C). Up-regulation in the CAT expression by ~3.4-fold (p=0.8329), ~4.5-fold (p=0.7598) and ~40.9-fold (p=0.0048) respectively was observed in osteoarthritic rats receiving *S. cordifolia*, *P. longum* and indomethacin (Fig. 23C). In addition, down-regulation in the synovial expression of

PON-1 by ~4.9-fold (p=0.6065) was observed in osteoarthritic control rats compared to control rats (Fig. 23D). While osteoarthritic rats receiving *S. cordifolia*, *P. longum* and indomethacin have shown increased mRNA level of PON-1 by ~2.8-fold (p=0.7883), ~1.1-fold (p=0.9901) and ~12.1-fold (p=0.1205) respectively (Fig. 23D).

In the knee synovium, osteoarthritic control rats showed up-regulation of MMP-3, MMP-9 and TIMP-1 by ~14.1- fold (p=0.0227), ~10.6-fold (p=0.1886) and ~1.2-fold (p=0.4845), respectively when compared to control rats (Fig.24). Down-regulation of expression of MMP-3 by ~2.8-fold (p=0.0929) and ~3.6-fold (p=0.0623) was observed in the osteoarthritic rats receiving *S. cordifolia* and indomethacin, respectively (Fig. 24A). *P. longum* treated group revealed significant down-regulation ~6.1-fold (p=0.0361) in MMP-3 expression by (Fig. 24A). In addition, both indomethacin and *S. cordifolia* treated osteoarthritic rats showed down-regulation in synovial expression of MMP-9 by ~9.9-fold (p=0.1918) and ~2.1-fold (p=0.4231), respectively (Fig. 24B). Osteoarthritic rats receiving *P. longum* did not exhibited down-regulation in the MMP-9 expression. Up-regulation of TIMP-1 was observed in rats treated with *S. cordifolia* and *P. longum* by ~1.5-fold (p=0.0934) and ~1.7-fold (p=0.03), respectively. On the contrary, indomethacin was unable to affect the level of synovial TIMP-1 in the osteoarthritic knee joints (p=0.4682) (Fig. 24C).

5.3 To comparatively evaluate anti-osteoarthritic potential of selected gums and formulations using *in vivo* model

5.3.1 Effect of gums and formulations on body weight

Body weight was decreased in osteoarthritic control group (CIOA) and in INDO group (Fig. 25). Treatment with TC (33.2 ± 7.52 gm) have significantly ($p<0.01$) increased body weight compared to CIOA (11.3 ± 3.47 gm) and INDO (4 ± 3.01 gm) (Fig. 25). Treatment with formulations, TG (15.5 ± 2.2 gm) and DA (19.8 ± 3.79 gm) also increased body weight. Gums, BC (24.3 ± 3.02 gm) and BS (23 ± 2.45 gm) also showed significant increase in body weight compared to INDO (Fig. 25). AN (11 ± 4.32 gm) failed to increase body weight.

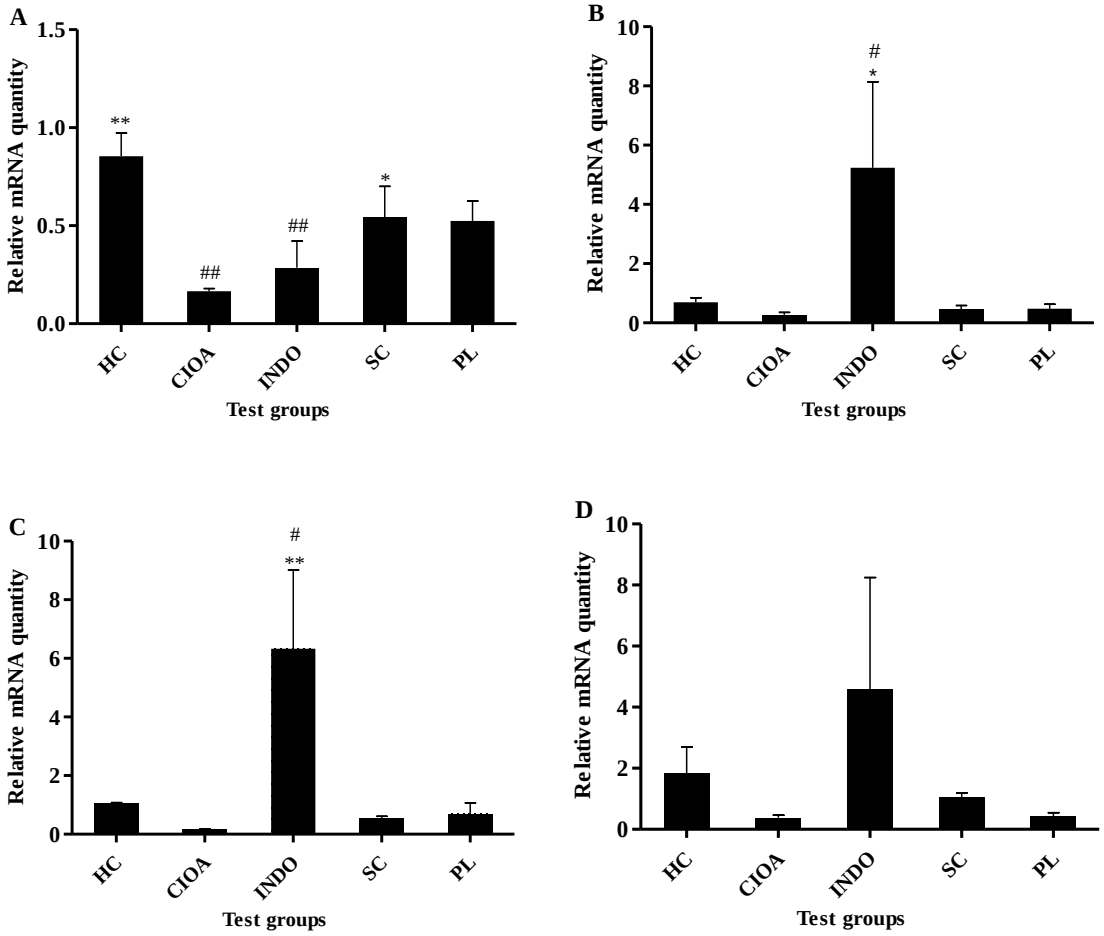

Figure 23: Effect of *S. cordifolia* (SC) and *P. longum* (PL) on the mRNA expression of A) SOD-1, B) GPx-1 C) CAT and D) PON-1 in the synovium of rats. All the values are expressed as Mean ± SEM (n=3). Comparisons were done between CIOA/HC and each individual treated group by Fisher's Least Significant Difference (LSD) test (*$p<0.05$, **$p<0.01$, $^{\#}p<0.05$, $^{\#\#}p<0.01$, * =Compared to CIOA, $^{\#}$ =Compared to HC). HC: Healthy control, CIOA: Osteoarthritic control, INDO: Positive control, SC: *S. cordifolia*, PL: *P. longum*.

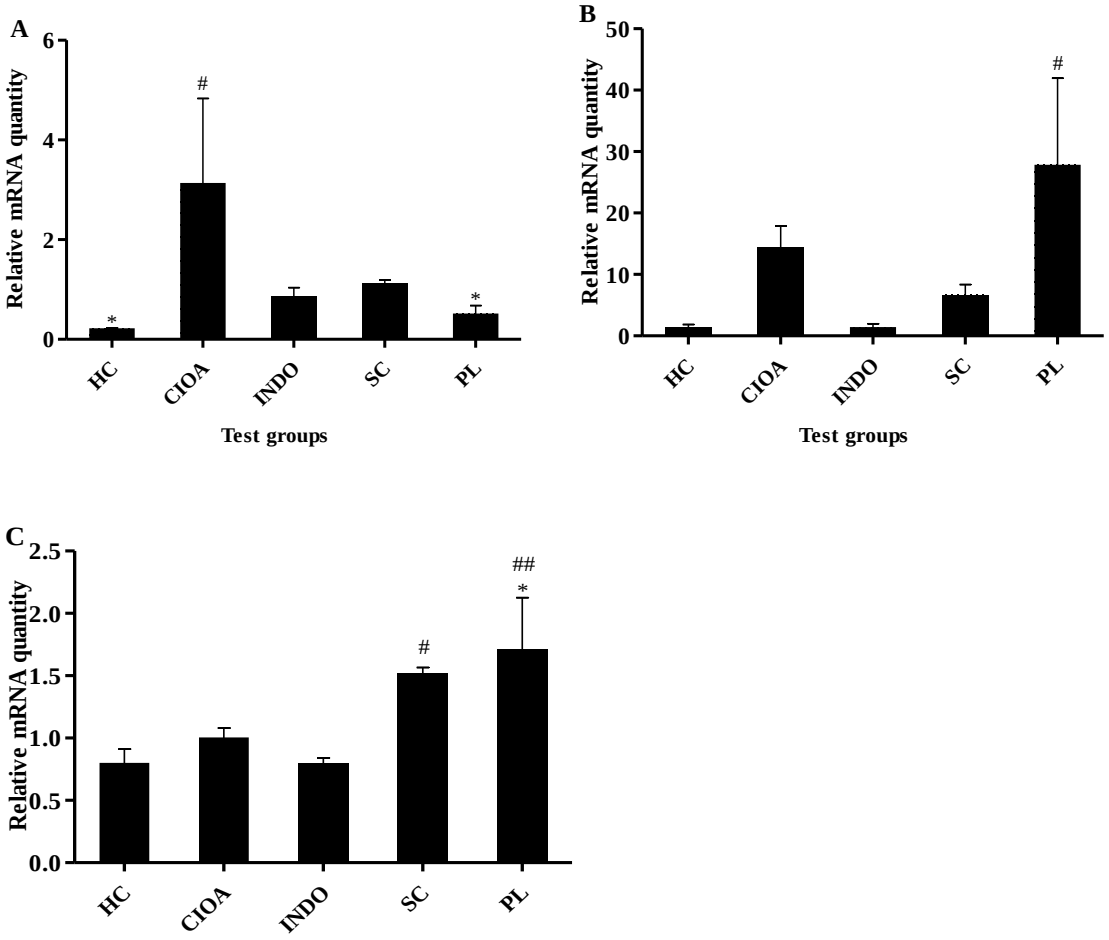

Figure 24: Effect of *S. cordifolia* (SC) and *P. longum* (PL) on the mRNA expression of A) MMP3, B) MMP9 and C) TIMP1 in the synovium of rats. All the values are expressed as Mean ± SEM (n=3). Comparisons were done between CIOA/HC and each individual treated group by Fisher's Least Significant Difference (LSD) test (*p<0.05, #p<0.05, ##p<0.01 *=Compared to CIOA, # =Compared to HC). HC: Healthy control, CIOA: Osteoarthritic control, INDO: Positive control, SC: *S. cordifolia*, PL: *P. longum*.

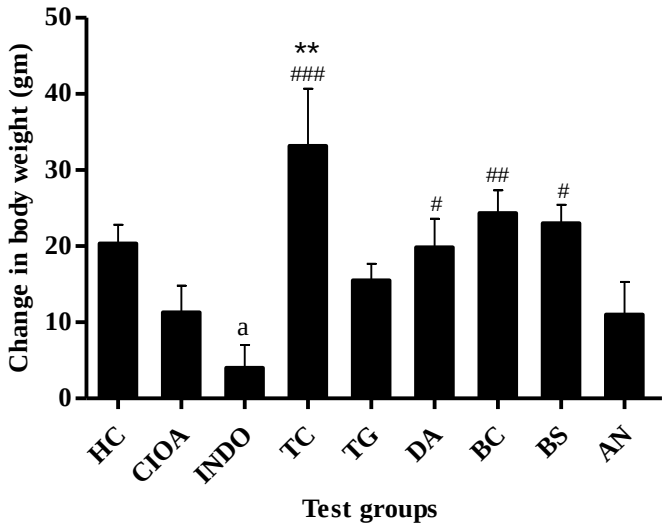

Figure 25: Effect of treatments on change in body weight.

Values are expressed as Mean ± SEM; n =6. $^{**}p<0.01$ compared to CIOA. $^{\#}p<0.05$; $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ compared to INDO. $^{a}p<0.05$ compared to HC. Data were analyzed by One-Way ANOVA followed by the Dunnett's multiple comparison test. CIOA: Osteoarthritic control, INDO: Positive control, TC: *Triphala*, TG: '*Triphala Guggul*', DA: *Dashamoolarishta*, BC: *B. ceiba*, BS: *B. serrata*, AN: *A. nilotica*.

5.3.2 Effect of gums and formulations on knee diameter

Osteoarthritic control group (CIOA) (1.75 ± 0.217 mm) demonstrated increase in knee swelling. Comparatively, TC (0.657 ± 0.329 mm) and AN (0.713 ± 0.263 mm) showed significant ($p<0.05$) reduction in knee swelling (Fig. 26). Treatment with TG, DA, BC and BS showed 1.13 ± 0.186 mm, 1.36 ± 0.359 mm, 1.37 ± 0.126 mm and 0.713 ± 0.263 mm knee swelling, respectively.

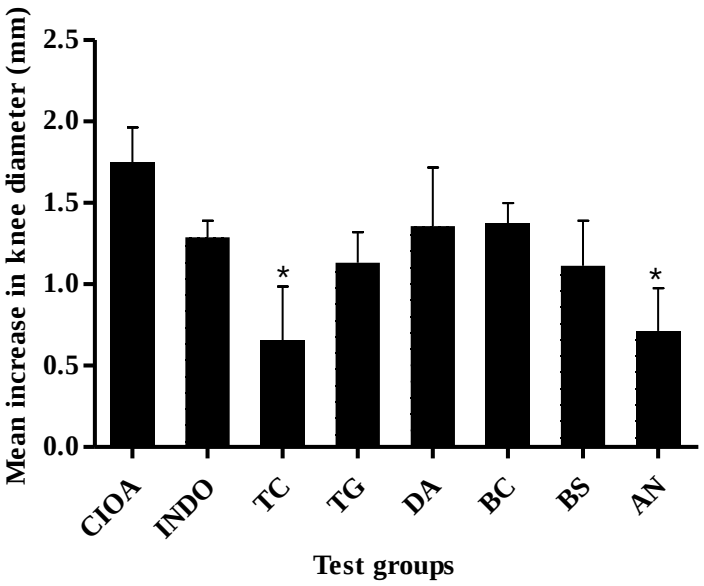

Figure 26: Effect of treatments on knee swelling.

Values are expressed as Mean ± SEM; n =6. $^{*}p<0.05$ compared to CIOA. Data were analysed by One-Way ANOVA followed by the Dunnett's multiple comparison test. CIOA: Osteoarthritic control, INDO: Positive control, TC: *Triphala*, TG: '*Triphala Guggul*', DA: *Dashamoolarishta*, BC: *B. ceiba*, BS: *B. serrata*, AN: *A. nilotica*.

5.3.3 Effect of gums and formulations on paw volume

INDO and TC produced highest percent inhibition i.e. 44.6 ± 2.7 and 39.8 ± 5.59, respectively (Fig. 27). While TG, DA, BC, BS and AN showed 15.9 ± 2, 21.4 ± 4.15, 12.9 ± 2.3, 11.5 ± 2.6 and 21.3 ± 1.42 percent inhibition, respectively. This shows anti-inflammatory activity of formulations and gums, comparatively TC produced highest inhibition in the rat paw edema.

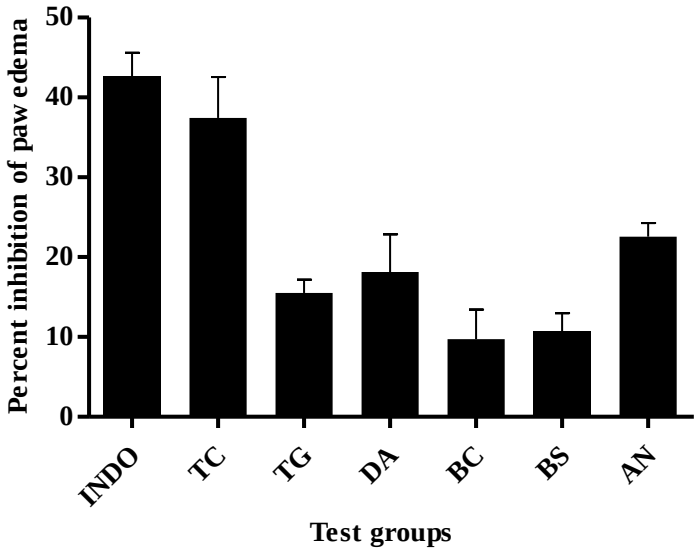

Figure 27: Effect of treatments on paw volume.

Values are expressed as Mean ± SEM; n =6. Data were analysed by One-Way ANOVA followed by the Dunnett's multiple comparison test. INDO: Positive control, TC: *Triphala*, TG: '*Triphala Guggul*', DA: *Dashamoolarishta*, BC: *B. ceiba*, BS: *B. serrata*, AN: *A. nilotica*.

5.3.4 Effect of gums and formulations on GAG

HC and CIOA group showed 47.3 ± 1.45 and 56.4 ± 2.58 µg/ml GAG release, respectively. Treatments with TC, BC and AN have significantly reduced GAG release in serum i.e. 20 ± 6.29 ($p<0.05$), 15 ± 12.6 ($p<0.01$) and 18.3 ± 4.32 ($p<0.01$) µg/ml, respectively compared to INDO (Fig. 28). TG, DA and BS showed GAG levels at 62.5 ± 25.3, 163 ± 14.6 and 57.1 ± 13 µg/ml, respectively.

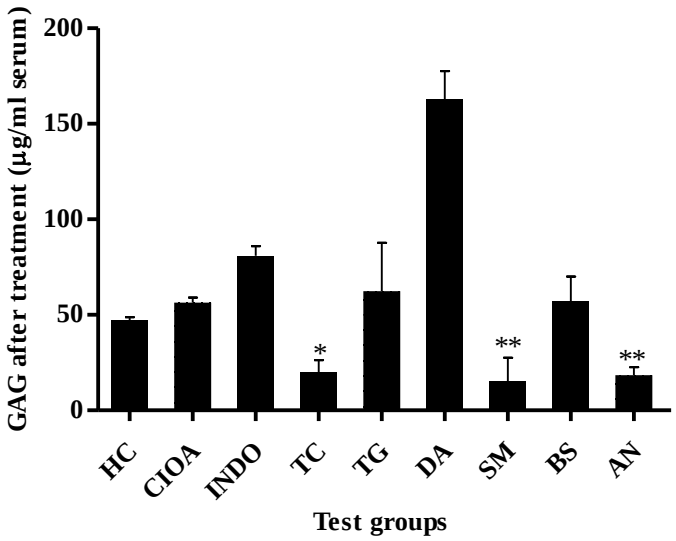

Figure 28: Effect of treatments on GAG release in the serum.

Values are expressed as Mean ± SEM; n =6. $^{*}p<0.05$; $^{**}p<0.01$ compared to INDO. Data were analysed by One-Way ANOVA followed by Dunnett's multiple comparison test. HC: Healthy control, CIOA: Osteoarthritic control, INDO: Positive control, TC: *Triphala*, TG: '*Triphala Guggul*', DA: *Dashamoolarishta*, BC: *B. ceiba*, BS: *B. serrata*, AN: *A. nilotica*.

