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# CORRELATION OF PHYSICOCHEMICAL PROPERTIES OF CHONDROITIN SULFATE TO ITS IN-VITRO ABSORPTION AND ANTI-INFLAMMATORY ACTIVITY

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# CORRELATION OF PHYSICOCHEMICAL PROPERTIES OF CHONDROITIN SULFATE TO ITS IN-VITRO ABSORPTION AND ANTI-INFLAMMATORY ACTIVITY

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ABSTRACT

The purpose of this study was to investigate if the molecular weight and degree of sulfation of chondroitin sulfate (CS) has an impact on its in vitro absorption and anti-inflammatory activity.

For absorption studies, Caco-2 cells were incubated with eight CS samples of differing molecular weights (7 kDa - 35 kDa). The amount of CS transported into the basolateral side of the Caco-2 monolayer was quantitatively determined to calculate the permeability coefficients ($P_{eff}$). The permeability coefficients of the eight different CS samples across Caco-2 cell monolayers were assessed. For anti-inflammatory studies, RAW 264.7 murine macrophage cells were pre incubated with the CS samples for an hour followed by addition of bacterial lipopolysaccharide (LPS). The anti-inflammatory activity of CS samples was measured by the ability of CS samples to inhibit expression of a panel of inflammatory cytokines- tumor necrosis factor-alpha (TNF-α), Interleukin-1 beta (IL-1β) and Interleukin-6 (IL-6). These inflammatory markers were quantitatively measured using ELISA and inhibition of nitric oxide (NO) production was measured using Griess reagent assay.

Of the eight samples evaluated, four had a $P_{eff}$ value of $15 \times 10^{-6}$ cm $sec^{-1}$ or higher indicating moderate to high absorption. Two of the four samples with higher $P_{eff}$ values were high molecular weight compounds (~ 35 kDa). At concentrations of 5 µg/ml as well as 15 µg/ml, CS samples significantly inhibited expression of LPS induced TNF-α. Expression of IL-6 was inhibited by some of the CS samples at 15 µg/ml concentration

but not at 5μg/ml. Under the experimental conditions, IL-1β and NO were not useful in estimating the anti-inflammatory activity of the CS samples. Statistical analysis which examined the relation between molecular weight and each of these inflammatory markers revealed no correlation ($p<0.05$).

Within the CS molecular weight range used in this experiment, the absorption of CS samples did not have a correlation with their molecular weights but, interestingly, correlation was observed between the absorption and percentage of 6-sulfated disaccharide in the CS samples. CS samples used in this study appeared to inhibit some of the inflammatory cytokines but no correlation seemed to exist between the molecular weights and anti-inflammatory activity of these samples.

# DEDICATION

This work is dedicated to all my teachers and mentors, who have encouraged and inspired me to think deeper.

ACKNOWLEDGMENTS

I would like to thank my advisers, Dr. Bodine and Dr. Haley, for their endless support and guidance. Their confidence in me and vision has helped me become a better researcher. I have learnt a lot from them and they continue to inspire me. Some of the work done in this dissertation would not have been possible without the co-operation of Dr. James R. Brooks, Pharmavite LLC. I would also like to thank Dr. Xiuping Jiang for agreeing to serve on my committee and guide me throughout my Ph.D. I would like to express my special thanks to the research and development team at Pharmavite who provided me invaluable guidance in completing this project. I am very grateful to Dr. Tzeng and Dr. Huang for their help with the anti-inflammatory part of this study.

Last but not least, I would like to thank my family for their unconditional support and unwavering faith in me. I thank all my good friends who have accompanied me on the road to a doctoral degree, and whose friendship will be treasured. My special thanks to Ramakrishna Podila and Pooja Puneet for their constant support and encouragement.

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CHAPTER 1

# INTRODUCTION

## 1.1. Arthritis:

Arthritis is a general term for inflammation of the joints (such as the knees, wrists, fingers, toes, and hips) and surrounding tissues. The two more common types of arthritis are osteoarthritis (OA) and rheumatoid arthritis (RA) (Figure 1.1).

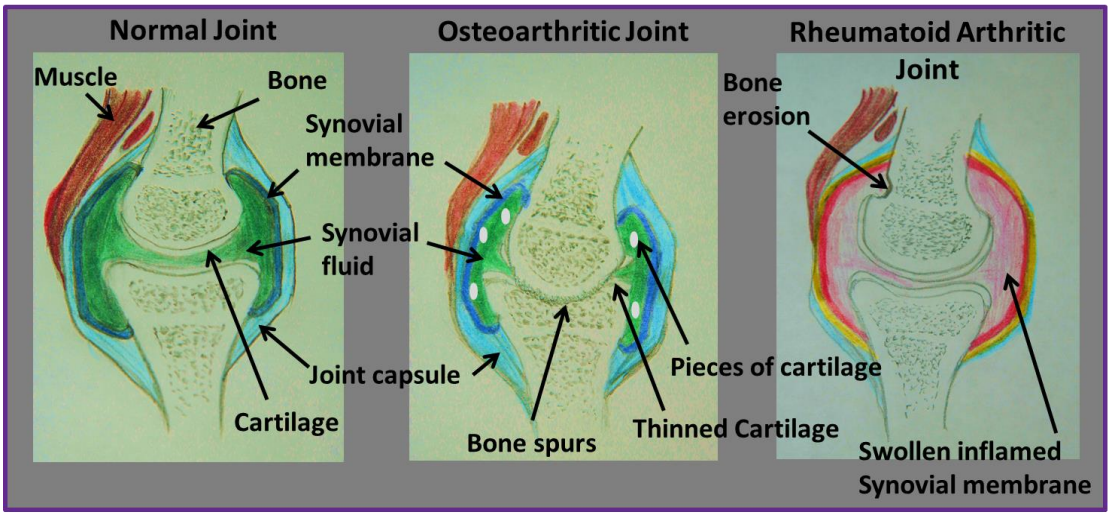


Figure 1.1: Normal and arthritic joints. In the case of osteoarthritis, cartilage is degenerated and bone ends rub together while in rheumatoid arthritis bones erode with simultaneous inflammation in the synovial membrane. (Illustration by Pooja Puneet)

### 1.1.1. Rheumatoid arthritis (RA):

Rheumatoid arthritis is an autoimmune inflammatory disease that usually involves joints in the fingers, thumbs, wrists, elbows, shoulders, knees, feet, and ankles. Patients with

RA may also have systemic symptoms including fatigue, fever, weight loss, eye inflammation, anemia, pleurisy (inflammation of lining of the lungs) or subcutaneous nodules (Ropes, Bennett, Cobb, Jacox, & Jessar, 1957; Scadding, 1969).

1.1.2. Osteoarthritis (OA):

Osteoarthritis (OA) is the most common form of arthritis, and is the single most important cause of disability in older adults (Issa & Sharma, 2006; Peat, McCarney, & Croft, 2001). OA is characterized by slow degeneration of joint cartilage and the underlying bone with possible development of bone spurs. The degeneration of these tissues eventually leads to pain, and possible limited mobility of the joint. OA is classified into idiopathic OA (primary) or secondary OA (traumatic, congenital, metabolic and other medical causes).

OA is defined as a condition of the synovial joints characterized by loss of cartilage, development of osteophytes and subchondral sclerosis which is also accompanied by a degree of synovitis (Brooks, 2003). OA has different clinical manifestations in different joints of the body. The more commonly affected joints are the hands, knees, hips, and spine. The onset of disease is gradual and usually begins after the age of 40 (Buckwalter, Saltzman, & Brown, 2004).

Incidence:

In 2008, approximately 27 million Americans were affected by OA. This increased from 21 million in 1990. About 13.9% adults over age 25 and 33.6% over age 65 are affected

(Lawrence et al., 2008). By 2030, a projected 67 million people will have physician-diagnosed arthritis (Hootman & Helmick, 2006). Before the age of 55, OA is observed to occur equally in men and women. Upon reaching the age of 55, OA is occurs more commonly in women (Buckwalter et al., 2004). With ageing of the population, the importance of osteoarthritis as a cause of disability is increasing in both industrialized countries as well as the developing world (Brooks, 2003). In 2004, an estimated expenditure of $60 billion was incurred as a result of OA in the form of traditional medical costs and indirect economic and wage losses (Buckwalter et al., 2004; D. W. Jackson, Simon, & Aberman, 2001). This expense is projected to increase as the incidence of OA is estimated to double by 2030.

Causes:

Understanding the exact cause of OA is complicated by the number of risk factors involved in the incidence as well as the progressive nature of the condition. It is the cumulative result of several risk factors. The importance and interaction of these risk factors also vary depending on the anatomical site of OA. Generalized or systemic risk factors include- age, gender, genetic predisposition, obesity, bone mineral density and nutrition; while localized or biomechanical risk factors include- physical activity, joint alignment and trauma to the tissue.

Symptoms:

The early symptoms of OA are stiffness, joint pain and swelling. The pain of osteoarthritis is often aggravated by activity and relieved by rest. As the disease

progresses, it may lead to extensive changes in joint morphology due to the growth of bone spurs, development of muscle weakness and loss of joint integrity which eventually results in decreased functionality of the joint. Radiographic studies and other diagnostic tools such as ultrasound, computed tomography (CT) and magnetic resonance imaging (MRI) are useful in evaluating joint pathology and determining the extent of damage to the cartilage and the associated tissues. OA was commonly described as a non-inflammatory disease in order to distinguish it from inflammatory arthritis such as RA (Bonnet & Walsh, 2005) . Despite this presumed distinction, there is an abundance of research showing close association of inflammation with symptoms and progress of OA.