5.3.5 Radiological analysis

Radiographic images of the joints were compared between the right knee (induced) and left knee for erosion of articular cartilage, reduced joint space and osteophyte formation. CIOA group showed severe osteoarthritic changes on induced knee, like marked erosion of articular cartilage and reduction in joint space due to loss of articular cartilage; suggesting disease was induced properly (Fig. 29B). In HC group, knee and patello-femoral joints appeared normal without obvious abnormalities in surrounding tissue (Fig. 29A). INDO treated group indicated moderate changes of OA with no improvement in joint space (Fig. 29C). Treatment with TG, BC and AN demonstrated minimal osteoarthritic changes and joint space was also improved (Fig. 29E, G, I). TG has also prevented joint surface erosions. Treatment with TC, DA and BS showed moderate osteoarthritic changes and has not improved joint space (Fig. 29D, F, H).

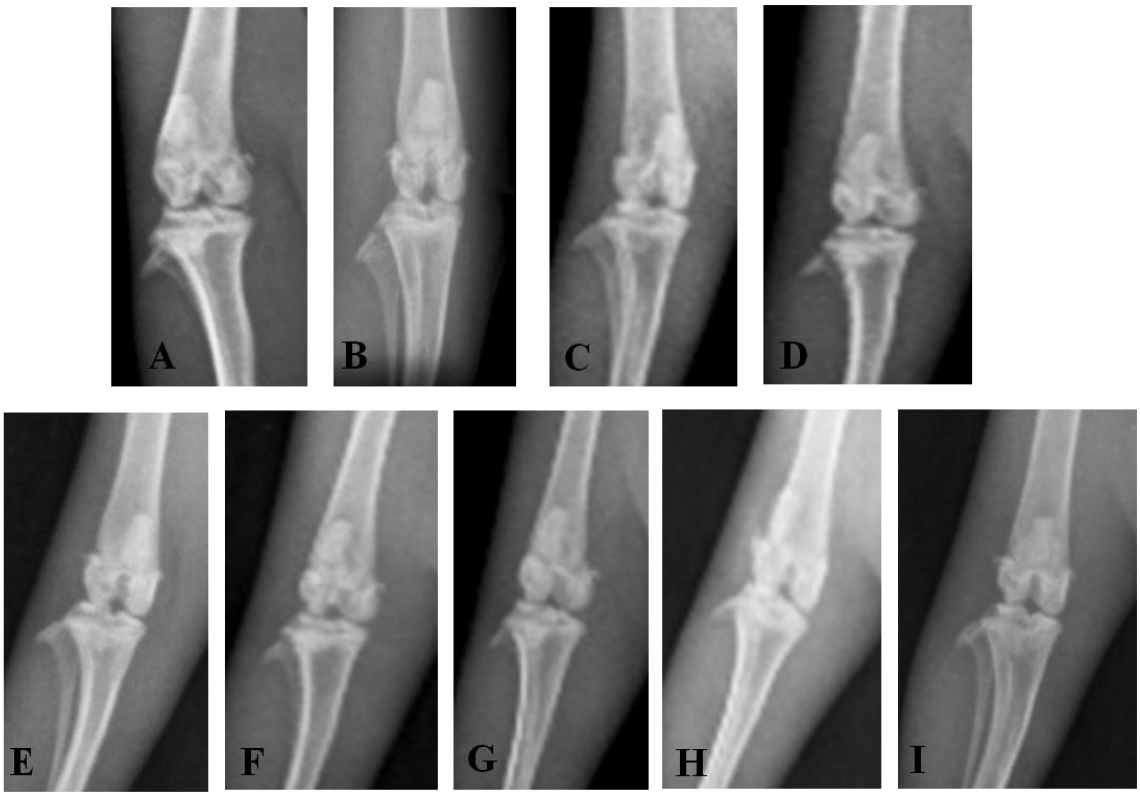

Figure 29: Radiography images of knee joint.

Right knee joint radiographs (in the Anterior Posterior view) of control and experimental animals. All radiographs were taken keeping object to x-ray source distance constant (100 cm) and scale is showing extent of zoom (1 unit is 0.5 cm).

HC (A), Saline injected groups displayed smooth articular surface while in (B) CIOA group, erosion of articular surface with significant joint space reduction; Comparatively (C) INDO group demonstrated minimal changes however joint space was not improved. (D) TC also showed erosions of joint surface while (E) TG has prevented joint surface erosions. Joint space erosions are observed in (F) DA as well. Joint space was slightly improved with (G) BC treatment, but not with (H) BS. (I) AN displayed minimal changes with increased joint space.

5.3.6 Histopathological analysis

Synovium: In the synovium of HC, 1-2 layers of cells were observed without cellular infiltration (Fig. 30A1). CIOA group showed 3-6 layers of cells with moderate cellular infiltrate (Fig. 30A2). INDO group also demonstrated increased lining of cells compared to HC (Fig. 30A3). TC and TG showed normal architecture of synovium tissue (Fig. 30A4, 30A5). However, DA and BS displayed increased cellular lining (Fig. 30A6, 30A8). BC also showed 3-6 layers of cells with short villi (Fig. 30A7). AN revealed normal architecture of synovium (Fig. 30A9). Histology of articular cartilage from HC group exhibited cartilage with normal volume, smooth surface with all zones intact. The chondrocytes appeared unremarkable. Tide mark was intact and distinct (Fig. 30B1). Collagen and Proteoglycan content also appeared normal (Fig. 30C1, 30D1). CIOA group showed decreased proteoglycan and collagen intensity in the deep zones, compared to INDO (Fig. 30D1, 30C1). TC treated rat tissue sections showed undulations of the articular cartilage with superficial fissures seen at focal areas. There is an increase intensity of chondrocytes in the superficial zones of cartilage. The tide mark was intact and distinct (Fig. 30B4). In TG treated group, chondrocytes were in small clusters of 2-4 cells with loss of tide mark which is crossed by blood vessels. Superficial fissures were also observed (Fig. 30B5). DA and BC treated sections displayed fissures extending deep in the mid zone of the articular cartilage. Chondrocytes were seen in small clusters of 2-4 cells with loss of tide mark which is crossed by blood vessels (Fig. 30B6, 30B7). BS sections showed fissures of the cartilage extending deep in the lower third of the cartilage with erosion. The chondrocytes were in large clusters of more than 5 cells. Tide mark appeared intact and distinct (Fig. 30B8). AN indicated chondrocyte pathology within normal limits (Fig. 30B9). Proteoglycan intensity was also increased after treatment with TC and AN, compared to the rest treatments (Fig. 30D4, 30D9). Similarly TC, BS and AN has shown more collagen deposion, comparatively (Fig. 30C4, 30C8, 30C9).

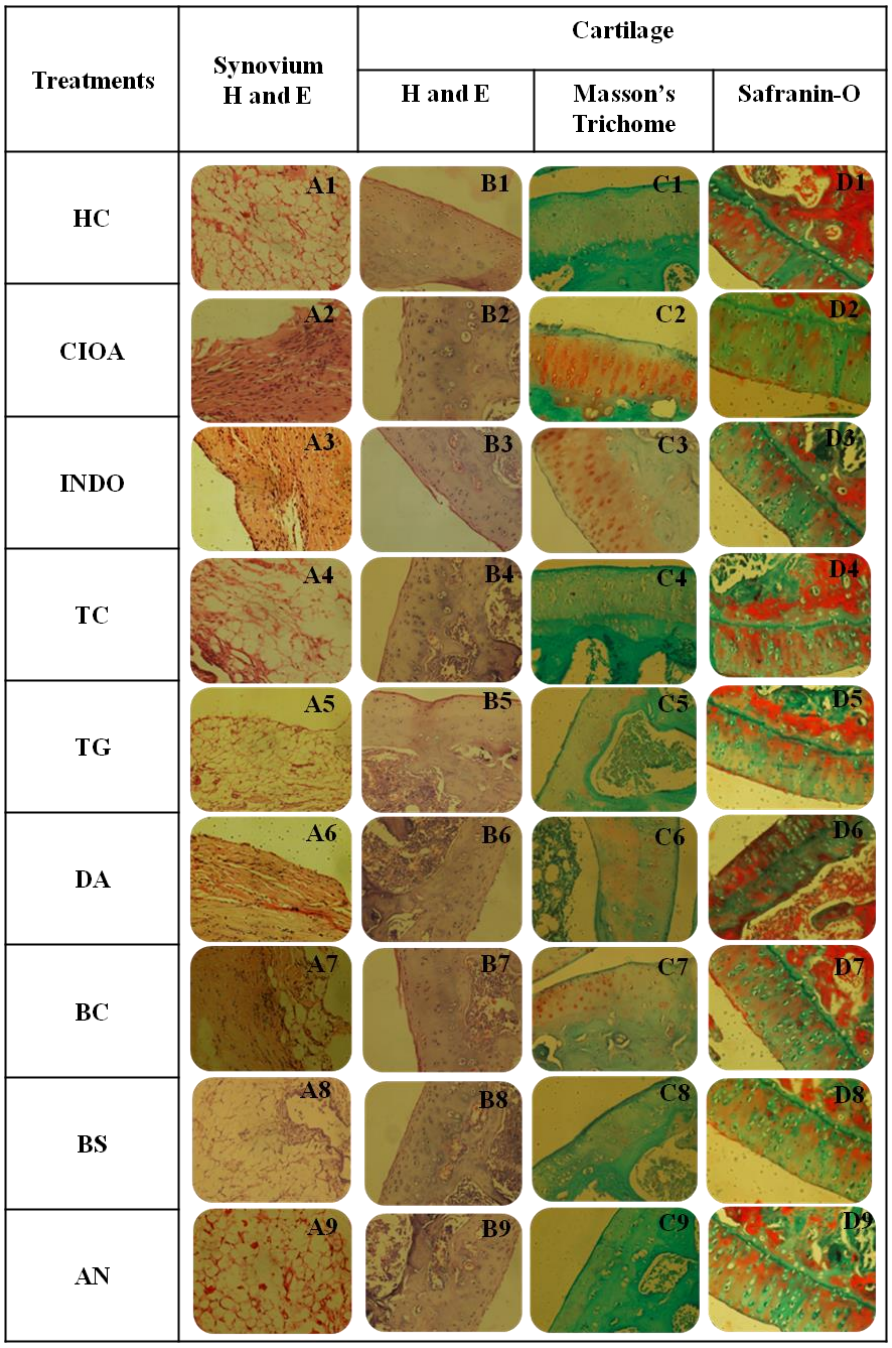

Figure 30: Histopathology score of synovial membrane and cartilage.

First column (A1- 9) showing photographs of synovium stained with H & E, and further are photographs of cartilage stained with H and E (B1- 9), Masson's trichome (C1- 9) and safranin-O (D1- 9) respectively. HC: Healthy control, CIOA: Osteoarthritic control, INDO: Positive control, TC: *Triphala*, TG: '*Triphala Guggul*', DA: *Dashamoolarishta*, BC: *B. ceiba*, BS: *B. serrata*, AN: *A. nilotica*.

5.3.7 qPCR analysis

Based on these results, qPCR analysis was carries out for *Triphala* and *A. nilotica*. Osteoarthritic control rats showed down-regulation of SOD, GPx, CAT and PON-1in the knee joint synovium by ~3.72-fold ($p<0.05$), ~2.78-fold and ~1.95-fold and ~2.26-fold respectively compared to control rats (Fig. 31). On the contrary, osteoarthritic rats receiving TC, AN and INDO, demonstrated up-regulation in the synovial expression of SOD by ~1.23-fold, ~2.38-fold and ~2.35-fold, respectively compared to osteoarthritic control rats (Fig. 31A). GPx expression was also elevated by ~1.07-fold, ~2.34-fold and ~2.27-fold in osteoarthritic rats receiving TC, AN and INDO respectively compared to osteoarthritic control rats (Fig. 31B). Similarly, Up-regulation in the CAT and PON-1 expression was observed by ~2.08-fold and ~1.77-fold, respectively in osteoarthritic rats receiving AN, compared to osteoarthritic control rats (Fig. 31C, 31D). CAT expression was found to be equal in TC and osteoarthritic control rats while it was upregulated by INDO (~2.67-fold) (Fig. 31C). PON-1 expression was also elevated by ~1.76-fold in osteoarthritic rats receiving TC compared to osteoarthritic control rats (Fig. 31D). In the knee synovium, osteoarthritic control rats showed up-regulation of MMP-3 (~2.64-fold) when compared to control rats (Fig. 32A). Down-regulation in expression of MMP-3 by ~1.93-fold, ~1.11-fold and ~6.99-fold was observed in the osteoarthritic rats receiving TC, AN and INDO respectively (Fig. 32A), compared to osteoarthritic control rats. Up-regulation of TIMP-1 was observed in rats treated with TC and AN by ~1.90-fold and ~3.72-fold ($p<0.01$), respectively when compared to osteoarthritic control rats (Fig. 32B). INDO was unable to upregulate TIMP-1 expression (Fig. 32B).

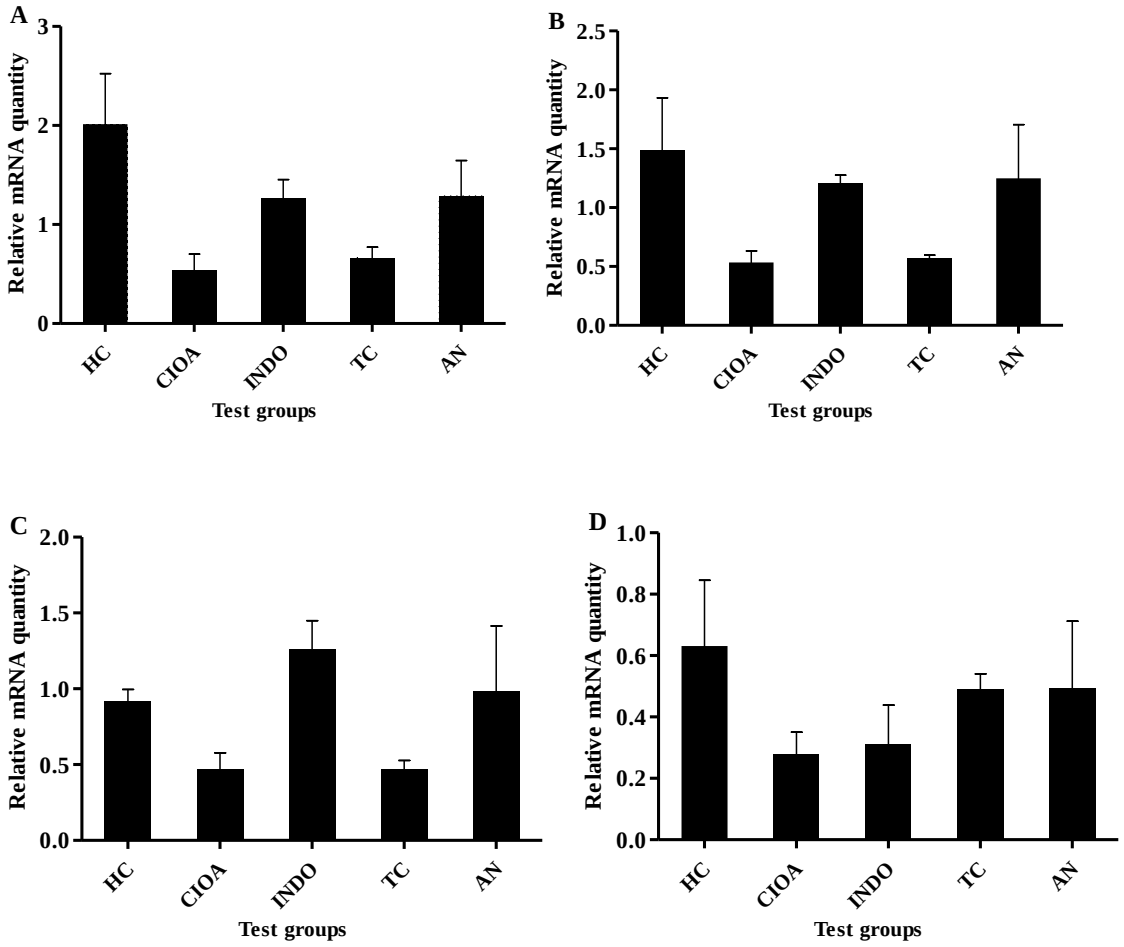

Figure 31: Effect of *Triphala* churna (TC) and *A. nilotica* gum (AN) on the mRNA expression of A) SOD-1, B) GPx-1 C) CAT and D) PON-1 in the synovium of rats. All the values are expressed as Mean ± SEM (n= 3). HC: Healthy control, CIOA: Osteoarthritic control, INDO: Positive control, TC: *Triphala*, AN: *A. nilotica*.

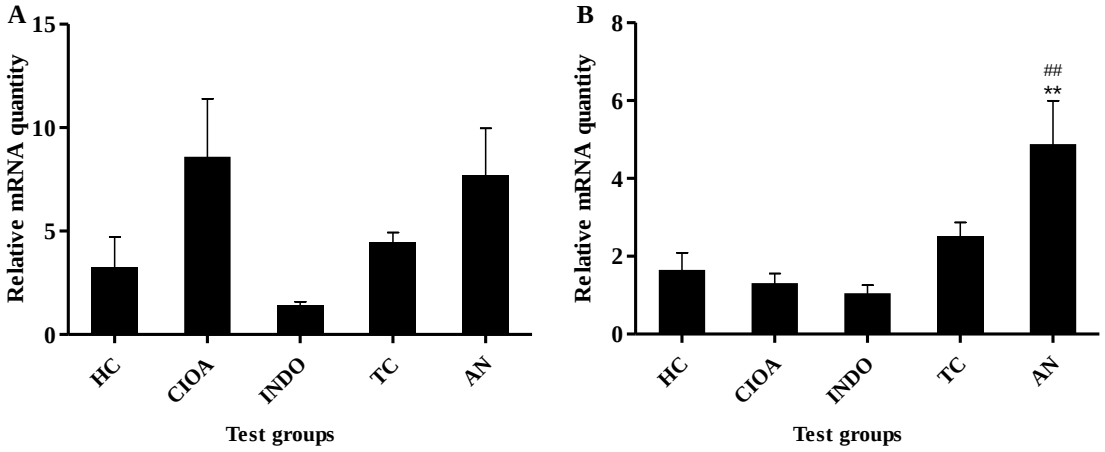

Figure 32: Effect of *Triphala* churna (TC) and *A. nilotica* gum (AN) on the mRNA expression of A) MMP3 and B) TIMP1 in the synovium of rats. All the values are expressed as Mean ± SEM (n= 3). Comparisons were done between CIOA/HC and each individual treated group by Dunnett's multiple comparison test (**$p<0.01$, $^{\#\#}p<0.01$ *=Compared to CIOA, # =Compared to HC). HC: Healthy control, CIOA: Osteoarthritic control, INDO: Positive control, TC: *Triphala*, AN: *A. nilotica*.

5.4 To develop and comparatively evaluate anti-osteoarthritic potential of selected new combination formulations using *in vivo* model

On the basis of results obtained in earlier studies, *S. cordifolia*, *P. longum* followed by *Z. officinale* has shown highest potential among selected plants. Further, we have developed four new combination formulations from these plants.