Pathophysiology:

OA affects chondrocytes which are the cells present in joint cartilage. In healthy cartilage, these cells are in metabolic equilibrium with constant catabolic and anabolic activities maintaining the structural and functional integrity of the cartilage extracellular matrix (ECM) (Poole, 2001). Any disturbance in this balance of anabolic and catabolic activities of chondrocytes is implicated in loss of the cartilage matrix that is correlated with development of OA (S. R. Goldring & Goldring, 2004; Shinmei & Nemoto, 1996). Multiple factors such as development of acquired or age-related alteration in chondrocyte function, effects of excessive mechanical loading, and the presence of dysregulated cytokine activities are likely involved in disruption of chondrocyte remodeling activities in OA (S. R. Goldring & Goldring, 2004).

Advanced studies on the pathophysiology of OA emphasize that it is not only a degenerative disease, but that there is an associated ongoing and underlying inflammatory process involved (S. B. Abramson, 2004; Brooks, 2003; Conrozier et al., 1998; D'Agostino et al., 2005; Ehrlich, 1975; Peyron, 1981; Spector et al., 1997). Inflammation may act as an important factor in promoting cartilage degradation by impairing its ability to repair ongoing damage (Mary B. Goldring & Otero, 2011). The inflammation present in OA occurs in both idiopathic OA and secondary OA (Altman & Gray, 1985).

Inflammatory cytokines are closely associated with the functional alterations of cartilage, synovium and the subchondral bone observed with arthritis (Fernandes, Martel-Pelletier, & Pelletier, 2002; S. R. Goldring & Goldring, 2004). These cytokines also inhibit chondrocyte compensatory synthetic processes required to reinstate the integrity of the damaged ECM (Fernandes et al., 2002). Thus, cytokines not only promote tissue damage and degradation but also hinder regeneration (Pelletier, Roughley, Dibattista, McCollum, & Martelpelletier, 1991) . Over expression of pro-inflammatory cytokines such as IL-1β and TNF-α seen in early stages of OA may contribute to progressive cartilage degradation by increased activation of interrelated pathophysiological pathways (Benito, Veale, Fitzgerald, van den Berg, & Bresnihan, 2005; M. B. Goldring, 2000). IL-1β and TNF-α also upregulate the chondrocyte production of metalloproteinases, inducible nitric oxide synthase (iNOS) and free radicals, the factors associated with cartilage matrix destruction (Hedbom & Hauselmann, 2002). Inflammation in OA is also associated with angiogenesis which may contribute to pain, the major symptom of OA (Bonnet & Walsh, 2005).

Treatment options:

Currently, there is no cure for osteoarthritis. Understanding the role of inflammation in OA may result in better management of the disease. Current treatments for OA focus on pain relief and preservation of the function of the joints involved. Anti-inflammatory drugs have been shown to not only reduce the pain but also reduce joint tenderness and stiffness in patients with OA (Dieppe, 1978). Analgesics, non-steroidal anti-inflammatory drugs (NSAIDs), cyclooxygenase-2 (COX-2) inhibitors, and local intra-articular steroid injections are commonly used pharmacological interventions in patients with OA. Therapies interfering with expression or action of proinflammatory cytokines IL-1β and TNF-α have been suggested as promising interventions in the management of OA (M. B. Goldring, 2000).

Although use of NSAIDs relieves symptoms of OA, continuous use of these drugs is limited by the potential side effect of gastrointestinal (GI) toxicity (Scanzello, Moskowitz, & Gibofsky, 2008). Furthermore, the higher price of COX-2 inhibitors compared to the over the counter NSAIDs sometimes outweighs their lowered risk of GI toxicity. Also, the use of COX-2 inhibitors requires caution in the case of patients with renal and cardiovascular conditions. This scenario demands the availability of an alternative therapy that provides the same advantages of the above mentioned drugs without the dangerous side effects of long term usage. Nutritional supplements containing glucosamine and chondroitin sulfate have shown promising results in reducing pain and preserving joint functionality in people and animals with OA (Black et al., 2009).

Chondroitin sulfate is present in virtually all of the commercially available joint health supplements.

### 1.2. Chondroitin sulfate:

Chondroitin sulfate (CS) belongs to a group of compounds called glycosaminoglycans (GAGs). Glucosamine, often paired with CS in supplements for OA is also a GAG. GAGs are long unbranched/linear polysaccharides consisting of repeating disaccharide units of a uronic acid and an amino sugar. GAGs are primarily located in the ECM of connective tissue (cartilage, bone, tendons, ligaments, bone and skin) as a part of proteoglycans (PGs). Multiple GAGs covalently attached to a core protein backbone form the PGs. These connective tissues also contain collagen. In articular cartilage tissues, collagen contributes to the tensile strength whereas the PGs give the tissue resilience.

Of particular interest to this dissertation is Chondroitin sulfate which is widely used in joint health supplements in the USA (McAlindon, LaValley, Gulin, & Felson, 2000). In most of the supplements it is present in combination with glucosamine and methylsulfonylmethane (MSM). CS has a potentially expanding market considering the tens of millions of Americans who suffer from OA. It is currently recommended as a SYSADOA (symptomatic slow acting drug for OA) by the European League Against Rheumatism (EULAR) based on research evidence, meta-analysis and numerous clinical studies and reviews (N. Volpi, 2009).

1.2.1. Structure & Characteristics:

CS is present in virtually all connective tissues of the body as part of PGs. CS represents a highly heterogeneous group of unbranched polysaccharides characterized by alternating disaccharide units of N-acetyl galactosamine and glucuronic acid as seen in Figure 1.2. D-glucuronic acid is β-1:3 linked to N-acetyl-D-galactosamine which is in turn β-1:4 linked to the D-glucuronic acid of adjacent disaccharide (Beaty & Mello, 1987). The more common and commercially available forms of CS consist of sulfated groups at positions C-4 or C-6 of galactosamine in the disaccharide unit. In addition to the variation in molecular weight, the presence of sulfate groups in different amounts and positions (2 and 3 of β-D-glucuronic acid, and 4 and 6 of N-acetyl-galactosamine residues) also greatly contributes to its heterogeneity. CS originated from different tissues varies in terms of amount and positions of sulfation and molecular mass (Nicola Volpi, 2004).

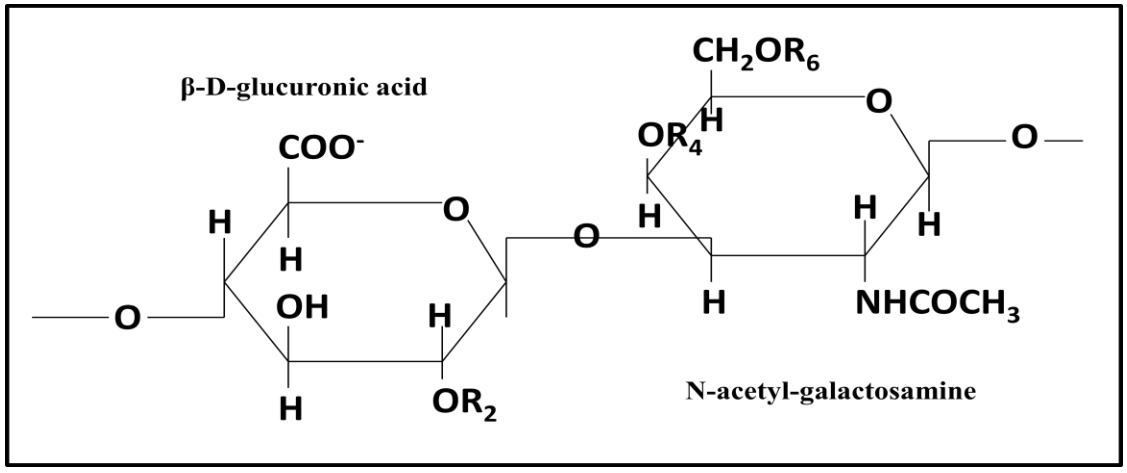


Figure 1.2: Nomenclature based on the position of sulfate group(s) in CS. i) When $R_2$=$R_4$=$R_6$= H: non-sulfated chondroitin (ΔDi-0s : 2-acetamido-2-deoxy-3-O-(β-D-gluco-

4-enepyranosyluronic acid)-D-galactose); ii) when $R_4=SO_3^-$ and $R_2=R_6=H$: Chondroitin-4-sulfate (ΔDi-4s: 2-acetamido-2-deoxy-3-O-(β -D-gluco-4-enepyranosyluronic acid)-4-O-sulpho-D-glactose ); and iii) when $R_6=SO_3^-$ and $R_2=R_4=H$: Chondroitin-6-sulfate (ΔDi-6s: 2-acetamido-2-deoxy-3-O-(β -D-gluco-4-enepyranosyluronic acid)-6-O-sulpho-D-glactose)

CS was first extracted and purified in the 1960s (Telser, Robinson, & Dorfman, 1965). Nuclear magnetic resonance (NMR) studies of CS from bovine trachea, porcine trachea and shark cartilage, characterized the disaccharide composition as presented in Table 1 (Mucci, Schenetti, & Volpi, 2000). Studies on CS obtained from other sources presented additional variations in molecular weight as well as amount and positions of sulfation confirming the heterogeneity of the compound (N. Volpi, Dondi, & Bolognani, 1998). In humans, the disaccharide composition of CS chains varies with age, position (topography) on the joint surface, and the zone of cartilage analyzed. The ratio of 6s-disaccharide to 4s disaccharide increased with age (Bayliss, Osborne, Woodhouse, & Davidson, 1999). Most commercially available CS for oral supplementation is obtained from bovine, porcine and shark sources, although several other sources of CS such as shark fin cartilage, crocodile hyoid cartilage, chicken keel cartilage, etc. have also been explored (Garnjanagoonchorn, Wongekalak, & Engkagul, 2007; Luo, Fosmire, & Leach, 2002; J.-S. Sim et al., 2007).

Table 1.1: Characteristics of CS from different sources. Adapted from (Mucci et al., 2000).

| Characteristics | Source | | |
|---|---|---|---|
| | Bovine trachea | Porcine trachea | Shark cartilage |
| Molecular mass(kDa) | 23.7±1.6 | 18.1±1.4 | >50.0 |
| ΔDi-0s(wt%) | 7.3±0.4 | 6.1±0.3 | 2.2±0.1 |
| ΔDi-4s(wt%) | 51.4±3.1 | 66.3±3.3 | 27.7±1.1 |
| ΔDi-6s(wt%) | 40.6±3.1 | 25.6±1.5 | 49.0±2.4 |
| ΔDi-dis+ΔDi-tris(wt%) | 0.7 | 1.9 | 22.0 |
| 4s/6s ratio | 1.24±0.06 | 2.41±0.12 | 0.47±0.02 |

1.2.2. CS in OA- Safety and efficacy:

Several *in vitro* studies, animal studies and clinical trials demonstrated the role and efficacy of CS in management and treatment of symptoms of OA as elaborated below. When bovine articular chondrocytes were exposed to stimulated conditions of in vivo stress (mechanical, thermal and cytokine), CS enhanced the protective metabolic response, suggesting its role as a biological response modifier (BRM) (Lippiello, 2003). In cultured rabbit chondrocytes , CS reduced IL-1β induced translocation of nuclear factor- κB (NF-κB) by about 8% but did not prevent increase in nitric oxide (NO) (Jomphe et al., 2008).

Numerous clinical studies provided evidence suggesting that orally administered CS acts as a slow acting symptomatic drug in patients with OA and is safe at doses as high as 800 – 1200 mg/day. Two different randomized, double blind, placebo-controlled studies (one 6-months and another one year), on patients with knee OA revealed that CS was well tolerated at a dosage of 800 mg/day. Further, CS showed efficacy in improving mobility and reducing pain (Bucsi & Poor, 1998; Uebelhart, Thonar, Delmas, Chantraine, & Vignon, 1998). In another 3 year randomized, double blind, placebo-controlled study conducted on patients with interphalangeal joint OA, CS treated patients showed delayed progression of OA corroborating its role as SYSADOA (Verbruggen, Goemaere, & Veys, 1998). In the same study, at an oral CS dosage of 1200 mg/day, no adverse effects were seen. This substantiates the safety of CS at doses present in commercially available joint health supplements.

In a twelve month, randomized, double blind, placebo controlled study conducted by Wildi et al., in patients with primary OA of the knee, the CS group showed significantly lower cartilage volume loss in as early as six months and fewer bone marrow lesions compared to the placebo group after twelve months (Wildi et al., 2011). CS was also shown to be effective in management of symptoms in the hand, knee and finger OA (Gabay, Medinger-Sadowski, Gascon, Kolo, & Finckh, 2011; Reginster, 2012; Uebelhart, Thonar, Delmas, et al., 1998; Verbruggen et al., 1998). CS, in combination with glucosamine, was also shown to be effective in management of OA of the knee in multiple clinical studies (Das & Hammad, 2000; Povoroznyuk, Grygoryeva, Dzerovych, & Karsevskaya, 2010).

Despite abundant evidence pointing to the efficacy of CS as a SYSADOA, there are some studies in which, the conclusions are not very encouraging. In a 24-week randomized, placebo controlled trial with 1000mg/day of CS treatment where symptoms (pain and functionality) and biochemical markers of bone, cartilage and synovial metabolism were measured, CS was not effective in improving the markers and only slightly better than placebo in management of pain (Mazieres, Hucher, Zaim, & Garnero, 2007). Another important multi center, double blind, placebo and Celecoxib (a non-steroidal anti-inflammatory drug) controlled study, the Glucosamine/Chondroitin Arthritis intervention Trial (GAIT), that was conducted to evaluate the efficacy and safety of CS and glucosamine as a treatment for knee pain from osteoarthritis also revealed that, overall, glucosamine or CS alone, or in combination, were not better than placebo in reducing knee pain by 20%. But, in sub group of patients with moderate to severe OA of knee, CS in combination with glucosamine significantly lowered pain compared to placebo (Clegg et al., 2006).

Interestingly, not only human clinical trials but also a number of animal studies provide evidence supporting the efficacy of CS treatment in OA. In horses with degenerative joint disease (DJD), CS in combination with glucosamine showed a trend towards the improvement in both objective and subjective outcomes, but the lack of a placebo group and the small number of animals limited statistical analysis of results (Hanson, Smalley, Huff, White, & Hammad, 1997). Orally administered CS was shown to have protective effects on chymopapain-induced cartilage damage in rabbits (Uebelhart, Thonar, Zhang, & Williams, 1998). Orally administered CS along with glucosamine was shown to be

beneficial in alleviating clinical, inflammatory and histological parameters of adjuvant-induced arthritis in rats (Chou et al., 2005). Long-term oral administration of glucosamine and CS reduced the destruction of cartilage and the upregulation of matrix metalloproteinase-3 (MMP-3) mRNA in a model of spontaneous osteoarthritis in Harley guinea pigs (Taniguchi et al., 2012).

In spite of numerous clinical trials confirming the efficacy and safety of CS at the dosages provided in supplements, questions have been raised regarding shorter durations and the lack of important demographic information of participants. According to an editorial in the Journal of the American Medical Association (JAMA), these two shortcomings of the clinical studies make it difficult to generalize the results of meta-analysis to individual patients (Towheed & Anastassiades, 2000). Most of the reviews and meta-analyses agree on the efficacy of CS in OA but considered the effect to be modest (Bruyere & Reginster, 2007; Huskisson, 2008; Jerosch, 2011; Kelly, 1998; Leeb, Schweitzer, Montag, & Smolen, 2000; McAlindon et al., 2000; Richy et al., 2003; Nicola Volpi, 2004). However, the conclusions of these meta-analyses were equivocal due to variations in methodological quality and efficacy, which made their reliability questionable (Black et al., 2009).

The role of CS as part of PG molecules is extended to crucial biological functions e.g., growth factor signaling, axonal growth and regeneration, neuritogenesis during CNS development, morphogenesis and cell division (Sugahara et al., 2003). It is also effective in lowering systemic inflammation associated with chronic inflammatory diseases like

the cardiovascular diseases and rheumatoid arthritis. Intra-peritoneal injection of CS administered to rabbits with atherosclerosis aggravated by induced chronic OA hindered progression of atherosclerosis by reducing the serum levels of pro-inflammatory cytokines C-reactive protein and IL-6. By altering other inflammatory and histological parameters, CS proved to be beneficial in slowing down the progression of atherosclerosis in these rabbits (Herrero-Beaumont et al., 2008). In adjuvant induced arthritis in a rodent model, administration of CS resulted in significant reduction in oxidative stress associated with inflammation. In these animals, 14 day pre-treatment with CS was proven to be more effective than administration of CS at the beginning of adjuvant induction of arthritis, which highlights the SYSADOA effect of CS (Bauerova et al., 2011).

1.2.3. Bioavailability of CS:

All the above evidence makes a strong case in favor of CS as a supplement for people with OA and for people with rheumatoid arthritis too, considering its systemic anti-inflammatory activity. As seen from its structure (Figure.1.1), however, CS is actually a heterogeneous group of compounds with variable molecular weights and sulfation patterns. Despite this structural variation, there is a dearth of research comparing CS samples of different molecular weights in terms of bioavailability as well as activity. Different studies showed different percentage absorption of CS (A. Adebowale, Du, Liang, Leslie, & Eddington, 2002; Barthe et al., 2004; Conte et al., 1991; J. Du, White, & Eddington, 2004; C. G. Jackson et al., 2010; N. Volpi, 2010).

In rats and dogs, about 10% of the orally administered 14 kDa MW CS was seen to be absorbed intact (Palmieri, Conte, Giovannini, Lualdi, & Ronca, 1990). Another study conducted in dogs also confirmed the absorption of intact CS (not digested into its component disaccharides) as well as CS's low molecular weight derivatives (A. Adebowale et al., 2002). In a study conducted in horses, both 8 kDa CS and 16.9 kDa orally administered CS were absorbed (J. Du et al., 2004). An in vitro study conducted using rat gut sacs also provided evidence of CS being absorbed in the GI tract (Barthe et al., 2004).

In humans, at an oral dose of 3 g, about 13% of CS with a mean molecular weight of 16 kDa appeared to be bioavailable (Conte et al., 1991). When partially depolymerized shark CS of mean molecular weight (MMW) 7.5 kDa was administered to human subjects at a dosage of 1.2 grams, about 10% was absorbed intact (G. Ronca & Conte, 1993). Although, in a study conducted on patients with symptomatic knee pain by Jackson et al., no significant increase in plasma concentration of CS was reported when single dose CS was administered orally, this finding has been attributed to the detection limits of analytical methods, fluorophore-associated carbohydrate electrophoresis (FACE) and superose 6 chromatography used to measure the plasma concentrations of CS (C. G. Jackson et al., 2010; N. Volpi, 2010).