5.4.1 Collagenase type II-induced osteoarthritis (CIOA) rat model

5.4.1.1 Effect of formulations on body weight

Body weight changes in HC, CIOA and DICLO group are 16.8 ± 0.91, 8.5 ± 0.885 and 15.2 ± 3.89 gm, respectively (Fig. 33). This shows decrease in body weight in osteoarthritic control rats. Body weight changes in SPL, M and H are 4.33 ± 4.1, 19.5 ± 4.06 and 12.7 ± 3.57 gm, respectively (Fig. 33). After treatment with PZ, changes in body weight found to be 13.3 ± 5.28, 14 ± 4.21 and 18 ± 2.35 gm, respectively by lower, middle and higher doses. Body weight change was 8.17 ± 2.86, 17 ± 1.24 and 18 ± 3.11

gm by SZL, M and H, respectively. Similarly, body weight change in SPZ treated group was 14.8 ± 1.54, 9.5 ± 1.28 and 9.17 ± 3.34 by lower, middle and higher doses, respectively (Fig. 33).

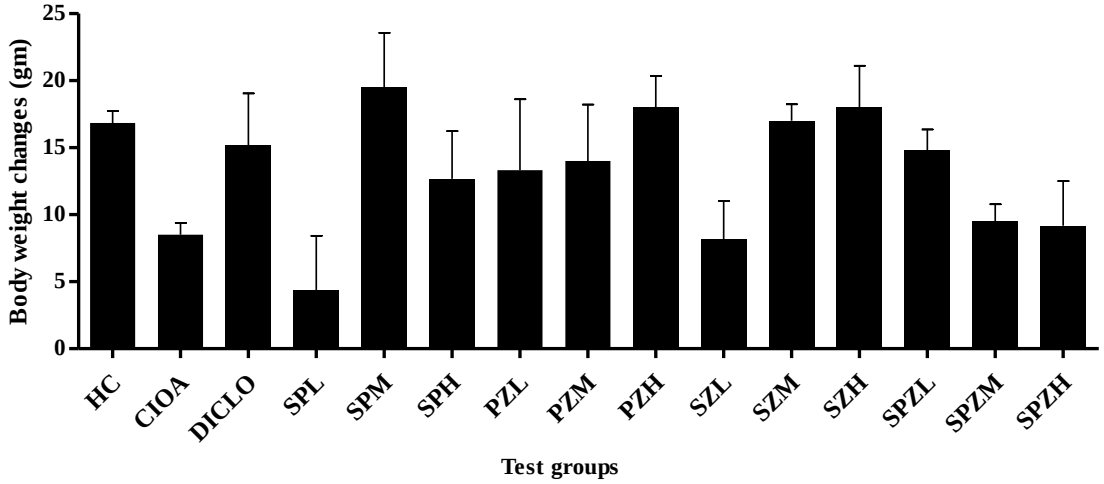

Figure 33: Effect of formulations on change in body weight.

Values are expressed as Mean ± SEM; n =6. HC: Healthy control, CIOA: Osteoarthritic control, DICLO: Positive control, SPL: SP at 135 mg/kg b. wt., SPM: SP at 270 mg/kg b. wt., SPH: SP at 540 mg/kg b. wt., PZL: PZ at 135 mg/kg b. wt., PZM: PZ at 270 mg/kg b. wt., PZH: PZ at 540 mg/kg b. wt., SZL: SZ at 135 mg/kg b. wt., SZM: SZ at 270 mg/kg b. wt., SZH: SZ at 540 mg/kg b. wt., SPZL: SPZ at 135 mg/kg b. wt., SPZM: SPZ at 270 mg/kg b. wt., SPZH: SPZ at 540 mg/kg b. wt.

5.4.1.2 Effect of formulations on knee diameter

CIOA group showed 0.806 ± 0.243 mm knee swelling and, comparatively DICLO ($p<0.05$), PZL ($p<0.01$), SZM ($p<0.05$) and SZH ($p<0.05$) significantly reduced knee swelling (Fig. 34). SPL, SPM, SPH, PZM, PZH and SZL showed 0.844 ± 0.148, 0.589 ± 0.171, 0.277 ± 0.162, 0.339 ± 0.111, 0.45 ± 0.204 and 0.6 ± 0.09 mm knee swelling, respectively (Fig. 34). Treatment with SPZ was not significant at any given doses.

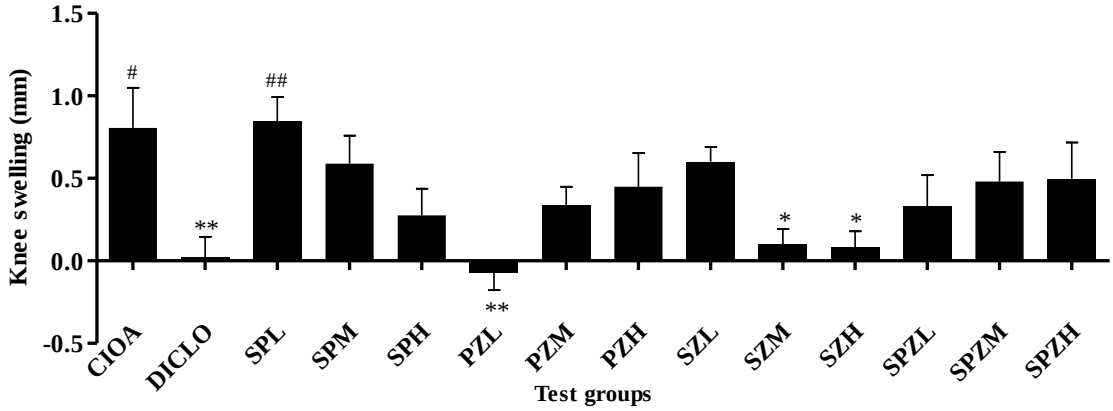

Figure 34: Effect of formulations on knee swelling.

Values are expressed as Mean ± SEM; n =6. $^{*}p<0.05$, $^{**}p<0.01$ compared to CIOA. $^{\#}p<0.05$, $^{\#\#}p<0.01$ compared to DICLO. Data were analysed by One-Way ANOVA followed by the Dunnett's multiple comparison test. CIOA: Osteoarthritic control, DICLO: Positive control, SPL: SP at 135 mg/kg b. wt., SPM: SP at 270 mg/kg b. wt., SPH: SP at 540 mg/kg b. wt., PZL: PZ at 135 mg/kg b. wt., PZM: PZ at 270 mg/kg b. wt., PZH: PZ at 540 mg/kg b. wt., SZL: SZ at 135 mg/kg b. wt., SZM: SZ at 270 mg/kg b. wt., SZH: SZ at 540 mg/kg b. wt., SPZL: SPZ at 135 mg/kg b. wt., SPZM: SPZ at 270 mg/kg b. wt., SPZH: SPZ at 540 mg/kg b. wt.

5.4.1.3 Effect of formulations on paw volume

INDO, SPL, SPM and SPH produced percent inhibition i.e. 27.4 ± 2.66 and 28.3 ± 2.32, 25.3 ± 2.34 and 24.5 ± 1.75 %, respectively (Fig. 34). While PZL, M and H showed 26.3 ± 2.56, 24 ± 1.3 and 24.6 ± 1.15 %, inhibition, respectively. % inhibition of paw edema by lower, middle and higher doses of SZ and SPZ were 28 ± 2.59, 24.2 ± 3.5, 22.5 ± 1.01, 14.5 ± 0.875, 23.8 ± 1.41 and 30.6 ± 1.94 %, respectively (Fig. 35).

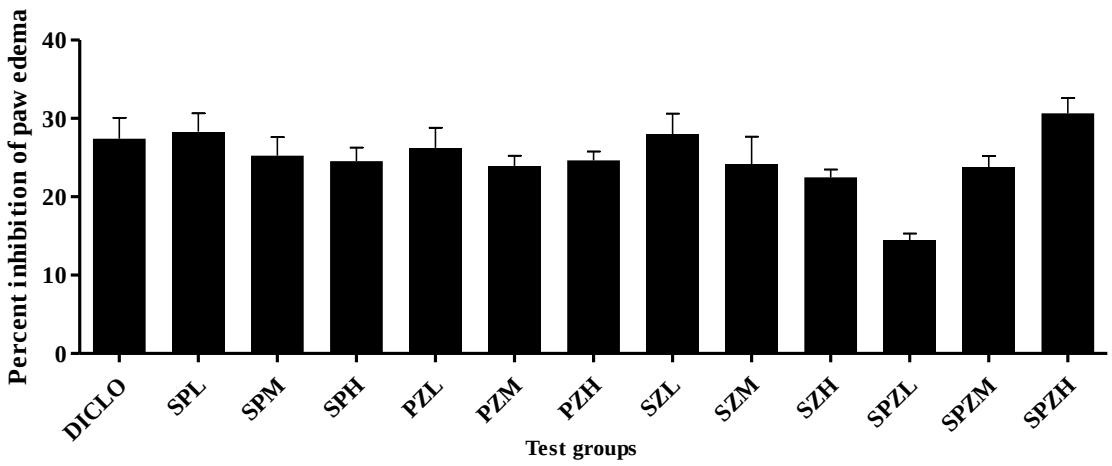

Figure 35: Effect of formulations on paw volume.

Values are expressed as Mean ± SEM; n =6. DICLO: Positive control, SPL: SP at 135 mg/kg b. wt., SPM: SP at 270 mg/kg b. wt., SPH: SP at 540 mg/kg b. wt., PZL: PZ at 135 mg/kg b. wt., PZM: PZ at 270 mg/kg b. wt., PZH: PZ at 540 mg/kg b. wt., SZL: SZ at 135 mg/kg b. wt., SZM: SZ at 270 mg/kg b. wt., SZH: SZ at 540 mg/kg b. wt., SPZL: SPZ at 135 mg/kg b. wt., SPZM: SPZ at 270 mg/kg b. wt., SPZH: SPZ at 540 mg/kg b. wt.

5.4.1.4 Effect of formulations on CRP

CRP levels in HC and CIOA groups were 0.0967 ± 0.003 and 0.363 ± 0.118 mg per litre, respectively (Fig. 36). SPL ($p<0.001$), SZL ($p<0.001$) and SZM ($p<0.05$) has significantly reduced CPR levels compared to CIOA (fig. 36). Middle and higher dose of SP showed CRP at 0.08 ± 0.16 and 0.17 ± 0.0764 mg per litre, respectively. PZL, PZM and PZH showed 0.28±0.11, 0.357±0.0841 and -0.097 ± 0.151 mg per litre CRP levels, respectively. CRP levels in SZH, SPZL, SPZM and SPZH groups are 0.0733 ± 0.103, 0.343 ± 0.0491, 0.34 ± 0.0208 and 0.35 ± 0.0115 mg per litre, respectively (fig. 36).

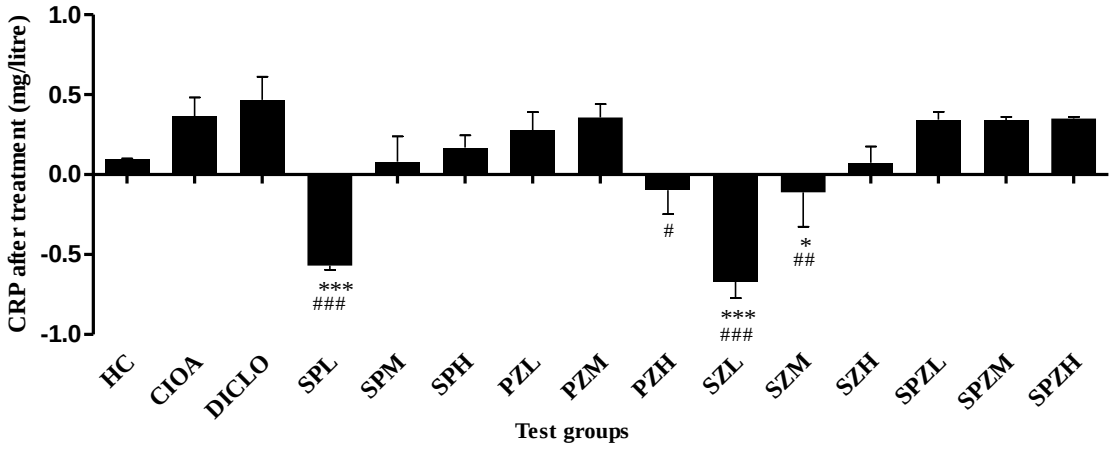

Figure 36: Effect of formulations on C-reactive protein (CRP).

Values are expressed as Mean ± SEM; n =6. Data were analysed by One-Way ANOVA followed by the Dunnett's multiple comparison test. $^{*}p<0.05$, $^{***}p<0.001$ compared to CIOA. $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ compared to DICLO. HC: Healthy control, CIOA: Osteoarthritic control, DICLO: Positive control, SPL: SP at 135 mg/kg b. wt., SPM: SP at 270 mg/kg b. wt., SPH: SP at 540 mg/kg b. wt., PZL: PZ at 135 mg/kg b. wt., PZM: PZ at 270 mg/kg b. wt., PZH: PZ at 540 mg/kg b. wt., SZL: SZ at 135 mg/kg b. wt., SZM: SZ at 270 mg/kg b. wt., SZH: SZ at 540 mg/kg b. wt., SPZL: SPZ at 135 mg/kg b. wt., SPZM: SPZ at 270 mg/kg b. wt., SPZH: SPZ at 540 mg/kg b. wt.

5.4.1.5 Effect of formulations on GAG

CIOA (285 ± 31.4 µg/ml) showed significant ($p<0.05$) increase in GAG levels compared to HC (Fig. 37). SZL ($p<0.001$), SZM ($p<0.001$), SZH ($p<0.001$), SPZM ($p<0.001$) and SPZH ($p<0.001$) has significantly reduced GAG levels compared to CIOA. SPZL has shown 216 ± 15.7 µg per ml GAG levels. Remaining groups have not indicated any significant decrease in GAG levels. Compared to DICLO (164 ± 35.5), SZL ($p<0.001$), SZM ($p<0.001$), SPZM ($p<0.01$) and SPZH ($p<0.001$) revealed significant reduction in GAG levels (Fig. 37).

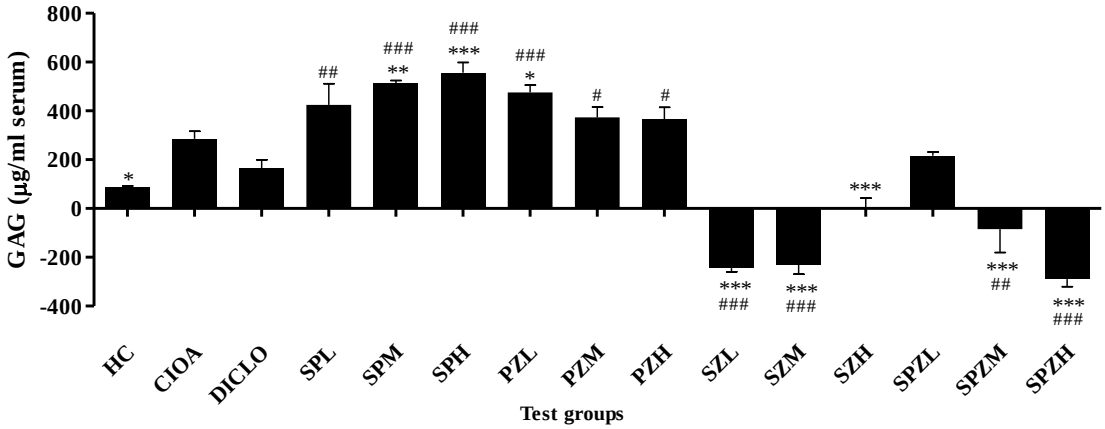

Figure 37: Effect of formulations on GAG.

Values are expressed as Mean ± SEM; n =6. Data were analysed by One-Way ANOVA followed by the Dunnett's multiple comparison test. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ compared to CIOA. $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ compared to DICLO. HC: Healthy control, CIOA: Osteoarthritic control, DICLO: Positive control, SPL: SP at 135 mg/kg b. wt., SPM: SP at 270 mg/kg b. wt., SPH: SP at 540 mg/kg b. wt., PZL: PZ at 135 mg/kg b. wt., PZM: PZ at 270 mg/kg b. wt., PZH: PZ at 540 mg/kg b. wt., SZL: SZ at 135 mg/kg b. wt., SZM: SZ at 270 mg/kg b. wt., SZH: SZ at 540 mg/kg b. wt., SPZL: SPZ at 135 mg/kg b. wt., SPZM: SPZ at 270 mg/kg b. wt., SPZH: SPZ at 540 mg/kg b. wt.

5.4.1.6 Effect of formulations on ALP

At the end of experiment ALP levels of osteoarthritic control group are 40.8 ± 4.94 KA unites. SZL ($p<0.001$), SZM ($p<0.001$), SZH ($p<0.001$), SPZL ($p<0.001$), SPZM ($p<0.001$) and SPZH ($p<0.01$) has significantly reduced ALP levels after treatment which was found to be increased in CIOA group (Fig. 38). ALP levels after treatment of SP-L, M and H are found to be 30 ± 2.25, 15.3 ± 0.731 and 23.1 ± 1.54 KA unites, respectively. Similarly with the treatment of PZ, ALP levels are 13.5 ± 2.84, 10.9 ± 4.09 and 21.7 ± 2.69 KA unites by PZL, M and H, respectively (Fig. 38).

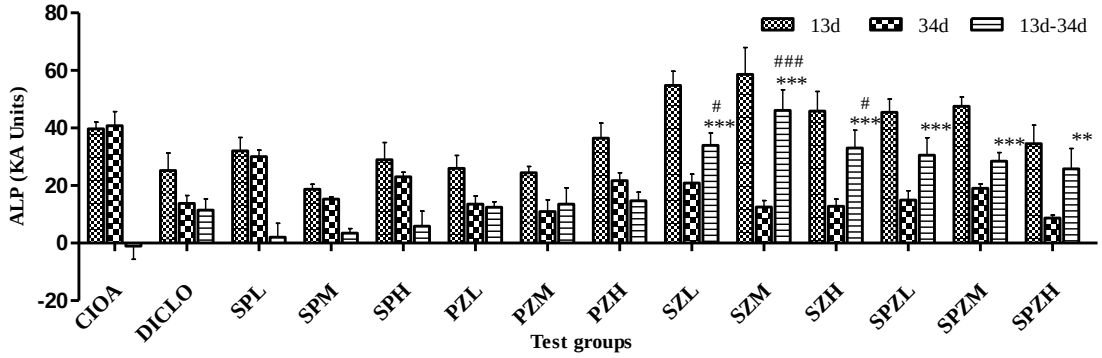

Figure 38: Effect of formulations on ALP.

Values are expressed as Mean ± SEM; n =6. Data were analysed by One-Way ANOVA followed by the Dunnett's multiple comparison test. $^{**}p<0.01$, $^{***}p<0.001$ compared to CIOA. $^{\#}p<0.05$, $^{\#\#\#}p<0.001$ compared to DICLO. CIOA: Osteoarthritic control, DICLO: Positive control, SPL: SP at 135 mg/kg b. wt., SPM: SP at 270 mg/kg b. wt., SPH: SP at 540 mg/kg b. wt., PZL: PZ at 135 mg/kg b. wt., PZM: PZ at 270 mg/kg b. wt., PZH: PZ at 540 mg/kg b. wt., SZL: SZ at 135 mg/kg b. wt., SZM: SZ at 270 mg/kg b. wt., SZH: SZ at 540 mg/kg b. wt., SPZL: SPZ at 135 mg/kg b. wt., SPZM: SPZ at 270 mg/kg b. wt., SPZH: SPZ at 540 mg/kg b. wt.

5.4.1.7 Radiological analysis

Radiographic images of the joints were compared between the right knee (induced) and left knee for erosion of articular cartilage, reduced joint space and osteophyte formation. CIOA group showed severe osteoarthritic changes on induced knee, like marked erosion of articular cartilage and reduction in joint space due to loss of articular cartilage (Fig. 39B). In HC group, knee and patello-femoral joints appeared normal without obvious abnormalities in surrounding tissue (Fig. 39A). DICLO treated group revealed severe changes of OA (Fig. 39C). After treatment with SP, osteophytes became blunt and joint space was also improved with regression in the erosion of cartilage surface. PZ has shown moderate changes of OA (Fig. 39E). After treatment with SZ, joint space was improved and erosion of articular surface was regressed (Fig. 39F). SPZ treated rat group has demonstrated severe changes of OA (Fig. 39G). Thus, SZ showed cartilage protection against OA. Based on all physiological, biochemical and

histopathological parameters studied, combination SZ has exhibited highest anti-osteoarthritic potential. Hence in histopathology and radiology analysis, data only for SZL, M and H have shown.

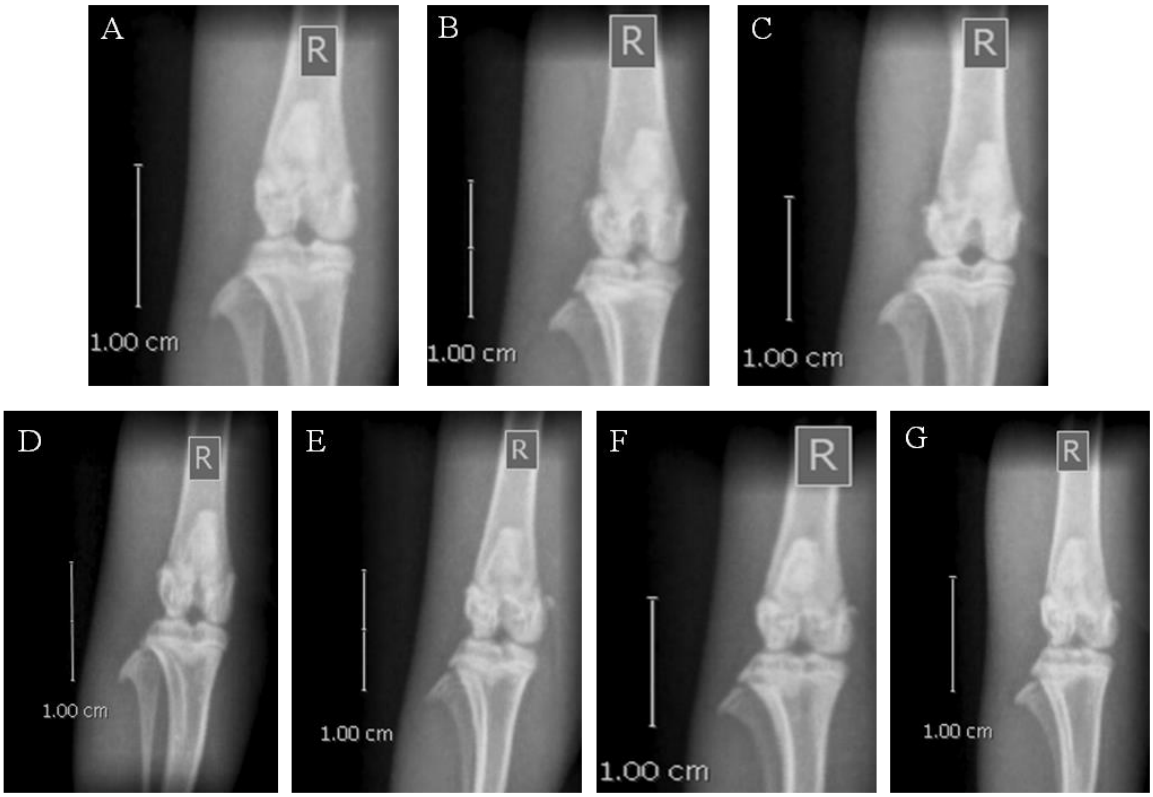

Figure 39: Radiography images of knee joint.

Right knee joint radiographs (in the Anterior Posterior view) of control and experimental animals. All radiographs were taken keeping object to x-ray source distance constant (100 cm) and scale is showing extent of zoom. (A) Saline injected groups displayed smooth articular surface (arrow) while in (B) CIOA and (C) DICLO group showed severe changes of OA; Comparatively (D) SP, (E) PZ, (F) SZ and (G) SZH group demonstrated minimal changes of OA.

5.4.1.8 Histological analysis

Histology of the synovium in HC group demonstrated 1-2 layers of cells without cellular infiltration (Fig. 40A1). In OAC group, lining cells showed mild increase in layer and stroma showed mild mononuclear infiltrate (Fig. 40A2). Compared to DICLO, all formulation treated group have displayed protection to synovium. Histology of the articular cartilage in HC group showed cartilage with normal volume, smooth surface with all zones intact (Fig. 40B1). Chondrocyte pathology was within normal limits (Fig. 40C1) and the matrix was densely stained red with Safranin (Fig. 40D1). Intra-articular injection of collagenase produced erosion of cartilage with irregular surface and mild fibrillation, chondrocytes were seen in clusters in joint tissues of CIOA and DICLO (Fig. 40C2, 40C3). Also in comparison with control group (HC), proteoglycans (PGs) integrity was decreased in the upper zone (Fig. 40D2, 40D3). Comparatively, SZ has protected cartilage degradation. Among selected formulations, SP and SZ also revealed more collagen deposition with reduced cleft formation compared to CIOA and DICLO (Fig. 40C4, 40C6). PGs layer density was also increased after formulations treatment, compared to OAC and DICLO (Fig. 40D4-D7). Thus, comparatively SZ protected chondroid matrix and cartilage surface degeneration.

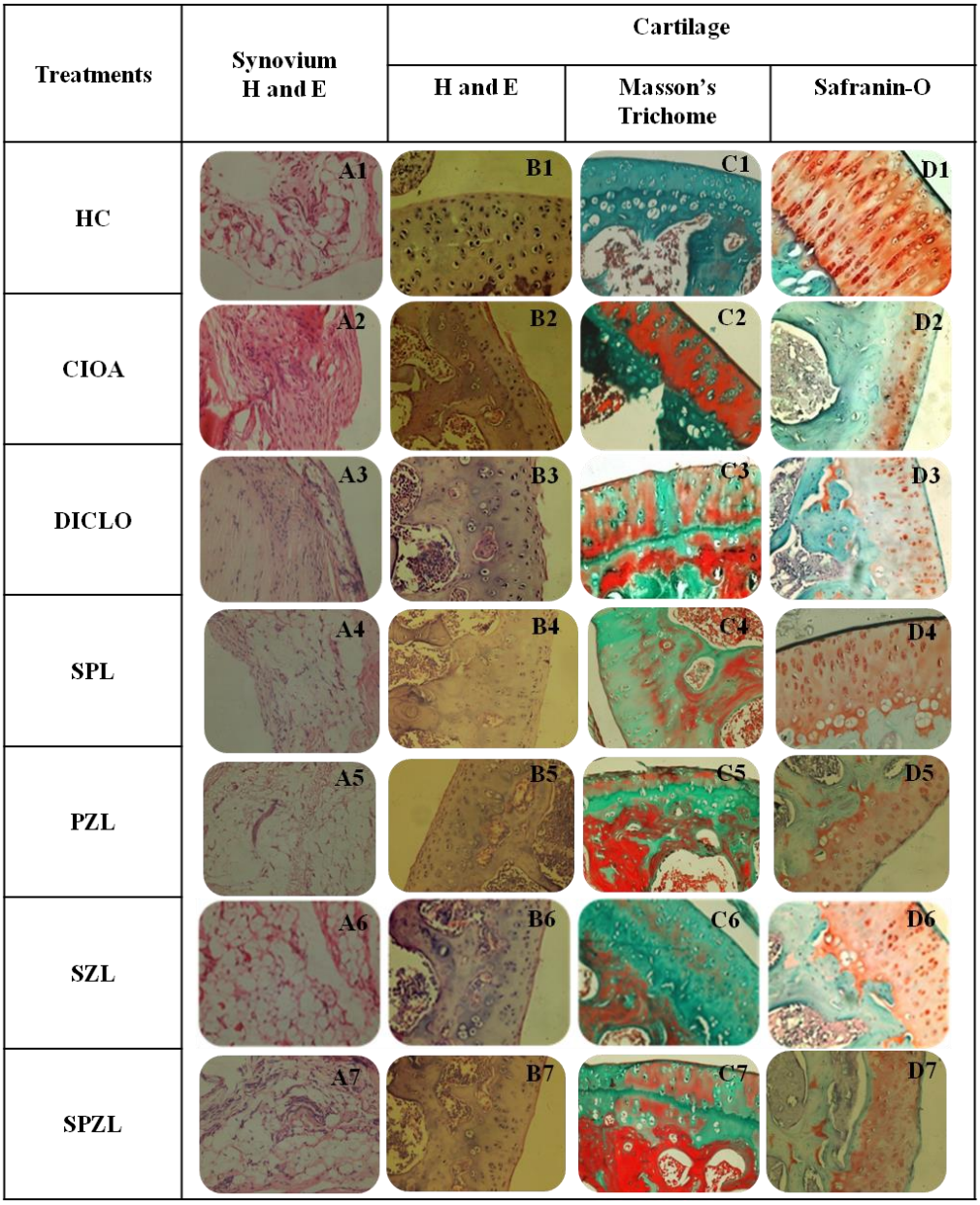

Figure 40: Histopathology pictures of synovial membrane and cartilage.

First column (A1-6) showing photographs of synovium stained with H and E and further are photographs of cartilage stained with H and E (B1-6), Masson's trichome (C1-6) and safranin-O (D1-6) respectively. HC: Healthy control, OAC: Osteoarthritic control, DICLO: Positive control, SPL: OA-F2 at 135 mg/kg b. wt., PZL: OA-F2 at 135 mg/kg b. wt., SZL: OA-F2 at 135 mg/kg b. wt., SPZL: OA-F2 at 135 mg/kg b. wt., H and E: hematoxyline and eosin.

5.4.1.9 qPCR analysis

Considering the studies parameters, overall prioritized formulation-SZ was taken further for qPCR analysis. Osteoarthritic control rats showed down-regulation of SOD, GPx and CAT in the knee joint synovium by ~1.22-fold, ~1.22-fold and ~1.33-fold, respectively compared to control rats (Fig. 41). On the contrary, osteoarthritic rats receiving SZL, M and H, showed up-regulation in the synovial expression of SOD by ~1.87-fold ($p<0.01$), ~1.22-fold and ~1.29-fold, respectively compared to osteoarthritic control rats (Fig. 41A). GPx expression was also elevated by ~2.51-fold ($p<0.01$), ~1.36-fold and ~1.22-fold in osteoarthritic rats receiving SZL, M and H respectively compared to osteoarthritic control rats (Fig. 41A). Similarly, Up-regulation in the CAT expression by ~1.29-fold and ~1.15-fold respectively was observed in osteoarthritic rats receiving SZM and H compared to osteoarthritic control rats (Fig. 41B). SZL did not demonstrated upregulation in CAT expression. Rats receiving DICLO did not show up-regulation in SOD, GPx or CAT expression. In the knee synovium, osteoarthritic control rats exhibited up-regulation of MMP-3, MMP-9 and TIMP-1 by ~9.34-fold ($p<0.05$), ~33.47-fold and ~1.57-fold when compared to control rats (Fig. 42). Significant down-regulation in expression of MMP-3 by ~4.45-fold ($p<0.05$), ~8.55-fold ($p<0.01$), ~3.58-fold ($p<0.05$) and ~4.29-fold ($p<0.05$) was observed in the osteoarthritic rats receiving SZL, M, H and DICLO respectively (Fig. 42A), compared to osteoarthritic control rats. These groups have also down-regulated MMP9 expression by ~7.85-fold, ~25.50-fold, ~19.38-fold and ~27.47-fold, respectively (Fig. 42A). Up-regulation of TIMP-1 was observed in rats treated with SZL, H and DICLO by ~1.52-fold, ~1.17-fold and ~1.02-fold, respectively when compared to osteoarthritic control rats (Fig. 42B). SZM was unable to affect the level of synovial TIMP-1 in the osteoarthritic knee joints.

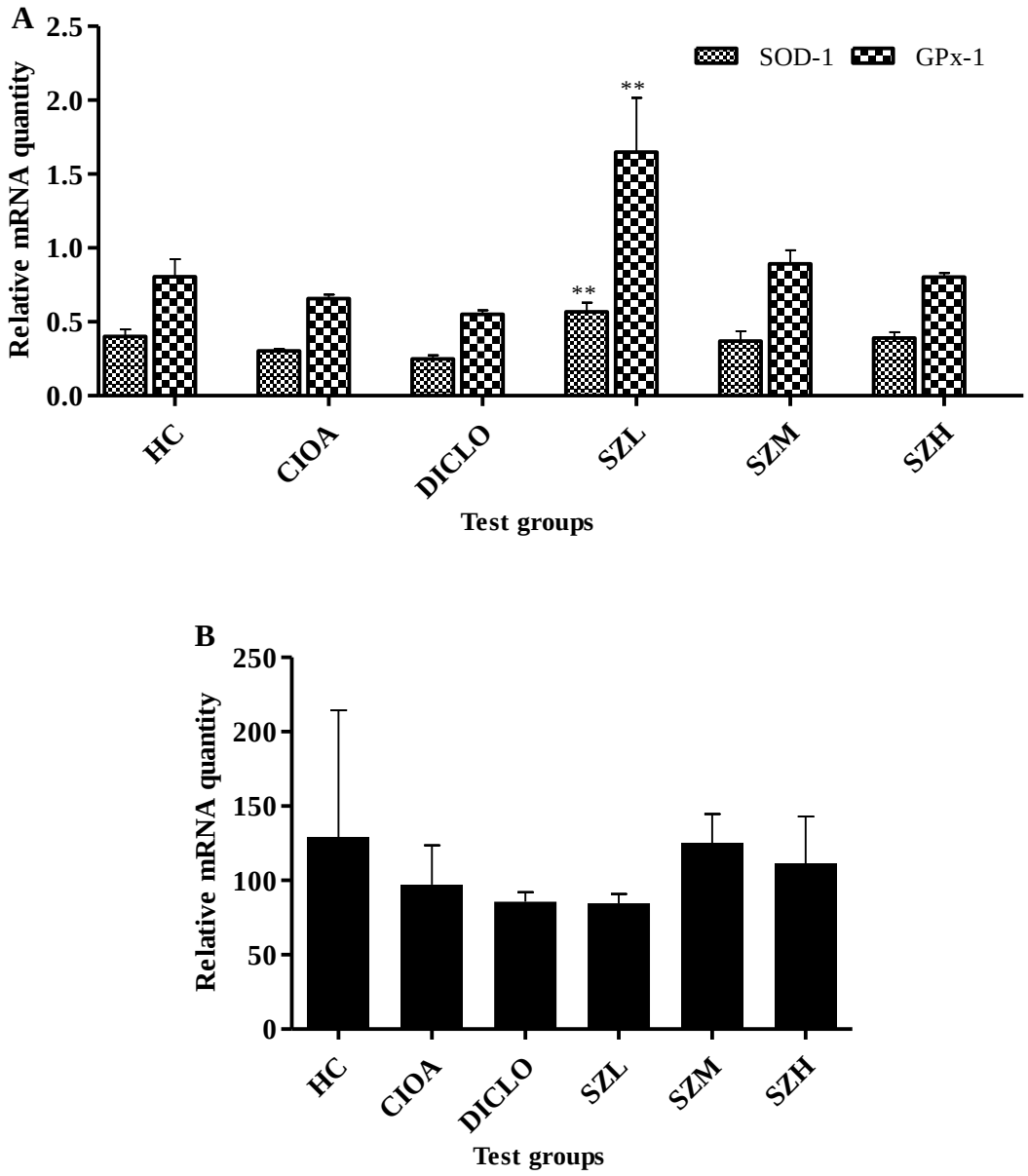

Figure 41: Effect of SZL, SZM and SZH on the mRNA expression of A) SOD-1, GPx-1 and B) CAT in the synovium of rats.

Values are expressed as Mean ± SEM (n=3). Comparisons were done between OAC/HC and each individual treated group by Dunnett's multiple comparison test. **p<0.01 Compared to OAC. HC: Healthy control, OAC: Osteoarthritic control, DICLO: Positive control, SZL: SZ at 135 mg/kg b. wt., SZM: SZ at 270 mg/kg b. wt., SZH: SZ at 540 mg/kg b. wt.