In a group of healthy male volunteers, oral administration of 4 g of CS with a mean molecular weight 24 kDa and of bovine origin, over a time period of 48 hrs, resulted in significant increase in plasma CS levels (more than 200%) detected qualitatively as well

as quantitatively by agarose-gel electrophoresis (N. Volpi, 2002). When ichthyic origin CS of MMW 44 kDa was orally administered to healthy male volunteers at the same dosage as above (4 g single dosage), it only showed a moderate increase (120%) in plasma CS concentrations (N. Volpi, 2003). In a study conducted in horses, 8 kDa CS showed higher bioavailability compared to CS of molecular weight 16.9 kDa (32% as opposed to 22% respectively), but this difference is found to be statistically insignificant, probably due to the small number of animals involved in the study (J. Du et al., 2004). All of this evidence supports the idea of high molecular weight CS being less bioavailable compared to the low molecular weight CS.

Although speculations have been made regarding the correlation between MW of CS sources and their bio availability, at present, no such targeted studies have been conducted either *in vitro* or *in vivo*. Hence, the focus of this study is to investigate the relationship of MW of CS with its *in vitro* absorption and anti-inflammatory activity. The research has been divided into three major objectives-

1. To determine the molecular weights and disaccharide composition of CS samples used in this study.
2. To determine the *in vitro* absorption of CS samples and its correlation with molecular weight.
3. To determine the *in vitro* anti-inflammatory activity of CS samples and its correlation with molecular weight.

# CHAPTER 2

## DETERMINATION OF MOLECULAR WEIGHTS AND DISACCHARIDE COMPOSITIONS OF CHONDROITIN SULFATE (CS) SAMPLES

CS is a highly heterogeneous molecule due to the variations in its molecular weight and sulfation patterns. Molecular weights and disaccharide compositions of CS from different animal sources were listed in Table 1.1 (Mucci et al., 2000). For this study, a total of 8 CS samples of unknown molecular weights were obtained from 4 different raw material suppliers. For confidentiality purposes, code names were assigned to the samples and their suppliers. Table 2.1 provides these assigned names, sources and suppliers of CS samples.

Table 2.1: Assigned names, sources, and suppliers of CS samples used

| **Name of the sample** | **Source** | **Supplier** |
|---|---|---|
| Sample A | Synthetic | Supplier 1 |
| Sample B | Synthetic | Supplier 1 |
| Sample C | Bovine cartilage | Supplier 2 |
| Sample D | Bovine cartilage | Supplier 3 |
| Sample E | Porcine | Supplier 4 |
| Sample F | Bovine trachea | Supplier 2 |
| Sample G | Synthetic | Supplier 1 |
| Sample H | Synthetic | Supplier 1 |

Samples A, B, G and H were provided by the same independent supplier, supplier 1. Samples D and E were provided by independent CS raw material suppliers 3 and 4 respectively. Samples C and F were purchased from Sigma-Aldrich (Sample C- Catalog # C6737 and Sample F- Catalog # C9819).

**2.1. Determination of Molecular weight (MW):**

Analytical methods such as fluorophore assisted carbohydrate electrophoresis (FACE) (Buzzega, Maccari, & Volpi, 2010), high performance size exclusion chromatography (HPSEC) (Mucci et al., 2000; N. Volpi & Bolognani, 1993; N. Volpi et al., 1998) and agarose gel-electrophoresis were utilized by researchers in the past to determine MW of CS. HPSEC method slightly modified from Volpi and Bolognani (1993) was used as the analytical method to determine the MWs of CS samples used in this study. Known molecular weight CS samples were used as standards.

2.1.1. Material and methods:

CS standards of known MW were provided by an independent supplier, supplier 1. Briefly, two Agilent Bio SEC-3 (150 x 7.8 mm) columns with pore sizes 150 Å and 100 Å (Agilent, Part # 5190-2507 and # 5190-2502) were connected in tandem. 80% buffer (30 mM Disodium hydrogen phosphate, 30 mM Sodium sulfate, pH 7) and 20% ethanol was used as mobile phase at a flow rate of 1.0 ml/min and run time of 15 minutes. 5 µl injection volumes of standards or samples were injected and chromatograms were recorded at 205 nm using a photo diode array (PDA) detector (Waters # 2998 PDA detector). A standard curve was plotted using retention times of standards and their corresponding molecular weights. The standard curve was then used to determine

molecular weights of CS samples whose retention times were known. The detailed protocol is attached as Appendix 1.

2.1.2. Results:

A CS standard curve was plotted as outlined in the materials and methods section and Appendix 1. The curve best fitting to the data was a third degree polynomial. Figure 2.1 shows the equation of the standard curve ($R^2 = 0.99$) that was used to calculate molecular weights of CS samples.

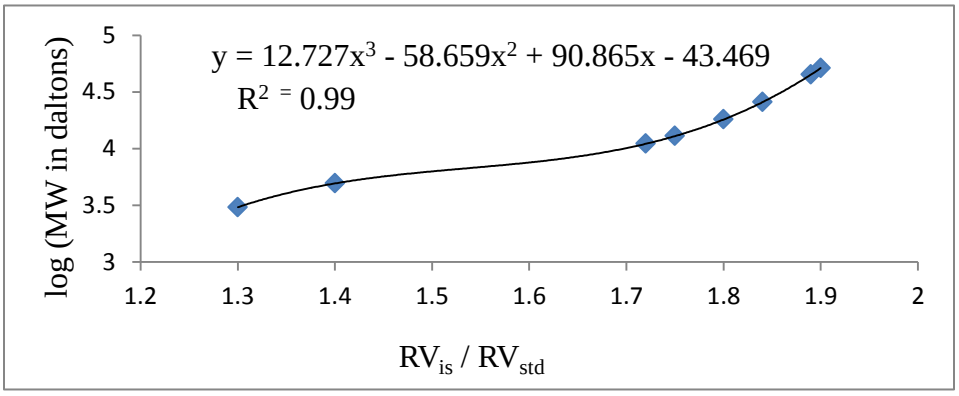


Figure 2.1: **CS standard curve**: Y- axis= log (molecular weight in daltons), X-axis= (retention volume of internal standard / retention volume of CS standard)

Table 2.2: Calculated molecular weights of CS samples

| CS sample | MW in kDa |
|---|---|
| Sample A | 7 |
| Sample B | 8 |
| Sample C | 21 |
| Sample D | 20 |
| Sample E | 31 |
| Sample F | 31 |
| Sample G | 35 |
| Sample H | 35 |

Molecular weights of CS samples were calculated using the standard curve equation shown in Figure 2.1. Table 2.2 presents the calculated molecular weights. Samples E and F were of same molecular weight (31 kDa). Same is the case with samples G and H (35 kDa). Molecular weights of samples A (7 kDa) and B (8 kDa) as well as C (21 kDa) and D (20 kDa) differ by only one kDa from each other.

**2.2 Determination of disaccharide composition:**

CS represents a highly heterogeneous group of unbranched polysaccharides characterized by alternating disaccharide units of N-acetyl galactosamine and glucuronic acid as seen in Figure 1.2. Heterogeneity of CS is partly due to the presence of sulfate groups in different amounts and in various positions (2 and 3 of uronic acid, and 4 and 6 of N-

acetyl-galactosamine residues) (Nicola Volpi, 2004). Chondroitinase ABC and chondroitinase AC II have been used to hydrolyze CS into its component disaccharides. The hydrolysis was then followed by quantitative determination of those disaccharides using capillary electrophoresis (Bayliss et al., 1999; Karamanos, Axelsson, Vanky, Tzanakakis, & Hjerpe, 1995; Malavaki et al., 2008; N. Volpi, 2004), strong anion exchange –HPLC (N. Volpi, 2003), precolumn derivatization and fluorescence detection (J. P. Du & Eddington, 2002), and HPLC with UV detection (J.-S. Sim et al., 2007). Chondroitinase ABC hydrolyzes both chondroitin sulfate and dermatan sulfate (sometimes referred to as chondroitin sulfate B) whereas chondroitinase AC II is specific for CS(Ji, Roman, Zhou, & Hildreth, 2007).

Disaccharide compositions of CS samples used in this study were determined by enzymatic hydrolysis of CS samples by chondroitinase AC II followed by ion-pairing reversed-phase liquid chromatography (LC) – UV method developed and validated by Ji et al (Ji et al., 2007).

2.2.1. Materials and methods

Briefly, CS samples were incubated with chondroitinase AC II, an enzyme specific to chondroitin sulfate for 3 hours at 37 $^{o}$C to hydrolyze CS into its component disaccharides. Disaccharides were then separated and quantified by ion-pairing liquid chromatography with ultraviolet detection at 240 nm. For detailed protocol, refer to Appendix 2.

2.2.2. Results:

Percentage disaccharides present in CS samples were determined as outlined in Appendix 2. Table 2.3 presents the disaccharide composition of CS samples used in this study. Percentage disaccharide compositions were reported as % mean ± standard error.