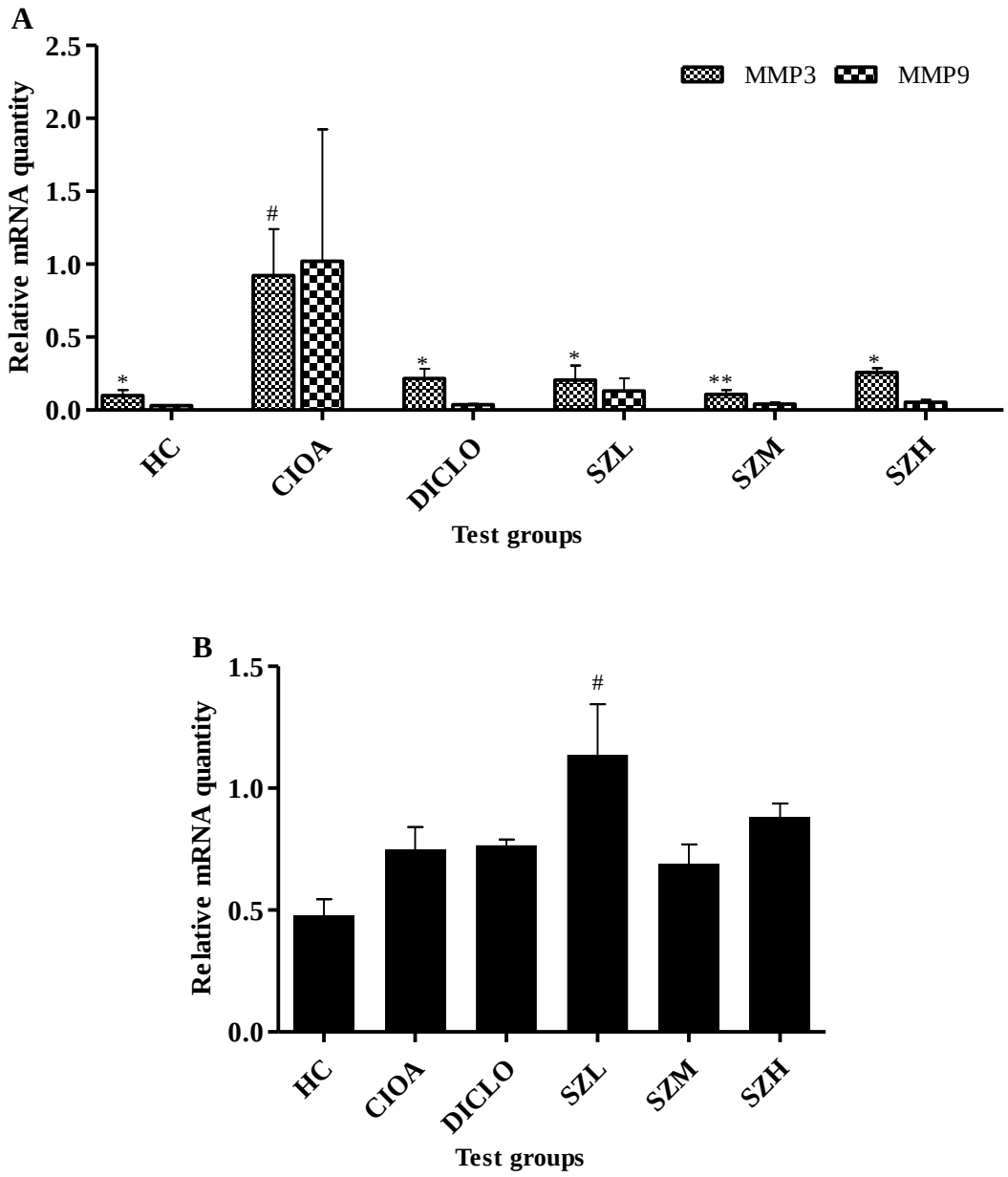

Fig 42: Effect of SZL, SZM and SZH on the mRNA expression of A) MMP3, MMP9 and B) TIMP in the synovium of rats.

Values are expressed as Mean ± SEM (n=3). Comparisons were done between OAC/HC and each individual treated group by Dunnett's multiple comparison test. $^{*}p<0.05$; $^{**}p<0.01$ Compared to OAC. $^{\#}p<0.05$ compared to HC. HC: Healthy control, OAC: Osteoarthritic control, DICLO: Positive control, SZL: SZ at 135 mg/kg b. wt., SZM: SZ at 270 mg/kg b. wt., SZH: SZ at 540 mg/kg b. wt.

5.4.2 Toxicity studies of prioritized new combination formulation, SZ

5.4.2.1 Acute toxicity tests

Oral administration of SZ did not produce any mortality or toxicity upto a dose level of 2000 mg/kg b. wt. Therefore, SZ is safe upto 2000 mg/kg and the approximate lethal dose (LD50) of SZ in the experimental rats was higher than 2000 mg/kg.

5.4.2.2 Sub-acute toxicity tests (Repeated dose 28 day oral toxicity study)

5.4.2.2.1 Effect of SZ on body weight

The rats treated with test SZ at 135, 270 and 540 mg/kg b. wt. doses, found to grow and gain body weight normally and there was no treatment related change found in the body weight gains (Table 18). Rats also showed significant increase in body weight compared to initial values (0^{th} day). However, there was no significant difference in body weight gain noted between the control group and treated groups, indicating no adverse effect of SZ on body weight.

5.4.2.2.2 Effect of SZ on food consumption

Changes in food intake of all rats are presented in Table 19. The food intake of treated group rats at different time points were compared with control group rats. All reversal groups are compared with control reversal group. No abnormal deviations were observed in the food consumption of all animals.

5.4.2.2.3 Effect of SZ on organ weights of animals

The macroscopic analysis of the treated animals (Brain, Liver, Kidney, Adrenals, Heart, Thymus, Spleen, Testis, Uterus, Ovaries and Lungs) did not show significant changes in colour, texture and weight compared with the control group. The results of organ weights are summarized in Table 20 and 21. This indicated that oral administration of SZ did not detrimentally affect the colour, texture and weight of organs. Except for epidydemis that increased significantly for SZ-L ($p<0.05$) and SZ-M ($p<0.05$) and SZ-REV ($p<0.001$) compared to their respective control.

5.4.2.2.4 Effect of SZ on hematological parameters

Hematological parameters of male and female rats were examined as presented in Table 22, 23, 24 and 25. The hemoglobin content has shown no significant changes in the treated groups as compared to the control groups. In all of the treated groups, the Haemoglobin, Red Blood Cell (RBC) count, Hematocrit contents, Mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), WBC, Neutrophils, Lymphocytes, Monocytes, Platelet count have shown no significant changes in comparison with that of the control groups. Except for Eosinophils that decreased significantly in SZ-REV ($p<0.05$) in males and in SZ-L ($p<0.001$) in females, compared to their respective controls.

5.4.2.2.5 Effect of SZ on serum biochemical parameters

Results of clinical biochemistry examinations are shown in Table 26 and 27. There were no significant changes in enzymes ALP, SGOT, SGPT, direct and total bilirubin and uric acid content of the SZ treated group compared with respective control group animals. However, significant increase in the Urea content of SZ-M ($p<0.001$) and SZ-H ($p<0.001$) male were observed in comparison with the control group animals.

5.4.2.2.6 Effect of SZ on organs

The gross macroscopic examinations of internal organs of treated groups did not show any morphological difference when compared with the control group animals. The shape, size and texture of these collected organs of treated rats were found to be normal.
Figure 43 represents the normal histopathological and cytological structure of liver, brain, lungs and kidney. The histopathological photographs have shown that no toxic effects of SZ were detected in organs of treated animals. The results of the histopathological studies provided evidence for supporting the findings of the biochemical analysis. No lesions, inflammation or pathological changes related to treatment with SZ were observed in the organs of the animals from the treatment groups compared to the untreated group. In general, the treatment-related results were very similar to those of the control group.

Table 18: Effect of SZ on body weight.

Test group	Body weight (gm)						Body weight changes (gm)			
	0th day		29th day		43rd day		29th day		43rd day	
	M	F	M	F	M	F	M	F	M	F
C	103±8.44	110±4.92	230±15.9	165±12.9	-	-	127±15.3	55±14.7	-	-
C-REV	101±7.89	128±5.25	228±4.21	197±16.1	249±7.03	216±23.2	127±6.89	68±14.3	149±9.48	86±20.7
SZ-L	158±2.42	139±4.86	272±13[a]	210±7.14[aaa]	-	-	114±12.4	71±3.7	-	-
SZ-M	161±2.90	138±4.81	272±8.64[a]	203±9.8 [aa]	-	-	111±7.22	64±8.03	-	-
SZ-H	161±3.42	143±3.57	261±9.53	201±5.42[a]	-	-	99.3±9.07	58±4.9	-	-
SZ-REV	164±3.76	140±7.02	306±14.3[aaa]	210±6.43	313±12.2[aaa]	219±5.93[aaa]	142±18	70±3.06	148±16	79±2.67

Values are expressed as mean ± SEM (n=5) using one way ANOVA followed by Dunnett's multiple comparison test. [a]= $p<0.05$, [aa]= $p<0.01$, [aaa]= $p<0.001$ when compared to 0th day. All reversal groups are compared with Control reversal group. C: Control, C-REV: Control Reversal, SZ-L: SZ at 135 mg/kg b. wt., SZ-M: SZ at 270 mg/kg b. wt., SZ-H: SZ at 540 mg/kg b. wt., SZ-REV: SZ Reversal. M: Male, F: Female.

Table 19: Effect of SZ on food consumption.

Test groups	Food consumption (gm)											
	1st week		2nd week		3rd week		4th week		5th week		6th week	
	M	F	M	F	M	F	M	F	M	F	M	F
C	18±0.6	16.1±0.4	30.7±0.2	25.2±0.2	31.7±0.4	25.8±0.3	31.3±0.7	13.2±0.8	-	-	-	-
C-REV	18.1±0.5	18.3±0.5	28.5±0.2	26±0.3	28.8±0.3	23.7±0.6	27.8±0.6	23.5±1	29±0.4	21.6±0.2	30.3±0.2	22.7±0.2
SZ-L	25±0.2^{aaa}	19.3±0.3	27.2±1.6	20.8±1.5^{aa}	24.2±0.5^{aaa}	19.2±0.2^{aaa}	23.3±0.8^{aaa}	19.3±0.6^{aa}	-	-	-	-
SZ-M	24.6±0.4^{aaa}	18.1±0.4	26.3±2.0	19.3±1^{aaa}	22.8±0.3^{aaa}	18.2±0.5^{aaa}	22.2±0.3^{aaa}	18.3±0.7^{aaa}	-	-	-	-
SZ-H	23.1±0.4^{aaa}	19.1±0.3	23.7±1.1^{aa}	17.5±1.1^{aaa}	21.8±0.6^{aaa}	17.8±0.5^{aaa}	22±0.6^{aaa}	18.2±0.6^{aaa}	-	-	-	-
SZ-REV	23.7±0.4^{aaa}	21.1±2.7	27.2±1.2	18.3±0.6^{aaa}	26.3±0.7^{aa}	17.5±2.2^{aaa}	25.8±0.4	16.7±0.7^{aaa}	26.7±0.2^{aaa}	16±0^{aaa}	27.7±0.4^{aaa}	16.7±0.2^{aaa}

Values are expressed as mean ± SEM (n=5) using one way ANOVA followed by Dunnett's multiple comparison test. ^{aa}= $p<0.01$, ^{aaa}= $p<0.001$ when compared to Control. All reversal groups are compared with Control reversal group. C: Control, C-REV: Control Reversal, SZ-L: SZ at 135 mg/kg b. wt., SZ-M: SZ at 270 mg/kg b. wt., SZ-H: SZ at 540 mg/kg b. wt., SZ-REV: SZ Reversal. M: Male, F: Female.

Table 20: Effect of SZ on weights of Brain, Liver, Kidney, Adrenals, Heart, Thymus.

Test group	Weight (gm)											
	Brain		Liver		Kidney		Adrenals		Heart		Thymus	
	M	F	M	F	M	F	M	F	M	F	M	F
C	1.7±0.08	1.7±0.06	9.3±0.82	6.7±0.55	1.8±0.14	1.4±0.12	0.04±0.004	0.05±0.003	0.9±0.08	0.7±0.05	0.5±0.06	0.4±0.07
C-REV	1.7±0.17	1.9±0.08	10±0.25	8.8±1.3	1.8±0.03	1.5±0.17	0.04±0.002	0.07±0.002	0.9±0.05	0.8±0.06	0.4±0.02	0.5±0.06
SZ-L	1.9±0.03	1.8±0.04	10±1.15	8.6±0.61	1.9±0.14	1.5±0.06	0.05±0.004	0.05±0.002	1.1±0.08	0.7±0.03	0.5±0.04	0.5±0.03
SZ-M	1.9±0.02	1.9±0.03	10±0.87	8.5±0.78	1.9±0.05	1.4±0.08	0.05±0.001	0.06±0.002	0.9±0.05	0.8±0.03	0.5±0.07	0.5±0.03
SZ-H	1.9±0.03	1.8±0.04	9.7±0.64	7.9±0.48	1.8±0.1	1.4±0.03	0.05±0.005	0.06±0.002	0.9±0.02	0.7±0.01	0.4±0.04	0.4±0.05
SZ-REV	1.9±0.04	1.9±0.05	14±0.34	9.7±0.56	2.2±0.14	1.8±0.1	0.05±0.003	0.07±0.01	1±0.01	0.8±0.02	0.5±0.03	0.4±0.02

Values are expressed as mean ± SEM (n=5). C: Control, C-REV: Control Reversal, SZ-L: SZ at 135 mg/kg b. wt., SZ-M: SZ at 270 mg/kg b. wt., SZ-H: SZ at 540 mg/kg b. wt., SZ-REV: SZ Reversal. M: Male, F: Female.

Table 21: Effect of SZ on organ weights of Spleen, Testis, Uterus, Epididymis, Ovaries and Lungs.

Test group	Weight (gm)							
	Spleen		Testis	Uterus	Epididymis	Ovaries	Lungs	
	M	F	M	F	M	F	M	F
C	1.4±0.15	1.5±0.2	2.6±0.16	0.36±0.14	0.6±0.04	0.08±0.01	1.8±0.14	1.3±0.09
C-REV	1.6±0.1	1.5±0.4	2.7±0.03	0.40±0.01	0.81±0.02	0.09±0.01	1.7±0.09	1.6±0.13
SZ-L	1.7±0.2	1.5±0.1	2.9±0.09	0.54±0.09	0.79±0.05[a]	0.08±0.01	2.0±0.11	1.5±0.05
SZ-M	1.7±0.1	1.3±0.1	2.8±0.13	0.3±0.04	0.77±0.02[a]	0.08±0.01	2.1±0.13	1.6±0.12
SZ-H	1.8±0.2	1.4±0.1	2.7±0.12	0.26±0.03	0.75±0.06	0.07±0.01	1.8±0.11	1.6±0.07
SZ-REV	1.8±0.06	1.4±0.1	3.1±0.06	0.48±0.04	1.11±0.03[aaa]	0.1±0.01	2.2±0.17	1.9±0.03

Values are expressed as mean ± SEM (n=5) using one way ANOVA followed by Dunnett's multiple comparison test. [a]= $p<0.05$, [aaa]= $p<0.001$when compared to Control. All reversal groups are compared with Control reversal group. C: Control, C-REV: Control Reversal, SZ-L: SZ at 135 mg/kg b. wt., SZ-M: SZ at 270 mg/kg b. wt., SZ-H: SZ at 540 mg/kg b. wt., SZ-REV: SZ Reversal. M: Male, F: Female.

Table 22: Effect of SZ on Haemoglobin, RBC and Hemocrit.

Test group	Haemoglobin (gm%)		RBC (10^6/µl)		Hemocrit (%)	
	Male	Female	Male	Female	Male	Female
C	15.1±0.65	14.5±0.25	7.86±0.19	7.32±0.14	43.1±3.45	39.9±0.60
C-REV	14.1±0.05	13.0±0.80	8.12±0.04	7.07±0.13	38.1±0.30	35.4±1.00
SZ-L	15.3±0.75	14.8±0.75	8.13±0.89	7.61±0.68	40.5±3.15	37.8±2.55
SZ-M	14.7±0.85	15.1±0.50	8.03±0.45	7.98±0.34	40.0±1.90	39.1±1.30
SZ-H	15.1±0.20	15.6±0.20	8.19±0.10	7.98±0.36	40.9±0.65	39.5±1.30
SZ-REV	14.5±0.30	14.3±0.15	8.77±0.11	8.24±0.16	39.3±0.35	37.8±0.70

Values are expressed as mean ± SEM (n=5). C: Control, C-REV: Control Reversal, SZ-L: SZ at 135 mg/kg b. wt., SZ-M: SZ at 270 mg/kg b. wt., SZ-H: SZ at 540 mg/kg b. wt., SZ-REV: SZ Reversal.

Table 23: Effect of SZ on MCV, MCH and WBC.

Test group	MCV (fl)		MCH (pg)		WBC total count (*10^3/cumm)	
	Male	Female	Male	Female	Male	Female
C	54.7±3.03	54.5±1.87	19.2±0.35	19.8±0.72	12170±4630	26750±6450
C-REV	46.9±0.60	50.1±0.46	17.3±0.20	18.4±0.78	22750±4750	16800±5300
SZ-L	49.9±1.59	49.7±1.13	18.9±1.15	19.5±0.76	25100±6600	20250±3150
SZ-M	49.8±0.42	49.0±0.46	18.2±0.03	18.9±0.18	21500±6600	18750±5450
SZ-H	49.9±0.18	49.6±0.64	18.4±0.47	19.6±0.65	23150±3650	21300±2100
SZ-REV	44.8±0.16	45.9±1.74	16.5±0.13	17.3±0.51	28150±1850	15850±5250

Values are expressed as mean ± SEM (n=5). C: Control, C-REV: Control Reversal, SZ-L: SZ at 135 mg/kg b. wt., SZ-M: SZ at 270 mg/kg b. wt., SZ-H: SZ at 540 mg/kg b. wt., SZ-REV: SZ Reversal.

Table 24: Effect of SZ on Neutrophils, Lymphocytes and Eosinophils.

Test group	Neutrophils (%)		Lymphocytes (%)		Eosinophils (%)	
	Male	Female	Male	Female	Male	Female
C	22.5±6.50	9.50±4.50	65.0±0.0	81.0±4.0	4.5±0.5	6.0±0.0
C-REV	13.0±7.00	9.00±0.00	76.5±6.5	79.0±1.0	6.0±0.0	6.0±0.0
SZ-L	14.0±8.00	8.50±2.50	77.5±6.5	82.0±2.0	3.0±1.0	2.5 ± 0.5^{aaa}
SZ-M	8.00±5.00	17.5±2.50	81.5±6.5	69.5±3.5	3.0±1.0	5.5±0.5
SZ-H	15.5±1.50	11.5±2.50	76.5±0.5	77.5±1.5	2.5±0.5	6.0±0.0
SZ-REV	22.0±2.00	8.50±0.50	72.5±2.5	80.0±1.0	2.0 ± 1.0^{a}	5.0±0.0

Values are expressed as mean ± SEM (n=5) Data were analysed by one way ANOVA followed by Dunnett's multiple comparison test. ^{a}= $p<0.05$, ^{aaa}= $p<0.001$ when compared to Control. All reversal groups are compared with Control reversal group. C: Control, C-REV: Control Reversal, SZ-L: SZ at 135 mg/kg b. wt., SZ-M: SZ at 270 mg/kg b. wt., SZ-H: SZ at 540 mg/kg b. wt., SZ-REV: SZ Reversal.

Table 25: Effect of SZ on Monocytes and Platelet count.

Test group	Monocytes (%)		Platelet count (*10^4/cumm)	
	Male	Female	Male	Female
C	8.0±6.0	3.5±0.5	739000±139000	763500±87500
C-REV	4.5±0.5	6.0±1.0	710000±103000	629500±87500
SZ-L	5.5±0.5	7.0±1.0	822500±40500	566000±32000
SZ-M	7.5±0.5	7.5±0.5	572500±30500	567000±5000
SZ-H	5.5±0.5	5.0±1.0	674500±8500	738500±21500
SZ-REV	3.5±1.5	6.5±1.5	659500±41500	538000±33000

Values are expressed as mean ± SEM (n=5). C: Control, C-REV: Control Reversal, SZ-L: SZ at 135 mg/kg b. wt., SZ-M: SZ at 270 mg/kg b. wt., SZ-H: SZ at 540 mg/kg b. wt., SZ-REV: SZ Reversal.

Table 26: Effect of SZ on ALP, SGOT and SGPT.