Table 2.3: Disaccharide composition of CS samples (n=4)

| **Sample** | **% ΔDi-0S (Mean±SE)** | **%ΔDi-4S (Mean±SE)** | **%ΔDi-6S (Mean ±SE)** |
|---|---|---|---|
| A | 6.91 ± 0.4 | 66.61 ± 3.7 | 26.49 ± 3.2 |
| B | 7.36 ± 0.7 | 00.00 ± 0.0 | 92.64 ± 0.7 |
| C | 9.06 ± 1.5 | 64.21 ± 2.0 | 26.73 ± 0.5 |
| D | 9.44 ± 1.7 | 63.73 ± 2.3 | 26.83 ± 0.6 |
| E | 10.14 ± 1.9 | 68.84 ± 3.3 | 21.01 ± 1.3 |
| F | 8.56 ± 1.4 | 75.58 ± 2.8 | 15.87 ± 1.3 |
| G | 11.11 ± 1.7 | 00.00 ± 0.0 | 88.89 ± 1.7 |
| H | 10.50 ± 1.5 | 00.00 ± 0.0 | 89.50 ± 1.6 |

Synthetic CS samples B, G and H had no measurable 4-sulfated disaccharide and had approximately 90% of 6-sulfated disaccharide. Sample F (from bovine trachea) had the highest percentage of 4-sulfated disaccharide. Percentage of 4 – sulfated disaccharide varied little between samples A, C, D and E. Samples C and D were from bovine cartilage, sample E was from porcine source and A was synthetic.

Objective 1 was completed by characterizing the CS samples used in this study in terms of their molecular weights and disaccharide compositions. In later chapters, the relationship between the above mentioned parameters and *in vitro* absorption and anti-inflammatory characteristics of CS samples were determined.

# CHAPTER 3

# DETERMINATION OF IN VITRO ABSORPTION OF CS SAMPLES AND ITS RELATIONSHIP WITH MOLECULAR WEIGHT

## 3.1 Introduction

The variation in molecular weights and sulfation patterns make CS a heterogeneous molecule. Although speculations have been made regarding differences in the bio-availability of CS (A. O. Adebowale, Cox, Liang, & Eddington, 2000; Cho et al., 2004; J. Du et al., 2004; Palmieri et al., 1990; N. Volpi, 2003), due to its structural variations, no detailed study has yet been conducted to investigate the effects of these variations on absorption. Even though clinical trials or animal studies may provide a deeper insight into the molecular weight: bioavailability relationship of CS, the tedious and expensive nature of such studies demands a preliminary screening tool which can identify candidates/samples that might have higher bioavailability and could potentially be included in a clinical trial. To this end, *in vitro* models of intestinal absorption can provide an alternative to *in vivo* studies.

Currently, *in-vitro* models are being used extensively by researchers to understand mechanisms of absorption of nutrients and transportation of drugs across the intestinal lining. As reviewed by Le Ferrec et al., (Le Ferrec et al., 2001) in a report on 'In vitro models of the intestinal barrier', mechanisms of transportation across the intestinal barrier include transcellular transportation (carrier mediated or active transportation, endocytosis and pinocytosis), paracellular transportation or passive diffusion through the tight

junctions (Figure 3.1). Mode of transportation depends on the physicochemical properties of the compound such as structure, molecular weight or size, charge distribution and hydrophobicity (Le Ferrec et al., 2001).

As identified in the report and recommendations of European Center for the Validation of Alternative Methods (ECVAM) some examples of in vitro models of intestinal absorption include- (i) everted gut sac of the rat small intestine, (ii) isolated and perfused intestinal segments, (iii) Ussing chambers, (iv) cell models, etc. Cell models include isolated human intestinal epithelial cells, small-intestine cell lines from fetal and neonatal rats, non-intestinal cell systems like Madin Darby canine kidney (MDCK) cells, and human adenocarcenoma cell lines like HT-29 and Caco-2 (Le Ferrec et al., 2001).

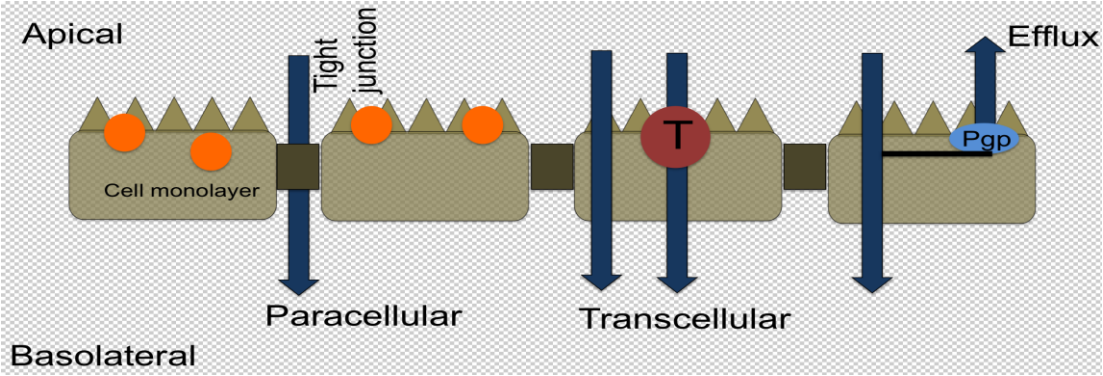


Figure 3.1: Mechanisms of transportation across small intestinal epithelial cells.

Caco-2 cells are isolated from a primary colonic tumor. When cultured under standard cell culture conditions, they spontaneously differentiate into polarized enterocytes characterized by apical and basolateral sides, presence of tight junctions (Figure 3.2) and

expression of most of the small intestinal brush border enzymes and transporters (Table 3.1).

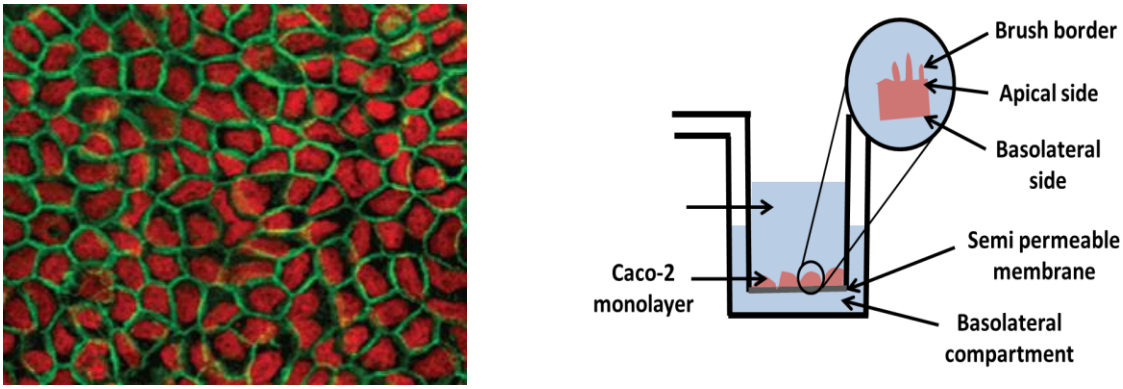


Figure 3.2: (Left) Confocal analysis of occludin staining (green) revealing tight junctions status in the CacoReady™ cell barrier (made available by ADMEcell, Emeryville, CA). (Right) Pictorial representation of a well from CacoReady™ plate.

Note: CacoReady™ is a trademark of ADVANCELL. ADMEcell is a licensed distributor of CacoReady™ 24-well kit by ADVANCELL.

Similar to intestinal transportation/absorption of nutrients, Caco-2 cell monolayer also facilitates transportation by transcellular and paracellular pathways. In a review article comparing drug transport in Caco-2 monolayers with intestinal drug transport *in vivo*, Artrusson et al. concluded that Caco-2 monolayers can be used to predict drug transport by different pathways across the intestinal epithelium. It was also reported that the best correlation to the in vivo situation was obtained for drugs that are absorbed by the paracellular transportation mechanism (Artursson, Palm, & Luthman, 2001).

Table 3.1: Characteristics of parental Caco-2 cells*

| | |
|---|---|
| Origin | Human colorectal adenocarcinoma |
| Differentiation | 14-21 days after confluence in standard cell culture medium |
| Morphology | Polarised cell monolayer with tight junctions and apical brush border |
| Electrical parameters | High electrical resistance |
| Digestive enzymes | Membranous peptidases and disaccharidases of the small intestine |
| Receptors/Transporters | Permeability glycoprotein (p-glycoprotein); multidrug resistant associated protein; glucose transporters (GLUT)- GLUT1, GLUT2, GLUT3, GLUT5; Sodium-glucose transporter (SGLT1); vitamin $B_{12}$; vitamin $D_3$ . |

*Adapted from (Le Ferrec et al., 2001)

Paracellular transportation was identified as the primary mechanism of transportation of CS disaccharides, and low molecular weight and intact CS (Jin et al., 2010; J. S. Sim et al., 2005). Hence, the Caco-2 monolayer model was chosen for this study to examine the relationship of absorption of CS with its molecular weight and/or sulfation patterns.

### 3.2. Materials and methods:

According to the protocol provided by ADMEcell, CacoReady™ is a new ready-to-use concept for *in vitro* intestinal absorption evaluation. This kit provided a 21-cell barrier in integrated HTS Transwell® -24 (A registered trademark of Corning Lifesciences) with an exclusive and proprietary shipping medium that is stable at room temperature. The kit of differentiated Caco-2 cell barriers was shipped on day 13 of differentiation and provided 14- day polarized cultures of Caco-2 cells on polycarbonate micro-porous filters in HTS Transwell® -24 plates (6.5 mm diameter, 0.33 $cm^2$ area and 0.4 μm pore diameter).

Plate receipt and preparation:

1. As per the suggestion of supplier, all permeability experiments were performed on Mondays after the CacoReady™ kits were received. Exactly two weeks (14 days) prior to shipping the Caco-2 cells were plated in the CacoReady™ plates on a Monday. Between days 14- 21, Caco-2 cells differentiate and become confluent to form a polarized monolayer, with tight junctions and apical brush border, morphologically resembling enterocytes. The Monday after receipt of CacoReady™ plates (day 21) is ideal for performing permeability experiments.
2. The CacoReady™ plates were received on day 18, Friday before permeability experiments. Upon receipt, the plates were unwrapped and inspected for leakages and then placed in a biosafety cabinet (BSC) (Labconco, Kansas city, MO. Catalog # 3430009) at room temperature until the time for changing the shipping medium.

3. Shipping medium in the plates was changed following a 4 hr incubation in a 37 $^{o}$C and 5% $CO_2$ incubator (Symphony$^{TM}$ Forced Air, VWR) which allowed transport media to liquefy completely.
4. Caco-2 media was prepared according to the ADMECell protocol. Briefly, 50 ml of fetal bovine serum (FBS) (Invitrogen, Catalog # 26140), 5 ml of 200 mM L-glutamine (Invitrogen, Catalog # 25030) and 5 ml of Penicillin/Streptomycin (Invitrogen, Catalog # 15150-148) were added to 440 ml of Dulbecco's modified eagle medium (DMEM) (Invitrogen, Catalog # 10567). Prepared Caco-2 media was labeled and stored at 2-8 $^{o}$C and warmed to 37 $^{o}$C prior to use.
5. After 4 hrs incubation, the CacoReady™ plates were placed in the BSC and a new Costar 24 well plate (Costar 3526) was placed next to the CacoReady™ plates.
6. Lids of the new Costar plate and the CacoReady™ plate were removed and placed side by side facing upwards, oriented in the same direction. The apical plate from the CacoReady™ plate was gently lifted and placed on top of the new plate to get access to the basal wells of the CacoReady™ plates.
7. The transport media in the basal wells of CacoReady™ plate was aspirated using an alcohol sterilized aspirator. Then, the media from apical wells of CacoReady™ plate placed on new plate was also aspirated. A manifold (Drummond, Catalog # 3-000-097) was used to aspirate media from the apical wells to avoid accidental damage to the cell monolayer. The manifold tips were positioned against the well

walls and media was aspirated for 5-7 seconds. By default, 70 µl of media remained in each well after aspiration.

8. Caco-2 media was added to apical wells (230 µl) and basal wells (750 µl). The apical plate with media was then returned to the top of the basal CacoReady™ plate with media and covered with its lid. This plate was then incubated at 37 $^{o}$C, 5% $CO_2$ until the day of permeability experiments (Monday).

Preparation of samples:

1. Final concentrations of 30 mg/ml CS working samples were prepared by dissolving 150 mg of each CS sample in 50 ml sterile 1 X Hank's balanced salt solution (HBSS) transport buffer. HBSS transport buffer had a composition of 1 X Hank's balanced salt solution (HBSS) (Lonza, Catalog #b04-315Q) with 1.1 mM $MgCl_2$. $6H_2O$ (Amresco, Catalog # E525) and 1.3 M $CaCl_2$. $2H_2O$ (Amresco, Catalog # E506).
2. CS is water soluble, the samples dissolved completely in transport buffer. The samples were then aliquoted and stored at 2-8 $^{o}$C until the day of permeability study.
3. 20 µM propranolol hydrochloride (Sigma, Catalog # P0884) stock solution was prepared and serial dilutions were performed to prepare a set of 5 standards ranging between 0 µM – 20 µM. The same stock solution was also used to prepare 10 µM working solution of propranolol hydrochloride to be used a high permeability control.

4. Stock, standards, and working solutions of sodium Fluorescein (Sigma, Catalog # 46960) were prepared in a method similar to that of propranolol hydrochloride. Sodium fluorescein was used as a low permeability control.
5. A 200 μM Lucifer yellow CH dilithium salt (Sigma, Catalog # L0259) stock solution was prepared and serial dilutions were performed to prepare a set of 5 standards (conc. 0 μM- 200 μM). A working sample of 100 μM conc. was also prepared from the stock. Lucifer yellow was used to test integrity of the Caco-2 cell monolayer.

Measurement of Transepithelial Electrical Resistance (TEER):

1. On the day of permeability experiments (Monday) TEER measurement was performed using an Epithelial Volt-Ohm meter (EVOM) (World precision instruments, Catalog # EVOM2) on the CacoReady™ plate prior to any further processing. According to the supplier, plates with TEER < 1000 Ohm. $cm^2$ were considered unacceptable on which to perform permeability assays. All the plates met the required criteria.
2. The electrodes (probe) (World precision instruments, Catalog # STX 100) were sterilized by submerging the probe in 70% ethanol for 30 minutes, then equilibrated by submerging for another 30 minutes in Caco-2 media at room temperature.
3. While equilibrating the probe, the CacoReady™ plate was also removed from the incubator and placed in the BSC to allow it to reach room temperature. TEER

measurements were performed at room temperature according to CacoReady™ supplier's instructions.

4. Briefly, the shorter electrode of the probe was slightly submerged inside the culture medium of the apical (upper) compartment and the longer arm was placed through the lateral hole of the CacoReady™ plate to submerge it in the medium of the basal (lower) compartment. Care was taken not to touch the cell monolayer with the electrode.
5. EVOM measures the resistance in Ohms. TEER was calculated using Equation 3.1.

$$\boxed{TEER = (R \times A)\ \Omega.\ cm^2} \quad \ldots\ldots\ldots\ldots\text{Equation 3.1}$$

Where, R is resistance measured by EVOM in Ohms

A is the area of cell monolayer, which is 0.33 $cm^2$

TEER > 1000 Ohm $cm^2$ was considered acceptable for permeability assay.

Apical to basal permeability protocol:

As mentioned earlier, all permeability assays were performed on a Monday. Working solutions of test compounds were prepared as outlined in the sample preparation section. The CS samples, blank transport buffer, high permeability and low permeability controls, and Lucifer yellow were all warmed to 37 $^oC$ prior to the experiments. The following steps were followed:

1. A sterile cell culture medium container placed in the BSC was filled with 50 ml of transport buffer at 37 $^{o}$C, kept warm in a water bath (Lab companion, Model BS-21).
2. The CacoReady™ plate and a new Costar basal plate were kept next to each other in same direction. The lids of both plates were removed and placed next to the plates.
3. The apical (upper) section of the CacoReady™ plate was gently lifted and placed on the new Costar basal plate.
4. The medium from the basal compartment of CacoReady™ plate was aspirated and discarded using an aspirator with a sterile tip.
5. The basal compartment of CacoReady™ plate was washed with 750 ul of transfer buffer per well. Following aspiration of the transport buffer wash, 750 µl of transport buffer was aliquoted per well again.
6. Cell culture medium from apical compartment wells was aspirated and discarded using a manifold. The apical wells were then gently washed by filling, aspirating and discarding 300 µl of transport buffer per well using a manifold.
7. As aspirating with a manifold leaves 70 µl of transport buffer in each well, the volume of apical samples was adjusted for a 300 µl total volume. Therefore, 230 µl each of samples, controls and blank transport buffer were filled in the apical wells to make a total apical volume of 300 µl according to the plate design (Figure 3.3).

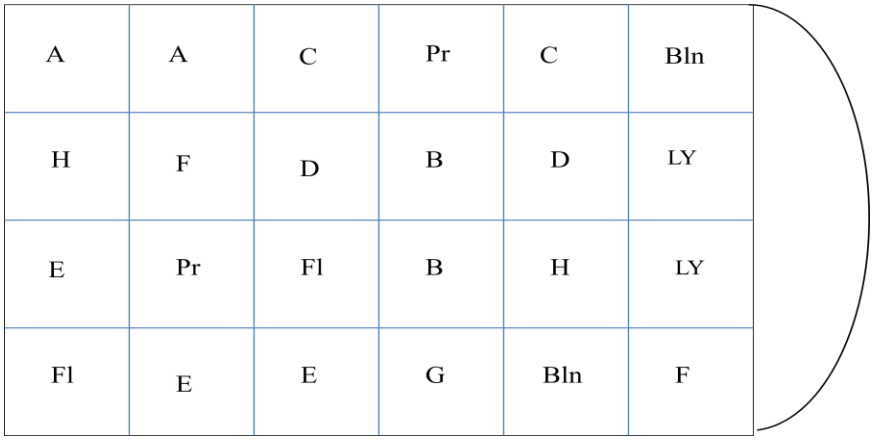


Figure 3.3: Caco-2 plate design. Samples are denoted by letters A-H. Bln=blank, Pr=propranolol hydrochloride, Fl=sodium fluorescein and LY=Lucifer yellow.

8. The apical section with samples was then gently lifted from the Costar basal plate and placed back on the CacoReady™ plate with transport buffer filled wells. The Costar plate was discarded after this.
9. CacoReady™ plate was then placed in the incubator at 37 $^{o}$C, 5% $CO_2$ for 2 hours.
10. After 2 hours, the CacoReady™ plate was removed from the incubator and placed in the BSC. A new Costar basal plate was placed next to it and lids of both plates were removed.
11. The apical section of CacoReady™ plate was gently lifted from the CacoReady plate and placed on top of the new Costar basal plate.
12. Samples from apical wells (now kept on top of the Costar plate) and basal wells of the CacoReady™ plate were then collected separately into 1 ml Eppendorf tubes, labeled and stored at -20 $^{o}$C until analysis.