Test group	ALP (K.A. unit)		SGOT (U/ml)		SGPT (U/ml)	
	Male	**Female**	**Male**	**Female**	**Male**	**Female**
C	13.3±2.90	14.9±2.48	131±12.0	141±8.82	37.4±5.64	34.2±7.24
C-REV	24.2±4.71	20.9±1.26	107±8.80	131±6.53	54.3±4.16	65.9±8.03
SZ-L	8.96±0.81	10.0±1.44	136±16.1	135±6.68	22.0±6.26	22.4±6.38
SZ-M	13.8±2.38	9.48±2.14	76.9±36.4	122±19.0	23.7±2.38	33.3±9.43
SZ-H	12.3±0.94	9.63±0.67	118±3.63	122±1.66	52.6±8.10	45.6±12.9
SZ-REV	15.3±4.03	17.5±1.70	96.0±11.1	93.8±3.49	62.9±9.61	54.6±6.43

Values are expressed as mean ± SEM (n=5). All reversal groups are compared with Control reversal group. C: Control, C-REV: Control Reversal, SZ-L: SZ at 135 mg/kg b. wt., SZ-M: SZ at 270 mg/kg b. wt., SZ-H: SZ at 540 mg/kg b. wt., SZ-REV: SZ Reversal.

Table 27: Effect of SZ on Direct Bilirubin, Total Bilirubin, Uric acid and Urea.

Test group	Direct Bilirubin (mg/dl)		Total Bilirubin (mg/L)		Uric acid (mg/dl)		Urea (mg/dl)	
	Male	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**
C	0.156±0.065	0.068±0.04	1.47±0.01	1.49±0.008	3.5±0.3	3.2±0.25	45.5±4	50.6±3.43
C-REV	0.088±0.03	0.104±0.04	1.53±0.05	1.25±0.42	3.05±0.12	3.6±0.2	62.1±0.7	59±3.22
SZ-L	0.065±0.013	0.182±0.166	1.55±0.12	1.54±0.1	3.13±0.28	2.69±0.08	42.5±0.66	57.1±4.04
SZ-M	1.07±1.04	0.177±0.104	1.6±0.04	1.52±0.04	2.9±0.17	4.05±0.05	62.7±1.39^{aaa}	61.6±1.52
SZ-H	0.507±0.351	0.113±0.035	1.47±0.15	1.34±0.06	3.55±0.15	3.97±0.27	64.4±2.45^{aaa}	59.7±4.79
SZ-REV	0.242±0.122	0.009±0.07	1.5±0.12	1.66±0.04	3.55±0.13	3.47±0.38	64.8±0.36	58.3±0.8

Values are expressed as mean ± SEM (n=5) using one way ANOVA followed by Dunnett's multiple comparison test. ^{aaa}= $p<0.001$ when compared to Control. All reversal groups are compared with Control reversal group. C: Control, C-REV: Control Reversal, SZ-L: SZ at 135 mg/kg b. wt., SZ-M: SZ at 270 mg/kg b. wt., SZ-H: SZ at 540 mg/kg b. wt., SZ-REV: SZ Reversal.

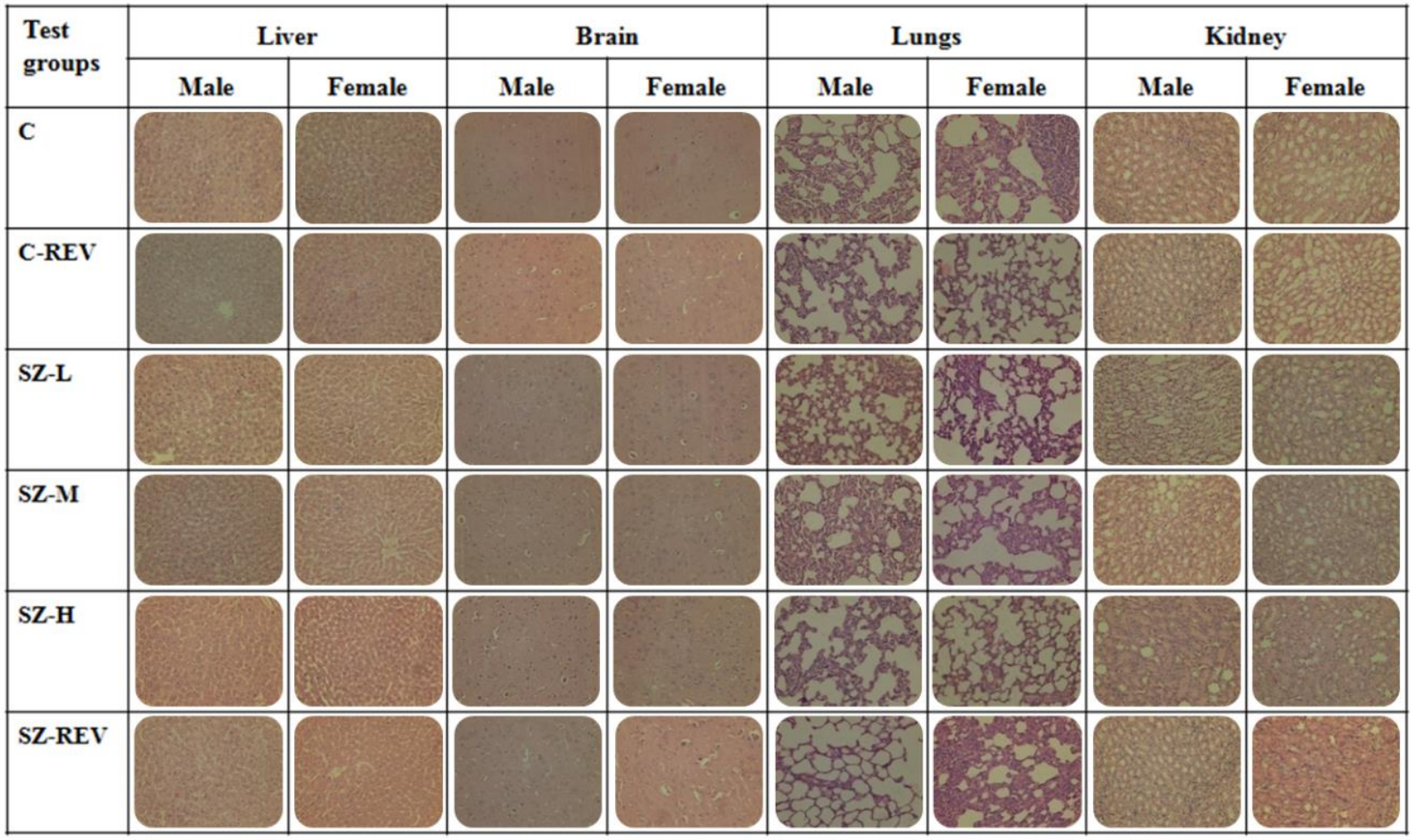

Figure 43: Histopathology images of liver, brain, lungs and kidney.

C: Control, C-REV: Control Reversal, SZ-L: SZ at 135 mg/kg b. wt., SZ-M: SZ at 270 mg/kg b. wt., SZ-H: SZ at 540 mg/kg b. wt., SZ-REV: SZ Reversal.

5.5 To evaluate anti-arthritic effects of new combination formulation using HIG-82, rabbit synoviocytes

5.5.1 Cell toxicity using MTT assay

As shown in figure (Fig. 44), synoviocytes viability showed that SZ had no effect on the viability of cells at the concentrations of 0-80 μg/ml.

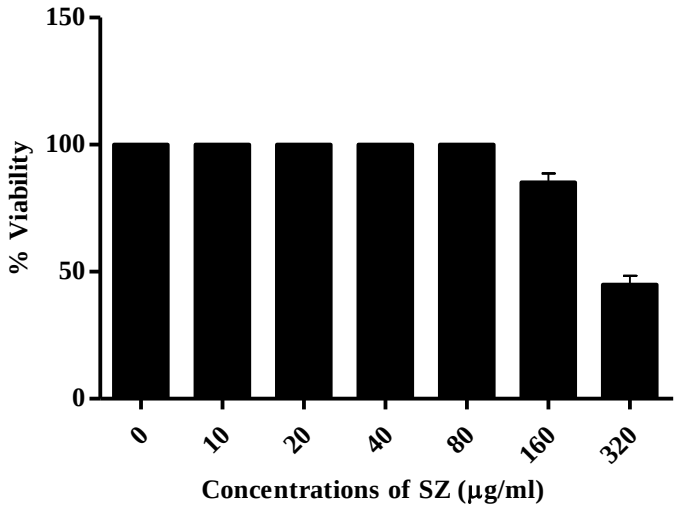

Figure 44: Effect of SZ on the cytotoxicity.

Values are expressed as Mean ± SEM.

5.5.2 LPS-stimulated rabbit synoviocytes sarcoma model

5.5.2.1 Determination of Nitric Oxide (NO) levels

The effect of SZ on NO production in LPS-stimulated synoviocytes is shown in (Fig. 45). LPS significantly increased NO production (114 ± 3.67 mM/ml) in synoviocytes compare to untreated cells (24.59 ± 9.603 mM/ml). In the presence of LPS, SZ at the concentration of 80 μg/ml significantly ($p<0.05$) reduced NO production (37.39 ± 18.26 mM/ml). SZ at the 20 and 160 μg/ml concentration showed 64.995 ± 15.846 and 85.68 ± 28.03 mM/ml NO levels (Fig. 45).

5.5.2.2 Determination of Superoxide dismutase (SOD) activity

As shown in figure (Fig. 46), the activity of SOD was significantly ($p<0.01$) decreased by LPS, with untreated cells having 48.5 ± 3.54, % activity while LPS-only cells had 30 ± 1.41, % activity. After addition of SZ, the activity of SOD was increased

i.e. 34.5 ± 4.95, 35.5 ± 2.12 and 28.5 ± 0.71 % with the addition of 20, 80 and 160 µg/ml SZ, respectively (Fig. 46).

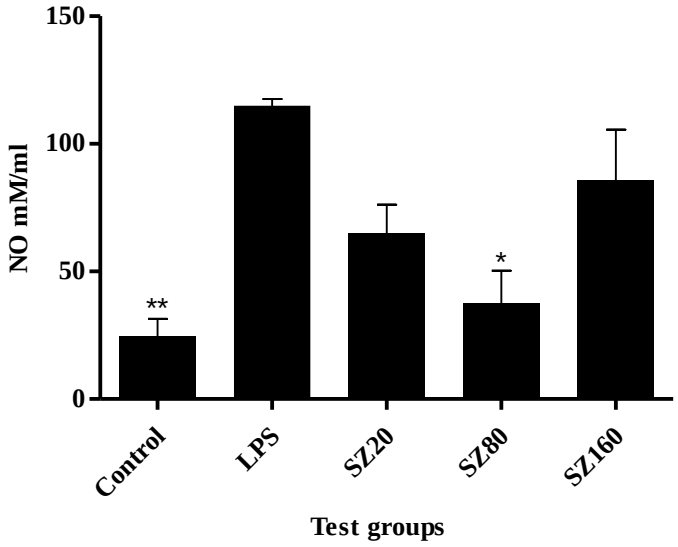

Figure 45: Effect of SZ on the level of NO in LPS-stimulated rabbit fibroblast like synoviocytes, HIG-82.

Values are expressed as Mean ± SEM. $^{*}p<0.05$, $^{**}p<0.01$ compared to LPS. SZ20: SZ at 20µg/ml, SZ80: SZ at 80µg/ml, SZ160: SZ at 160µg/ml.

5.5.2.3 Determination of Catalase activity

As shown in figure (Fig. 47), the activity of catalase was decreased by LPS (5.083 ± 0.105 nmol/min/ml), with untreated cells having 8.015 ± 0.597 nmol/min/ml. After addition of 80 and 160 µg/ml SZ the activity of SOD was increased i.e. 5.155 ± 0.078 and 7.162 ± 4.327 nmol/min/ml, respectively (Fig. 47).

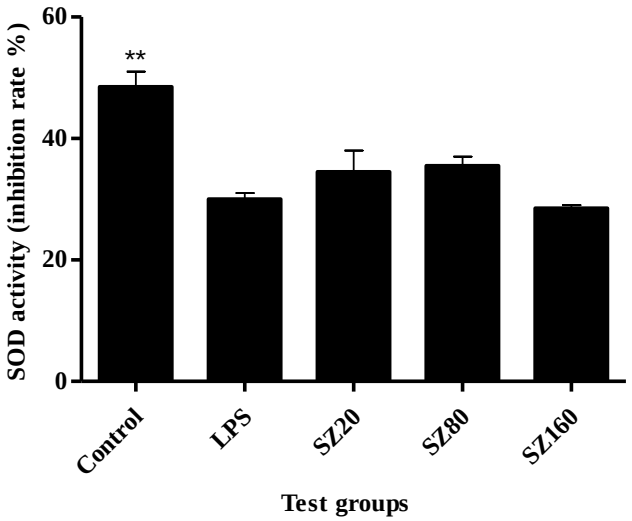

Figure 46: Effect of SZ on SOD activity in LPS-stimulated rabbit fibroblast like synoviocytes, HIG-82.

Values are expressed as Mean ± SEM. $^{**}p<0.01$ compared to LPS. SZ20: SZ at 20µg/ml, SZ80: SZ at 80µg/ml, SZ160: SZ at 160µg/ml.

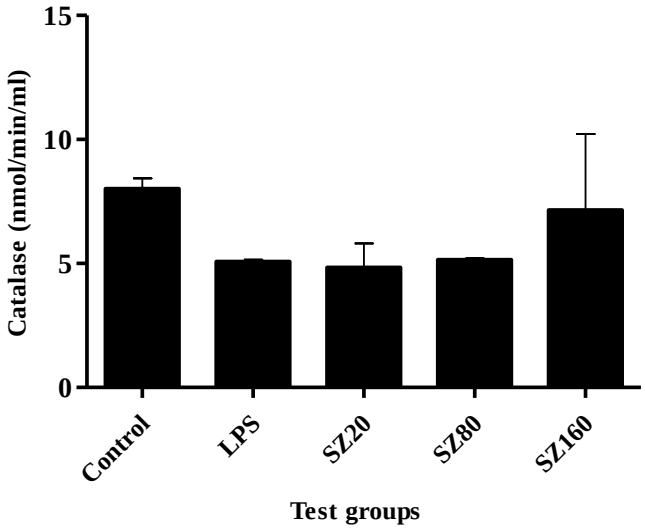

Figure 47: Effect of SZ on catalase activity in LPS-stimulated rabbit fibroblast like synoviocytes, HIG-82.

Values are expressed as Mean ± SEM. SZ20: SZ at 20µg/ml, SZ80: SZ at 80µg/ml, SZ160: SZ at 160µg/ml.

5.5.2.4 Estimation of Matrix metalloproteinase-3 (MMP-3) levels

As shown in figure (Fig. 48) MMP levels were significantly increased in LPS treated cells (1.07 ± 0.147 ng/ml) compared to untreated cells (0.066 ± 0.0 ng/ml). SZ20, SZ80 and SZ160 also significantly ($p<0.001$) decreased MMP-3 levels in LPS-stimulated synoviocytes (Fig. 48).

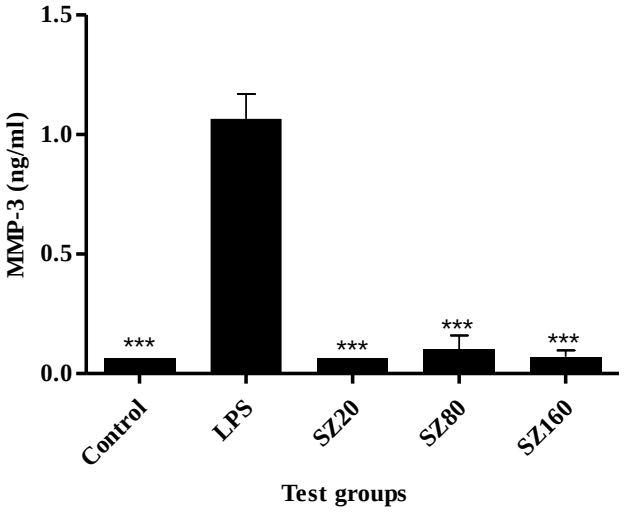

Figure 48: Effect of SZ on the level of matrix metalloproteinase 3 (MMP-3) in LPS-stimulated rabbit fibroblast like synoviocytes, HIG-82.

Values are expressed as Mean ± SEM. $^{***}p<0.001$ compared to LPS. SZ20: SZ at 20µg/ml, SZ80: SZ at 80µg/ml, SZ160: SZ at 160µg/ml.

5.5.2.5 Estimation of Matrix metalloproteinase-9 (MMP-9) levels

As shown in figure (Fig. 49) SZ80 (0.492 ± 0.116 ng/ml) and SZ160 (0.114 ± 0.042 ng/ml) was significantly ($p<0.001$) reduced MMP-9 levels compare to LPS-stimulated cells (1.15 ± 0.012 ng/ml) (Fig. 49).

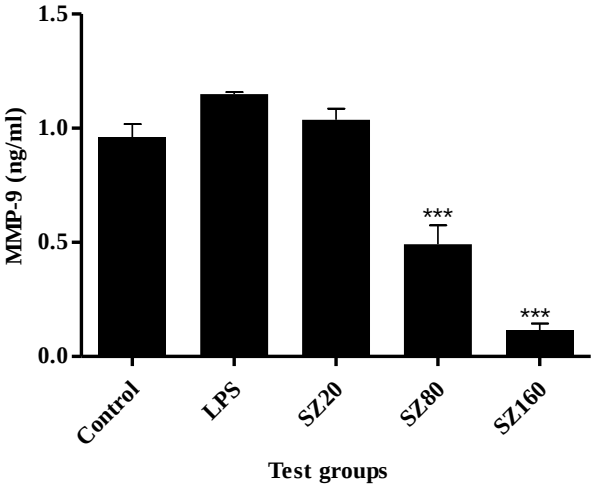

Figure 49: Effect of SZ on the level of matrix metalloproteinase 9 (MMP-9) in LPS-stimulated rabbit fibroblast like synoviocytes, HIG-82.

Values are expressed as Mean ± SEM. $^{***}p<0.001$ compared to LPS. SZ20: SZ at 20µg/ml, SZ80: SZ at 80µg/ml, SZ160: SZ at 160µg/ml.

Discussion

In elderly population, osteoarthritis (OA) of knee is a major cause of pain and disability throughout the world. It involves progressive degeneration of articular cartilage, synovitis, formation of osteophytes, loss of collagen fibers and fibrillation, due to inflamed knee joints (Patel et al., 2015). Since degeneration of cartilage is the result of biochemical and mechanical factors, modulation of such factors viz. MMP-TIMP may provide an important therapeutic approach for its treatment (Brown et al., 2008).

Current therapeutic options fail to block progression of OA and are also associated with many adverse events. Therefore, there is an increasing interest in use of compounds derived from natural sources for the treatment of OA, as they have shown clinical efficacy with minimal side effects, as compared to routine pharmacological strategies (Wu et al., 2014).

A layer of type II collagen in extracellular matrix (ECM) of cartilage imparts higher stability to articular cartilage in a typical healthy joint. Animal model of OA involves intra-articular injection of collagenase that degrades the collagen and Proteoglycans (PGs) of the ECM to produce cartilage damage as well as increases cellular infiltration in synovium and thus mimics human OA. This damage leads to release of certain degradation products, which are responsible for induction of inflammation in the joint (Yeh et al., 2008). PGs consist of a protein core with GAG side chains. During inflammation and cartilage degradation, GAG monomers are released into the surrounding fluid serving as the clinical symptom of OA (Kim et al., 2012). Yeh et al. (2008) demonstrated that intra-articular administration of collagenase, induced osteoarthritic changes, including degradation of cartilage, synovial inflammation, remodelling of subchondral bone and osteophyte formation in the facet joints of rats. Moreover, physical parameters like knee diameter, paw volume and paw withdrawal latency are easy to access therapeutic effects of drug on inflammation in OA (Bansod et al., 2011).