13. Quantitative analysis of CS in apical and basal samples was done by enzyme hydrolysis and RP-HPLC outlined in Appendix 2.
14. Apical and basal concentrations of propranolol, sodium fluorescein and lucifer yellow were analyzed by Fluorescence spectrophotometry (SpectraMax M2e, Molecular Devices, LLC., Sunnyvale, CA). Briefly, standard curves were obtained for each compound by measuring their OD values of respective standards of known concentrations. OD values for propranolol (sodium fluorescein and lucifer yellow) were measured at absorption wavelength ($\lambda_a$) of 280 nm (460 and430 nm) and emission wavelength ($\lambda_e$) of 320 nm (515 and 540 nm). Standard curves were plotted with concentration of sample (propranolol or fluorescein or Lucifer yellow) on y-axis against their OD values on x-axis. The standard curves equations were then used to calculate concentrations of propranolol, sodium fluorescein and lucifer yellow samples from the permeability experiment.
15. Once the concentrations of all the apical and basal samples were determined, coefficients of effective permeability $P_{eff}$ ($cm.s^{-1}$) were calculated using the following relationships:

$$\boxed{P_{eff} = V_R.\,(dC/dT) / A.\,C_0} \quad \text{.....Equation 3.2}$$

Where $V_R$ = Volume of transport buffer in basal or receiving compartment ($cm^3$)
A= area available for absorption (surface area of the cell monolayer = 0.33 $cm^2$)
$C_0$= Initial concentration of the sample in apical compartment ($mg.mL^{-1}$)

(dC/dT ) = Change in concentration of sample with respect to time.

In this case, dC/dT was calculated as $(C_{f-} C_i)/\Delta T$. Where $C_f$ and $C_i$ were initial and final concentrations of sample in the basal compartment. And, $\Delta T$ was duration of permeability which was 2 hours (7200 seconds).

16. The coefficients of effective permeability of all 8 samples as well as control samples (propranolol hydrochloride and sodium fluorescein) were determined using equation 2.1. Values were based on the means of two replicates per plate in two plates (a total of four replicates).
17. Analysis of variance (ANOVA) test was used (JMP Pro 10) to test for differences between samples at (p-value $\leq 0.05$) as well as inter plate and intra plate variability.
18. A one way student t-test was performed to compare sample means. Differences were considered significant at p-value $\leq 0.05$.

**3.3 Results:**

Absorption of CS samples was expressed as coefficients of effective permeability ($P_{eff}$) as outlined in methods. The $P_{eff}$ values for the samples A through H, propranolol hydrochloride (high permeability control), sodium fluorescein (low permeability control) and lucifer yellow (indicator of cell monolayer integrity, not shown in figure) are presented in Figure 3.4. The $P_{eff}$ values of lucifer yellow and sodium fluorescein were both zero. This, in addition to the TEER value >1000 $\Omega$ $cm^2$, guarantees the integrity of Caco-2 monolayers used in this study.

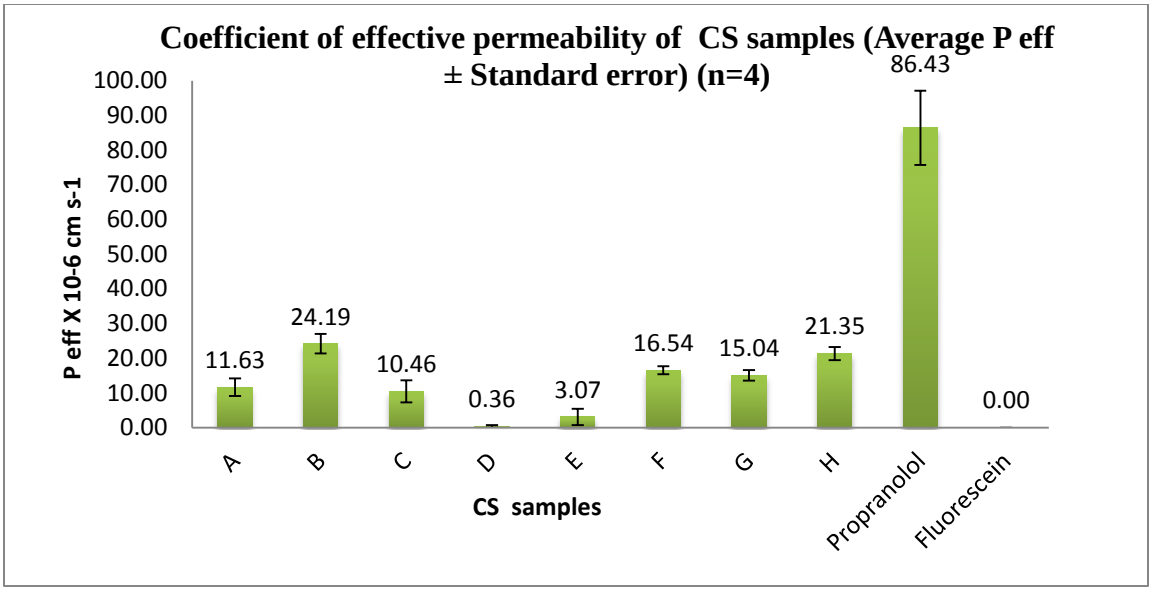


Figure 3.4: Coefficients of effective permeability ($P_{eff}$) for CS samples and control samples. Mean $P_{eff}$ value for each sample is represented on top of column for each sample.

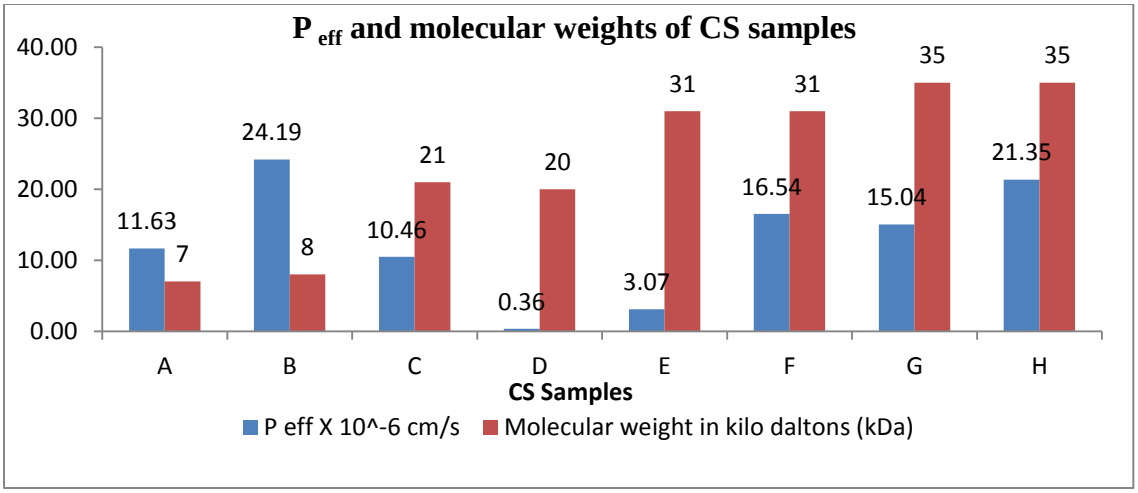


Figure 3.5: Trends of MW of CS samples and their absorption in terms of $P_{eff}$. Molecular weights (in kDa) and of $P_{eff}$ (in $cm.s^{-1}$) are represented on top of each column for respective samples.

As seen from Fig. 3.5, which shows the variation in coefficient of effective permeability of samples with the MW, no correlation is apparent concurring with the coefficient of effective permeability and molecular weight relationship presented in Fig. 3.4. Table 3.1 presents ranking of CS samples in descending order of their $P_{eff}$ values. Samples D and E had relatively low permeability coefficients and were significantly different from rest of the samples. Samples A, C, F and G had permeability coefficients ranging between 10-16 x $10^{-6}$ $cm.s^{-1}$, but did not significantly differ from each other. Samples F, G and H whose permeability coefficients were between 15- 21 x $10^{-6}$ $cm.s^{-1}$ were also not significantly different from each other ($p>0.05$). Importantly, the $P_{eff}$ of sample B appeared to be significantly higher than rest of the samples except for sample H. Of the

four synthesized samples (A, B, G and H), sample A had a significantly lower permeability than the others.

Table 3.2: Ranking of CS samples based on coefficient of permeability

| **Sample** | **Mean (P eff x $10^{-6}$ cm. $s^{-1}$ )** |
|---|---|
| Sample B | $24.19^{(\alpha)}$ |
| Sample H | $21.34^{(\alpha)(\beta)}$ |
| Sample F | $16.53^{(\beta)(\gamma)}$ |
| Sample G | $15.04^{(\beta)(\gamma)}$ |
| Sample A | $11.63^{(\gamma)}$ |
| Sample C | $10.46^{(\gamma)}$ |
| Sample E | $3.06^{(\delta)}$ |
| Sample D | $0.35^{(\delta)}$ |

Note: Levels not connected by same Greek letter are significantly different ($P<0.05$)

Bivariate fits were obtained to determine any correlations between absorption and molecular weight, percentage 6-sulfated fraction and, total sulfated fraction of CS in samples. Pearson correlation factor '$R^2$' was used to assess the above mentioned relationships. $R^2$ between and 0.5 to 1.0 were considered high.