Traditionally, various herbs are used as a source of therapeutic compounds. The chemical substances present in these herbs are thought to act on targeted disease in human body. Ample studies from the literature have reported how individual component of plants may be the active compound in amelioration of various disease conditions including OA

(Fouda and Berika, 2009). In the present thesis work, we have used the whole extract of plant parts as it contains mixtures of compounds which are often more active.

6.1 Most potent anti-osteoarthritic plants in CIOA rat model

To begin with, six herbs were tested for their comparative anti-osteoarthritic potential using various physiological, biochemical, radiology and histopathology studies. Standard drug, indomethacin inhibits COX-2 expression, which results in prolonged weight loss (Granado et al., 2007), our results endorse this assumption as indomethacin caused decreased body weight (Fig. 17). Absorption of 14C-glucose and 14C-leucine in rat's intestine was reported to be reduced due to inflammation (Chaudhari et al., 2012). Similarly poor absorption of nutrients may have resulted in weight loss in the study animals. Concomitantly, systemic action of cytokines may also be attributed for systemic inflammation leading to poor uptake of nutrients from inflamed gut and stress caused from their action (Kabra et al., 2011). This weight loss can be prevented by selected herb treatment (Caceres et al., 2000). Increased body weight during treatment of herb's may be due to the restoration of intestinal inflammation, improved absorption through intestine, and resolution of stress (Rajendran and Krishnakumar, 2010).

Among the test groups, oral administration of *S. cordifolia* significantly decreased knee swelling ($p<0.0001$) and paw swelling, compared to osteoarthritic control group. *S. cordifolia* also significantly prevented GAG release ($p<0.01$), suggesting cartilage protection which was also confirmed by radiology. H and E staining showed that *S. cordifolia* protected synovium during OA. According to previous reports, this bioactivity of *S. cordifolia* may be due to the inhibition of COX enzyme leading to inhibition of prostaglandin synthesis (Anilkumar, 2010).

P. longum demonstrated superior protection against knee swelling ($p<0.0001$) compared to all herbs, which is in accordance with the study reported anti-inflammatory action (Chauhan et al., 2011). *P. longum* also plays a role in inhibiting paw swelling and cartilage protection, as reduced GAG release was observed along with recovery in joint space. Modification of ECM and increased articular chondrocyte proliferation are characteristics of OA (Dealy, 2012). In the present study, *P. longum* protected chondroid matrix. This anti-osteoarthritic activity of *P. longum* may be due to piperine, amide

isolated from piper species, which is demonstrated using synoviocytes (Bang et al., 2009).

Z. officinale decreased knee swelling significantly ($p<0.05$) as well as paw edema, as compared to osteoarthritic control. It also significantly prevented loss of PG as reflected from lowered GAG release ($p<0.05$). Based on the previous reports, this action of *Z. officinale* may be due to its action of decreasing IL1, NO, PGE2 and LTB4 production in osteoarthritic cartilage (Shen et al., 2003, Anilkumar, 2010). Histopathology revealed that *Z. officinale* protected cartilage and prevented chondrocyte degeneration, which may be explained by inhibition of TNF-α. It is reported in literature that *Z. officinale* inhibited TNF-α production via inhibition of gene expression in human osteoarthritic synoviocytes and chondrocytes (Setty and Sigal, 2005).

Treatment with *R. communis* significantly reduced knee swelling ($p<0.0001$) as well as paw edema. This action may be due to presence of flavonoids, which are reported for their anti-inflammatory and anti-arthritic action (Anilkumar, 2010). *R. communis* prevented GAG release from osteoarthritic cartilage ($p<0.01$) and also protected chondrocytes and cartilage surface degradation.

V. negundo reduced knee swelling significantly ($p<0.0001$). It also showed highest reduction of paw edema compared to other herbs studied, supporting its therapeutic use in OA. *V. negundo* showed maximum efficacy in cartilage protection ($p<0.001$) on comparison with all herbs by preventing GAG release. This activity is possibly due to presence of agnuside, a compound isolated from the leaves of *V. negundo* which suppresses inflammatory mediators and T-cell-mediated cytokines (Pandey et al., 2012). Previous studies also support the action of *V. negundo* as its leaves possess anti-arthritic activity with evidence of inhibition for secretory PLA2 (Tandon, 2005; Vyawahare et al., 2008; Vinuchakkaravarthy et al., 2011). Although, *V. negundo* has demonstrated highest activity against some parameters, it has not shown any protection for cartilage and chondrocyte matrix degeneration.

T. terrestris significantly reduced knee swelling ($p<0.0001$) as well as paw edema. This activity of *T. terrestris* may be due to its COX-2 inhibition potential (Srivastava et al., 2005), *T. terrestris* also exhibited utmost activity in preventing GAG release ($p<0.001$)

from cartilage, compared to all herbs but it failed to protect chondrocyte and cartilage degradation as observed in radiography & histopathology studies.

Thus from the studied parameters, *S. cordifolia*, *P. longum*, followed by *Z. officinale* has shown highest anti-osteoarthritic potential on different parameters and merits further investigation. Hence *S. cordifolia* and *P. longum* were further evaluated for their mechanism of action using qPCR study of treated rat synovium tissue using pertinent OA biomarkers.

6.1.1 *S. cordifolia* and *P. longum* works through modulating levels of anti-oxidants and MMPs-TIMP

The antioxidant capacity of phenolic compounds possesses ability to scavenge radicals thereby protecting cells against oxidative stress (Ndhlala et al., 2010). In OA, oxidative stress leads to structural and functional damage to chondrocytes as well as cartilage degradation via aggrecan and collagen (Aydogan et al., 2008; Watari et al., 2011). Release of Nitric oxide (NO) and superoxide anion ($O2^-$) are the stress responses produced by chondrocytes (Henrotin et al., 2003). Interestingly, our results showed that inherent anti-oxidant defence was strengthened after oral administration of *S. cordifolia* and *P. longum*. Treatment with *S. cordifolia*, *P. longum* and indomethacin up-regulated SOD, CAT, GPx and PON-1 expression in synovium of osteoarthritic knee joint, the major anti-oxidant enzymes against ROS (Anu et al., 2013), thus protecting tissue from deleterious effects of free radicals. This difference however was not statistically significant due to high standard deviation in our study.

In OA, cartilage cumulative oxidative stress induces hypoxia-inducible factor 1-α in chondrocytes and cartilage, leading to over expression of interleukin 1β, tumor necrosis factor alpha (Kulkarni et al., 2014), which accelerates cartilage degeneration (Alcaraz et al., 2010). SOD protects the tissue by catalyzing transformation of superoxide; CAT and GPx detoxifies H_2O_2 (Aydogan et al., 2008). PON1 is a high-density lipoprotein (HDL)-associated enzyme and found to plays a protective role against lipid peroxidation of low-density lipoprotein (LDL) as well as HDL; activity of PON1 is found to decreases in OA (Aydin et al., 2012). Free radical scavengers and oxidative defense genes, including genes for superoxide dismutase (SOD) 2, SOD 3 and glutathione peroxidase 3 (GPx)

have been identified as potential therapeutic agents for the protection of articular cartilage against progression of OA (Alcaraz et al., 2010). Thus, administration of *S. cordifolia* and *P. longum* has upregulated anti-oxidant enzyme expression knee synovial tissue.

ROS contributes to cartilage degradation through activating latent collagenase and by up-regulating the expression of genes coding matrix metalloproteinases (MMPs) (Henrotin et al., 2003). MMPs derived from chondrocytes, synovium and polymorphonuclear leukocytes have been proposed to play a major role in cartilage degradation in OA (Alam et al., 2011). In our results, *P. longum* has significantly ($p=0.0361$) downregulated levels of MMP3. MMP-3 is the key enzyme involved in degradation of non-fibrillar collagen and other components like PGs of the ECM in cartilage (Sandya et al., 2009). It has been also reported that increased levels of MMP-3 shift the balance towards catabolic side and leads to cartilage damage (Huh et al., 2008). MMP mediated cartilage damage is initiated by synovial macrophage activation. These macrophages produce pro-MMP-9, which is activated via MMP-3 or MMP-13 pathway to active MMP-9 (Sellam and Berenbaum, 2010). *S. cordifolia* has effectively downregulated both MMPs compared to osteoarthritic control rats. Increased levels of MMPs compared to tissue inhibitors of metalloproteinases (TIMPs), is closely associated with degradation of ECM of joint tissues in OA (Yoshihara et al., 2000). TIMP-1 has greater effectiveness in the inhibition of MMP-1, -3 and -9 (Lee et al., 2008). This explains our results, wherein administration of *P. longum* has downregulated MMP-3 with corresponding significant ($p=0.03$) upregulation in TIMP. *S. cordifolia* administration also shows upregulation of TIMP with corresponding reduction in MMP-3 and -9, an ideal situation to restore MMP-TIMP balance.

This activity of both the plants may be potentiated taking into account their ability to inhibit cyclooxygenase (COX) leading to the inhibition of prostaglandin (PGE) synthesis (Anilkumar, 2010; Bang et al., 2009), thereby protected cartilage degeneration. Down-regulation of MMPs and Up-regulation of TIMPs prevents degradation of articular cartilage in OA. Therefore, inhibition of MMPs represents an attractive target in OA (Alcaraz et al., 2010). Down-regulation of both MMPs by *S. cordifolia* explains its GAG reducing role. Indomethacin is a known COX inhibitor. Prolonged treatment with PGE2 blocking agent decreases PGE2 production in synovial membrane of osteoarthritic rats

(Alvarez-Soria et al., 2008). So, PGE2 may have reduced the observed expression of MMPs as well as paw edema in indomethacin treated group. However, this action has not restored the balance in cartilage remodeling since indomethacin did not upregulate TIMP. Our result suggests that both the plants attenuated OA-augmented expression of MMPs; and restored the OA-reduced expression of TIMPs and anti-oxidants in the synovium.

6.2 Most potent anti-osteoarthritic gum in CIOA rat model

This sections deals with comparative evaluation of selected gums. Although gum of *B. ceiba* has not shown any significant reduction in knee swelling, it has demonstrated reduction in paw edema and in GAG ($p<0.01$) release. The reduction in paw edema could be because of its anti-inflammatory activity as reported by Anandarajagopal et al. (2013). Gum of *B. ceiba* has also protected loss of joint space. However, gum of *B. serrata* has not shown any significant results in studied physiological parameters. Whereas, gum of *A. nilotica* exhibited significant reduction in knee swelling ($p<0.05$) and GAG release ($p<0.01$). It has also demonstrated inhibition in paw edema, synovium inflammation and prevented joint space reduction. This anti-inflammatory effect of *A. nilotica* could be due to a diterpene niloticane, which inhibits cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) (Eldeen et al., 2010). *A. nilotica* also protected proteoglycan layer of cartilage, which can be correlated to GAG lowering activity of *A. nilotica*. Thus, comparatively among the three selected gums, *A. nilotica* gum exhibited highest anti-osteoarthritic activity. It was then further evaluated for molecular mechanism of action.

6.2.1 qPCR study from synovium of osteoarthritic rats treated with *A. nilotica*

Oral administration of *A. nilotica* has effectively tackled oxidative stress by upregulating SOD, GPx, CAT and PON-1 expression in the rat synovium. It has significantly upregulated the TIMP expression, thus protected cartilage from deleterious effects of MMPs.

However, compared to all studied plants, *S. cordifolia*, *P. longum* and *Z. officinale* have shown highest cartilage protective effects.

6.3 Comparative anti-osteoarthritic potential of formulations in CIOA rat model

In comparative evaluation of selected formulations, *Triphala* demonstrated superior protection against knee swelling ($p<0.05$), paw edema as well as GAG ($p<0.05$), compared to osteoarthritic control rats. It has shown highest activity in reducing knee swelling, paw edema and GAG levels, compared to '*Triphala Guggul*' and *Dashamoolarishta*. *Triphala* has also recovered joint space reduction. Histopathology of cartilage indicated that PG layer of cartilage was also increased after treatment with *Triphala*. This activity of *Triphala* could be due to anti-osteoarthritic activity of its ingredient which are *Phyllanthus emblica* and *Terminalia chebula* (Sumantran et al., 2007a, 2007b), which exhibited chondroprotection by inhibiting hyaluronidase and collagenase type II. PG contains core protein to which GAG chains are covalently attached (Ishiguro et al., 2002). *Triphala* protected cartilage through preventing PG damage and GAG release from cartilage. With '*Triphala Guggul*' and *Dashamoolarishta*, there is no significant reduction in knee swelling, paw edema and GAG release. As revealed in radiography, '*Triphala Guggul*' has shown cartilage protection which could be due to its potential of inhibiting hyaluronidase and collagenase (Sumantran et al., 2007b). Thus, among selected formulations, *Triphala* has highest anti-osteoarthritic potential which is further supported by evaluating its mechanism of action using qPCR studies.

6.3.1 qPCR study from synovium of osteoarthritic rats treated with *Triphala*

Triphala acts through modulating the levels of anti-oxidant enzymes, MMP-TIMP in rat synovium.

6.4 Comparative anti-osteoarthritic activity of developed formulations in CIOA rat model

On the basis of results observed in earlier studies, by using herbs which showed highest potential, we have developed new combination formulations for cartilage protection in OA. Literature states that plants of varying potency when combined may theoretically produce an additive or even synergistic effect, as compared to individual use

of the plant due to the sum of their individual effect (Parasuraman et al., 2014). In earlier experiments, *S. cordifolia*, *P. longum* followed by *Z. officinale* have revealed highest potential among selected plants. Hence, further studies were carried out to find out the best combination from these prioritized plants. We have prepared four different combinations using three prioritized plants which were comparatively evaluated their anti-osteoarthritic activity. For formulation preparation, 1:1 proportion of selected plants was used on the basis of *Ayurvedic* literature as for any drug, separate ratio of its ingredients is not mentioned, therefore all the ingredients are were taken in equal proportion (Sharangdhar samhita, 2011).

Four different, new combination formulations were prepared viz. SP, PZ, SZ and SPZ. Combination SP containing *S. cordifolia* and *P. longum*, does not have significant reduction in knee swelling. However it has reduced paw edema, this activity could be due to anti-inflammatory activity of both the plants (Anilkumar, 2010; Chauhan et al., 2011). SP has also shown anti-inflammatory activity through lowering CRP levels in serum, compared to osteoarthritic control rats. However, SP has failed to prevent GAG release and in reducing ALP levels in serum. From these results, it was observed that both the single plants were more effective than their combination, for treating OA and therefore were not an effective combination.

Combination PZ, of *P. longum* and *Z. officinale* has effectively reduced knee swelling and paw edema, compared to osteoarthritic control rats. Compared to other formulations, PZ has not significantly reduced CRP and ALP levels. PZ also failed to reduce GAG release in serum. Though, PZ has shown anti-inflammatory activity, however it has not shown significant anti-osteoarthritic activity as compared to other combinations.

Combination SPZ, of *S. cordifolia*, *P. longum* and *Z. officinale* have not shown any significant reduction in knee swelling, although it has shown reduction in paw edema, GAG and ALP levels. However, it has not significantly reduced CRP levels than osteoarthritic control rats.

Oral administration of SZ, combination of *S. cordifolia* and *Z. officinale*, has significantly attenuated the inflammation by reducing knee swelling ($p<0.05$) and paw edema compared to osteoarthritic control group. MMPs are the enzymes which when released, damage the cartilage. The cartilage degradation products in turn are potent inducers of

inflammation, thus ultimately enhance the inflammatory process by releasing more cytokines and MMPs (Patel et al., 2015). Hence, the reduction in knee swelling by SZ could also be attributed to downregulation of MMPs. Serum CRP, probably the most widely used clinical marker of systemic inflammation, has been shown to correlate well with CRP in synovial fluid in patients with OA or RA (Sturmer et al., 2004). In OA patients, elevated levels of CRP and ALP have been found compared to healthy individuals (Wolfe, 1997; Sarkar et al., 2013). In the present study, CRP and ALP levels were significantly decreased by SZ, compared to osteoarthritic control rats and diclofenac treated group. GAG is a major component of joint cartilage, joint fluid and other soft connective tissues. GAGs are released from the degrading cartilage matrix in large amounts during inflammation of the joints. After treatment with SZ, lower GAG release was observed along with conservation of the joint space. Thus, SZ showed cartilage protection which was also confirmed in radiography. H and E staining indicated that SZ protected synovium and cartilage degeneration during OA. Modification of ECM and increased articular chondrocyte proliferation are characteristics of OA (Dealy, 2012). SZ also protected chondroid matrix and prevented PG loss of ECM. Histopathological observation suggests correlation of SZ intervention which improved muscle degeneration, fibrillation, erosion of cartilage and chondroid matrix; this could be due to the potential downregulation of MMPs. Thus, among the tested four combinations, SZ exhibited highest potential in the management of OA. It was then evaluated for its underlying molecular mechanism using qPCR studies.

6.4.1 qPCR study from synovium of osteoarthritic rats treated with prioritized new combination formulation

As observed from histopathology, biochemical and physiological parameters, the deteriorating changes observed in OA induced rats were in most probability are the outcome of increased release of MMPs. It could also be due to secretion of pro-inflammatory cytokines with surge in reactive oxygen species (ROS) either from the inflamed synovium or from activated chondrocytes (Brown et al., 2008; Patel et al., 2015). ROS are also known to be involved in the regulation of IL-1 effects mediated by NF-kB. In the progression of OA, transcription factors like NF-κB can be triggered by a

number of stimuli, such as cytokines, excessive mechanical stress and degradation products of ECM. Activated NF-κB regulates several enzymes involved in matrix degradation, including MMPs (Wu et al., 2014). MMPs play a primary role in the downstream signaling pathways in OA and cartilage degradation (Jeong et al., 2015). MMP-3 is responsible to break down a number of ECM proteins, including fibronectin, laminin, denatured collagens and proteoglycans (Dealy, 2012). In addition to ECM degradation, MMP-3 is also involved in the activation cascades of MMP-13 and gelatinases. Since MMP-1 and MMP-3 play vital roles in ECM turnover, their regulation has been suggested to be useful in the treatment of OA. TIMPs, the endogenous regulators of MMPs, play important roles in maintaining homoeostasis with MMPs. Imbalance between MMPs and TIMPs is a salient feature in OA progression, leading to disruption of the balance between ECM biosynthesis and degradation (Wu et al., 2014). Therefore, exploration of the mechanisms through which the proteolytic activity, production and expression of MMPs are inhibited by plant-derived natural products used as arthritis remedies will support the effective treatment of OA and may lead to new therapeutic strategies (Park et al., 2015).

To determine whether SZ can affect the destruction of articular cartilage, we have examined the expression of OA biomarkers MMP-3, MMP-9 and TIMP-1 in the synovium of collagenase-induced OA in experimental rats. Interestingly, our results have demonstrated that the up-regulated MMP-3 and -9 expressions in osteoarthritic control rats were all markedly decreased by SZ. Since the activities of MMPs can be inhibited by TIMPs, we have investigated the effects of SZ on TIMP-1 expression. Interestingly, our results revealed up-regulation of TIMP-1 in the synovia of SZL and SZH treated osteoarthritic rats.

Previous studies attributed the bioactivity of *S. cordifolia* to inhibit COX enzyme and prostaglandin synthesis (Anilkumar, 2010). The action of *Z. officinale* could be due to its action of decreasing IL1, NO and PGE2 and LTB4 production as well as inhibition of TNF-α (Shen et al., 2003; Setty and Sigal, 2005; Anilkumar, 2010). In majority of the parameters studied, the dose specific differences were not significant, suggesting the activity range of selected doses is similar. It was evident that that activity shown by SZ is higher than *S. cordifolia* and *Z. officinale* alone, in vital physiological and inflammatory

OA parameters. Similarly, SZ has revealed better activity compared to *Triphala* in crucial OA parameters like knee swelling, GAG release and MMPs. As the developed formulation SZ has shown better activity. We have further evaluated its safety in acute and sub-acute toxicity studies.

6.5 Toxicity studies of prioritized, new combination formulation

Traditional medicines have maintained greater popularity all over the developing world and their use is rapidly increasing (Daswani et al., 2006). Therefore, assessment of the safety of herbal medicine has become very crucial. Both the plants of SZ formulation have proven to be effective against inflammation and their safety profile is reported. Acute toxicity studies using ethanol extract of roots of *S. cordifolia* revealed that LD_{50} was more than 3 gm/kg b. wt. dose (Sumanth and Mustafa, 2009). Aqueous root extract was also found to be safe up to a maximum dose of 2000 mg/ kg b. wt. (Bhatia et al., 2012). The acute toxicity studies of methanolic and aqueous ginger extract revealed LD_{50} was 10.25 and 11.75 gm/kg, respectively, by oral administration in mice (Shalaby and Hamowieh, 2010). Although, both the plants of SZ are routinely used by traditional practitioners, their effect in combination has not been studied. Therefore, we have evaluated toxic effects of SZ in acute and subacute toxicity studies in rats.

6.5.1 Acute toxicity test

In the acute toxicity study, no signs of toxicity or deaths were observed after oral administration of single large dose of SZ. These results showed that, SZ demonstrated high safety margin since the animals tolerated upto 2000 mg/kg b. wt. orally.

6.5.2 Sub-acute toxicity test (Repeated dose 28 day oral toxicity study)

Repeated oral administration of SZ at doses of 135, 270 and 540 mg/kg b. wt. for 28 days did not cause lethality or any significant sign of toxic effect in male as well as female rats. All the animals showed normal body weight gain till the end of the study, indicated that treatment with SZ did not affect normal health status of animals. Nutritional status of all animals was also found to be maintained as there were no

significant increase in the food intake and it was independent with the body weight changes. There was no abnormal deviations in the food intake of rats were observed. Thus from observed weight gain and food intake pattern of animals, SZ could be claimed to be non-toxic to the animals.

The gross examination of internal organs, colour and texture has not indicated any detectable changes compared with the control groups. The organs weights of SZ treated groups have not revealed any significant difference compared to control groups. Among the organs, liver and kidney weights were important in determining toxicity as their sensitivity predicts toxicity (Miechael et al., 2007).

The hematological evaluation has not shown any significant difference except for Eosinophils. The haematopoietic system is one of the most sensitive targets of toxic compounds and is an important index of physiological and pathological status in man and animals (Adeneye et al., 2006). There were no treatment-related changes in haematological parameters between control and treated groups, indicating that SZ is neither toxic to circulating red cells nor interfered with their production and that of platelets.

The biochemical parameters studied from serum revealed that SZ did not alter the liver enzymes as well as the kidney function test. ALP, SGOT and SGPT are the marker enzymes of cellular injury. ALP is responsible for functioning of hepatocytes and increased levels of ALP most of the time represents the liver damage and inflammatory conditions. ALP levels also indicated bile duct functioning (Manjunatha et al., 2005). Transaminases (SGOT and SGPT) are reliable indicators of liver function and biomarkers to predict the possible toxicity of drugs (Mayne and Mayne, 1994). SGPT and SGOT are released in significant amounts into the blood stream at the time of hepatic injury (Rajina and Shini, 2013). In our study, there were no changes in the SGPT, SGOT and ALP levels in treatment groups revealing that SZ did not affect liver function. Other biochemical parameters such as direct & total bilirubin, and uric acid also remained normal without any significant difference. The SZ produced significant increase in urea levels at the middle and higher doses; warranting further investigation, the reason is not exactly known but one possibility could be that the different active principles present in SZ are acting differently at the doses tested.

Furthermore, gross examination of internal organs from SZ treated animals and control animals showed normal architecture, indicating no detrimental changes and morphological disturbances. Gross and histopathological examinations of internal organs of all animals further confirmed that the SZ did not cause any tissue damage. The internal organs showed no pathological abnormality in comparison with that of control group.

Thus, the present study is an adequate evidence of safety of SZ formulation. It has also demonstrated that SZ have not exerted any undesirable effects on the functional state of the main organs of the animals after 28 days of treatment.

6.6 Anti-arthritic effect of prioritized, new combination formulation in inflammation induced synoviocytes

To validate the obtained results of *in vivo* model, activity of SZ was determined in inflammation induced rabbit synoviocytes, HIG-82. Proliferative fibroblast-like synoviocytes (FLSs) play crucial roles in both propagation of inflammation and joint damage because they produce a great amount of proinflammatory mediators such as matrix metalloproteinses (MMPs), interleukin 6 (IL-6), IL-8 and prostaglandin E2 (PGE2) (Bang et al., 2009). We have evaluated the anti-arthritic potential of new combination formulation SZ, by examining its effect on synoviocyte toxicity and inflammatory responses. Nitric oxide (NO) is a pluripotent signaling molecule synthesized by a family of nitric oxide synthase found in most of the tissues (Lee et al., 2013). NO is an important mediator and regulator of inflammatory responses and its increased levels are harmful which leads to chronic inflammatory condition (Krol et al., 1995). In an inflammatory response, the overproduction of NO reacts with superoxide creating cytotoxicity and tissue damage in an organism (Lee et al., 2013). The effect of SZ on NO inhibition was determined by treating the HIG-82 cells of LPS stimulation. SZ successfully decreased NO production, indicating SZ might be useful to suppress the inflammation.

Disorders of the organism's antioxidant-prooxidant balance appear in the course of an inflammatory process, which can accompany OA. The consequence of predominant prooxidative processes is, increased concentrations of products of protein and lipid oxidative damage as well as decreased antioxidant system activity. This leads to an

increased production of reactive oxygen species (ROS) and the intensification of oxidative stress (Olszewska-Slonina et al., 2010). Inflammatory reactions induce production of ROS, in turn can induce a chronic inflammatory reaction (Halliwell et al., 1988; Kapp, 1990). ROS have been implicated in destruction of cartilage matrix and inhibition of the synthesis of new cartilage material. To prevent toxicity by ROS, knee joint cells possess a well-coordinated antioxidant enzyme system formed by superoxide dismutase (SOD), catalase, and glutathione peroxidase (Yao et al., 2005; Olszewska-Slonina et al., 2010). Antioxidant enzymes prevent oxidative stress by scavenging of ROS. SOD scavenges the superoxide free-radical (Bulkey, 1983), in which SOD catalyzes the conversion of superoxide anion (O2 -) to hydrogen peroxide (H_2O_2), and H_2O_2 is further reduced to H_2O by catalase or glutathione peroxidase. SOD acts as a secondary messenger in the production of inflammatory cytokines (Yasui and Baba, 2006). Catalase exhibits anti-inflammatory effects by destroying H_2O_2 and by preventing formation of other cytotoxic oxygen species. Catalase also affects the expression of genes responsible for inflammation (Benhamou et al., 1998). Preventive antioxidant enzymes like SOD and catalase are the first line of defense against ROS. In our work, we have studied inflammatory mediators from cells and supernatants. Observations indicated that treatment of LPS stimulated HIG-82 cells with SZ enhances antioxidant enzyme activities that may be helpful in reducing inflammation.

FLSs are the source of a broad range of MMPs, including MMP-1, MMP-3, and MMP-13. MMP-3 is associated with the degradation of different types of collagen & proteoglycans and activates other MMPs such as MMP-2 and MMP-9 (Zeisel et al., 2005). In our study, SZ has significantly decreased LPS-induced MMPs production. These results suggest that SZ might effectively attenuate arthritis by inhibiting the production of proinflammatory mediators, MMP-3 and MMP-9 from synoviocytes. Hence it can be concluded that, SZ is a safe and effective treatment for osteoarthritis.

Summary and Conclusions

In the present study, we have demonstrated osteoarthritic changes like cartilage degradation, synovial inflammation and osteophyte formation in collagenase induced osteoarthritic rats. Further, we have comparatively evaluated the role of nine plants, three known and four new combination formulations in treating OA in rats, which is done for the first time so far. Protective effects were evaluated by studying key components of OA pathogenesis; like knee and paw swelling, biochemical parameters studied from serum and, cartilage degradation as assessed by radiographs and histopathological investigation. In testing therapeutic modalities for OA, it is valuable to study their role in synovitis. Our data has demonstrated anti-oxidant and anti-inflammatory role of treatments with respect to synovium and cartilage protection.

Most potent anti-osteoarthritic plants

- Compared to selected herbs, *S. cordifolia*, *P. longum* and *V. negundo* have shown significant reduction in knee diameter. All test plants have significantly reduced paw volume. NSAIDs cause disruption of GAG synthesis, which can accelerate the articular damage in arthritic conditions but selected herbs have significantly decreased the GAG release keeping the cartilage in good condition, which suggest the efficacy of these herbs not only in OA treatment but also to reduce the side effects of other treatment in which NSAIDs are used. This may be useful in treatment of OA patients targeting reduced oxidative stress, alleviating synovial inflammation and ultimately slowing down cartilage degeneration. It may be pertinent to note here that no current treatment for OA modulates these therapeutic targets. *T. terrestris* and *V. negundo* have shown better prevention for GAG release compared to *S. cordifolia* and *P. longum*. Although, these two plants have not shown highest activity but protected cartilage degradation compared to osteoarthritic and positive control. Suggesting that any combination of plants that although may have significant effect individually does not necessarily prove effective.
- According to radiological report, *S. cordifolia* and *P. longum* have prevented structural loss of knee joint and joint space was also as good as control group. Histological observations strongly support the protective effects of herbs, *S. cordifolia* and *P. longum* where they have protected synovium and cartilage matrix

followed by *R. communis* and *Z. officinale*. Results clearly indicate that *S. cordifolia* and *P. longum* are responsible for preventing structural loss of knee joint and joint space which is an important aspect in this disease. These changes in *S. cordifolia* and *P. longum* treated groups were certainly effective in curing OA but in rest groups, osteoarthritic knee joint was not recovered.

- Among studied gums, *B. ceiba* demonstrated protection to cartilage by preventing GAG loss. However *B. serrata* has not shown any protective role. Comparatively, *A. nilotica* has exhibited highest anti-osteoarthritic activity by reducing knee swelling, GAG as well as paw edema. It has preserved PG layer degradation. Histopathology and radiography results also support its cartilage protective effect.
- Though, *S. cordifolia*, *P. longum* and *Z. officinale* have not shown highest activity in some parameters tested but they proved at par and consistent activity and hence these herbs may have exploitable protection on cartilage degradation. Thus, among selected nine plants, *S. cordifolia*, *P. longum* followed by *Z. officinale* posses a significant anti-osteoarthritic activity as suggested by physiological and biochemical parameters as well as histopathology.

***Triphala*: Most potent anti-osteoarthritic formulation**

In formulation treated groups, comparatively *Triphala* has significantly reduced knee swelling, has shown highest percent inhibition of paw edema as well as significantly reduced GAG levels. *Triphala* and '*Triphala Guggul*' have protected synovium inflammation and other associated physiological changes. In radiography, '*Triphala Guggul*' has shown protection to cartilage, too. Thus, oral administration of *Triphala* exhibited strong anti-osteoarthritic activity through controlling key components of OA viz. reduced knee swelling, paw edema and GAG levels, compared to '*Triphala Guggul*' and *Dashamoolarishta*. Anti-osteoarthritic activity of *Triphala* is further supported by evaluating its mechanism of action using qPCR studies, which revealed that *Triphala* acts through modulating levels of MMP-TIMP in rat synovium.

SZ: Most potent new combination formulation

New combinations-SP, PZ and SPZ have revealed anti-inflammatory effect but not any protective role for osteoarthritic cartilage. Combination SZ, containing a proprietary blend of two medicinal plants viz. roots of *S. cordifolia* and rhizomes of *Z. officinale*. SZ notably tackled physiological problems and decreased levels of inflammatory markers such as CRP, ALP and GAG in the serum, which might have reduced the expression of biomarkers associated with OA. We have demonstrated at the mRNA level that SZ inhibited the expression of MMP −3 & −9, and increased the expression of TIMP-1, all of which are classic biomarkers of inflammation and cartilage degradation in OA. In toxicity studies, SZ is found to be safe for use.

***In vitro* anti-osteoarthritic activity of most potent new combination formulation**

We have demonstrated that new combination formulation-SZ has anti-arthritic effects in LPS-stimulated rabbit synoviocytes. SZ effectively controlled NO production, anti-oxidant activity was enhanced, and also attenuated levels of MMP-3 & MMP-9, which plays dominant role in osteoarthritis. Hereby suggesting that, SZ has a potential as therapeutic drug for OA.

Conclusions

This study suggests that antioxidant therapy could be helpful in treating the structural changes of joint tissues. Reducing synovial oxidative stress and inflammation could be a major target for cartilage protection in OA. In comparative evaluation of plants, *P. longum*, *S. cordifolia* and *Z. officinale* exhibited highest chondroprotective potential through modulation of studied physiological and biochemical parameters. Gum of *A. nilotica* has shown highest activity in terms of cartilage protection. Similarly, *Triphala* has demonstrated highest cartilage protective effects among the tested formulations. Our study has shown for the first time that, the plants viz. *A. nilotica*, *P. longum, S. cordifolia* and formulation namely, *Triphala* and new combination formulation SZ, possesses chondroprotective activity through modulation of biochemical parameters and MMPs-TIMP genes in knee synovial tissue. However, SZ merits consideration as an alternative therapy from natural sources for the treatment of OA. Results indicate that SZ improves OA-induced cartilage damage suggesting that SZ may be a potential therapeutic agent for OA and merits further preclinical and human clinical studies to take our leads forward.

Highlights, Societal relevance, Limitations and Future directions

Highlights of thesis

- Illustrated mechanism of action of plants and formulations with special reference to the pathologies related to synovium and cartilage.
- Development of new combination formulation for the management of osteoarthritis.
- Set a model to find out safer and better alternative for the management of osteoarthritis.

Societal relevance of the study

- Validation of alternative medicine for most prevalent disease, which leads to disability.
- New combination formulation is effective for OA specific pathologies and safer for use.
- Traditionally practiced yet underexplored modalities of preventive and curative measures merits promote for their global use.

Limitations of thesis

- Inflammatory mediators were not detected in qPCR studies of *in vivo* model.
- Additional anti-oxidant markers, inflammatory mediators and TIMP from synovial cells were not studied.
- New combination formulation is not validated clinically.

Future directions of thesis

- Developed formulation could be evaluated in different ratios.
- Activity of developed formulation could be confirmed using other OA models.
- Exact molecular mechanism of developed formulation against OA could be elucidated.
- Clinical trials of developed formulation could be initiated.
- Developed formulation could be further checked for activity in combination with other known nutraceuticals.
- Developed formulation could also be validated using cell lines like chondrocyte, mesenchymal stem cells.
- Formulation technology transfer could be carried out to concerned manufacturing industry.

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Publications

A) Patent: 01

1. Jagtap S., **Nirmal P.** and Harsulkar A. (2014). "A novel herbal formulation (OA-F2) for use in osteoarthritis" Indian patent application No.: 2509/MUM/2014.

B) Publications:

- **Published: 02**

1. **Nirmal P.**, Jagtap S., Devarshi P., Narkhede A., Koppikar S., Ingale D. and Harsulkar A. (2015)"Cartilage Protective Effect of *Sida cordifolia* L. and *Piper longum* L. is Through Modulation of MMPs and TIMP" International Journal of Advanced Research 3(11): 480-488.
2. **Nirmal P.**, Koppikar S., Bhondave P., Narkhede A., Nagarkar B., Kulkarni V., Wagh N., Kulkarni O., Harsulkar A. and Jagtap S. (2013)"Influence of Six Medicinal Herbs on Collagenase-induced Osteoarthritis in Rats"American Journal Chinese Medicine 41(6): 1407-1425.

- **Under revision: 01**

1. **Nirmal P.**, Jagtap S., Narkhede A., Nagarkar B. and Harsulkar A. "New Herbal Composition (OA-F2) Protects Cartilage Degeneration in a Rat Model of Collagenase Induced Osteoarthritis" BMC Complementary and Alternative Medicine.

- **Communicated: 01**

1. **Nirmal P.**, Jagtap S., Narkhede A., Nagarkar B. and Harsulkar A. "*Triphala* Protects Cartilage Degeneration in a Rat Model of Collagenase Induced Osteoarthritis" (European Journal of Pharmacology).

Awards

Awards

1 **Best oral presentation award** for paper entitled "Anti-osteoarthritic Effects of New Formulations in *in vivo* Cartilage Degeneration Model" at 3rd Global sustainable biotech congress (GSBC) – 2014 held at North Maharashtra University, Jalgaon, India on 1st to 5th December 2014.

2 **Best Young Researcher** Award by GRABS Educational Charitable Trust at Chennai on 10th May 2016.

Presentations

International conferences

1. Presented paper on "Anti-osteoarthritic Effects of New Formulations using *in vivo* and *in vitro* Model" at 2nd International conference on Biotechnology and Bioinformatics (ICBB-2015) held at Pune, India on 6th to 8th February 2015.
2. Presented paper on "Anti-osteoarthritic Effects of New Formulations in *in vivo* Cartilage Degeneration Model" at 3rd Global sustainable biotech congress (GSBC) – 2014 held at North Maharashtra University, Jalgaon, India on 1st to 5th December 2014.
3. Attended international Conference On "Current Trends in Medicinal Plants Research" organised by Department of Botany, University of Pune held at University of Pune, Pune on 10th to 12th January 2012.

National conferences

1. Presented paper on "Evaluation of Anti-osteoarthritic Potential of Selected Medicinal Plants using Collagenase Induced Osteoarthritis (CIA) Model" at UGC sponsored national seminar on "Recent Trends in Life Sciences" held at Yashwantrao Mohite College, Pune on 21st and 22nd December 2012.
2. Presented paper on "Therapeutic Effects of New Combination Formulation on Cartilage Protection in Collagenase Induced Osteoarthritis Model" at Conference on Collaborative Research in Ayurveda & Modern Biology (CCRAMB) held at The Ayurvedic Trust Campus, Ramanathapuram PO- Coimbatore -45 on 3rd& 4th February 2014.