Relation between coefficient of effective permeability and molecular weight:

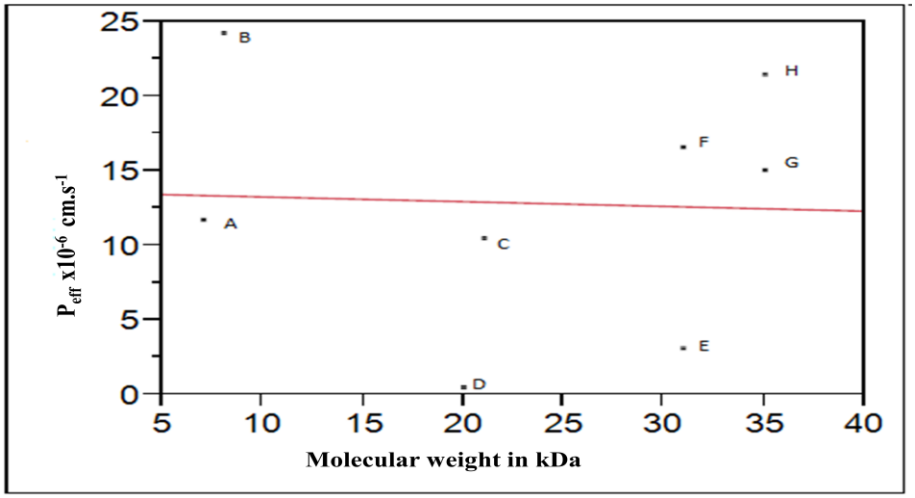


Figure 3.6: Bivariate fit of $P_{eff}$ x $10^{-6}$ (cm.$s^{-1}$) and molecular weight (kDa)

As seen in Fig. 3.6, there appears to be no correlation ($R^2$<0.5, P>0.05) between the permeability coefficient and molecular weight of CS samples within the molecular weight range of samples used in this study (7 kDa to 35 kDa).

Relation between coefficient of effective permeability and percentage of 6-sulfated disaccharide in CS sample:

The bivariate fit of $P_{eff}$ and % ΔDi- 6S in CS sample resulted in *R*-square value of 0.5, P = 0.0477 which revealed that these two parameters were correlated, albeit weakly.

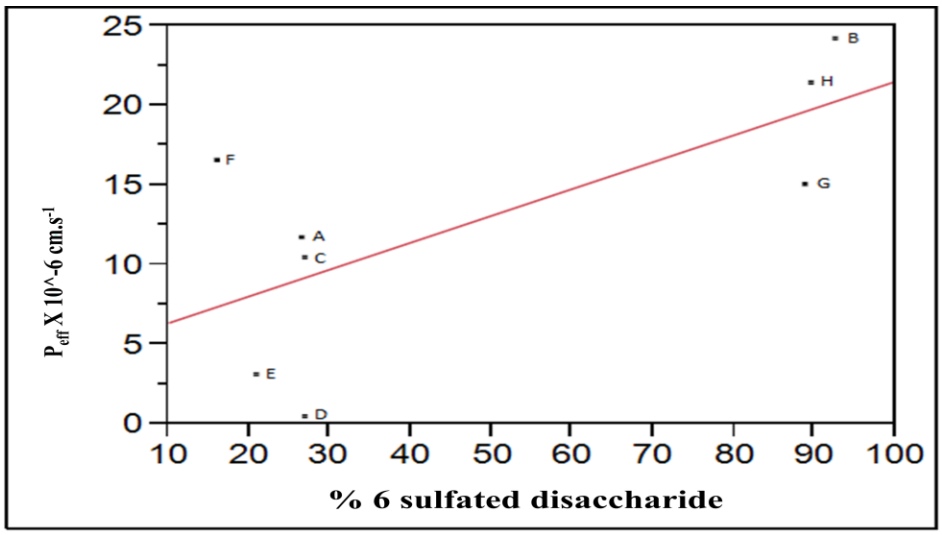


Figure 3.7: Bivariate fit of $P_{eff}$ x $10^{-6}$ ($cm.s^{-1}$) and % ΔDi-6S

As seen in Fig. 3.7, except for sample F, all the samples with % 6 S–disaccharide < 30% had a $P_{eff}$ value < 15 x $10^{-6}$ $cm.s^{-1}$. Among synthetic CS samples (A, B, G and H), only sample A had percentage 6-sulfated disaccharide less than 30% and incidentally, had the lowest $P_{eff}$ value. Samples B and H that are higher in 6-sulfated disaccharides also have higher permeability coefficients.

Relation between coefficient of effective permeability and total percentage of disaccharides in CS sample:

The bivariate fit of $P_{eff}$ and percentage of total sulfated disaccharides (sum of % 4S and %6S, reminder unsulfated) in CS samples resulted in an $R^2$ value of 0.03 strongly suggested that no correlation existed between percentage of total sulfated disaccharides (also termed as amount of sulfation) and coefficient of effective permeability (absorption) of CS samples tested. However, as evident from Fig. 3.8, percentage of total sulfated disaccharides varied little between the samples (range 89%- 93%). Samples with wider range of variable sulfated disaccharide percentages are needed to verify this observation.

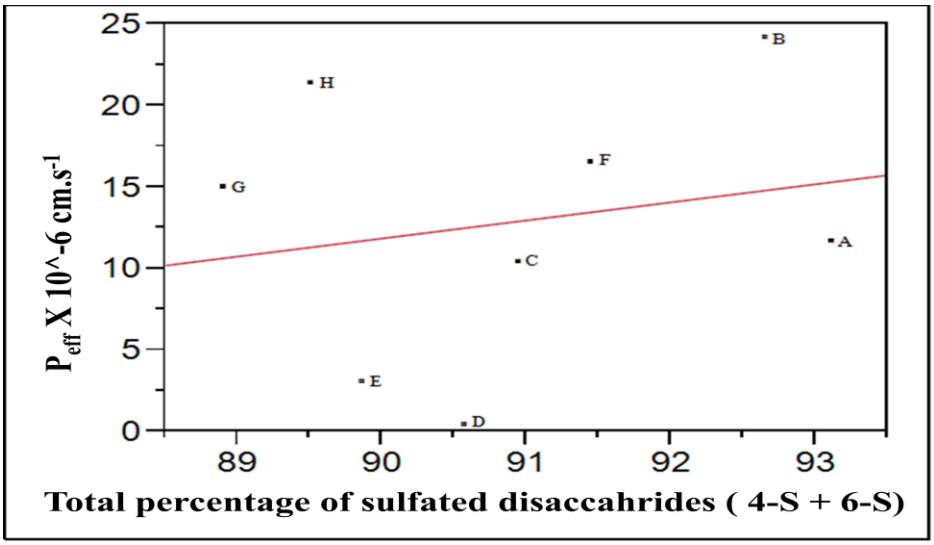


Figure 3.8: Bivariate fit of $P_{eff}$ x $10^{-6}$ ($cm.s^{-1}$) and total % of sulfated disaccharides

# CHAPTER 4

# DETERMINATION OF IN VITRO ANTI-INFLAMMATORY ACTIVITY AND ITS RELATIONSHIP WITH MOLECULAR WEIGHT OF CS SAMPLES

## 4.1 Introduction

Inflammation plays a major role in the progression of osteoarthritis (OA) (Bonnet & Walsh, 2005; Brooks, 2003; Dieppe, 1978; Ehrlich, 1975; Peyron, 1981). Synovitis (the inflammation of synovial membranes) is often present at the site of the joint affected by OA. Inflammation is known to be more common in advanced stages of OA rather than the early stages (D'Agostino et al., 2005). Synovial fluid from patients with OA often exhibits increased volume, reduced viscosity, and elevated white blood cells which are predominantly mononuclear (monocytes and lymphocytes) (Altman & Gray, 1985).

Cytokines are cell-produced chemical signals that play a crucial role in cell-cell interactions. They are often classified as catabolic, anti-catabolic, modulatory or anabolic with respect to their role in regulation of chondrocyte function. In the case of OA, catabolic cytokines promote degradation of cartilage; while anti-catabolic cytokines inhibit or antagonize the activity of catabolic cytokines. Anabolic cytokines act as growth and differentiating factors on chondrocytes and increase their activity. Modulatory cytokines are the ones that regulate the activity of other cytokines. IL-6 is a modulatory cytokine whereas TNF-$\alpha$ and IL-1$\beta$ are catabolic cytokines (S. R. Goldring & Goldring, 2004).

C-reactive protein is an acute phase protein and is a marker of systemic inflammation. In a clinical study comparing rapid versus slow progressive hip arthritis, the serum C-reactive protein (CRP) levels in a rapid progressive hip arthritis group were reported to be significantly higher (over 2 times) than individuals having the slow progressive form (Conrozier et al., 1998). In another clinical study of subjects with early knee OA, slight but definite, increased serum CRP levels were reported in subjects whose disease progressed over the next four years suggesting that low grade inflammation may be a significant aspect of early OA and could be a target for pharmacological intervention (Spector et al., 1997).

These results are supported by several review articles which discuss the role of cytokines in OA pathophysiology and emphasize the importance of pro-inflammatory (catabolic) cytokines in the initiation and progression of the disease (Fernandes et al., 2002; M. B. Goldring, 2000; Mary B. Goldring & Otero, 2011; Hedbom & Hauselmann, 2002). In synovial tissue obtained from patients with early and late OA, an over expression of pro-inflammatory cytokines TNF-α and IL-1β were seen in early OA, compared with late OA. Increased monocyte infiltration was also reported to be present in early OA (Benito et al., 2005). Macrophages derived from these monocytes are a source of inflammatory cytokines. Nitric oxide (NO) is another signaling molecule that plays a role in the pathogenesis of inflammation. NO is synthesized when arginine is converted to citrulline. In a review by Abramson on the role of NO and reactive nitrogen species (RNOS) in OA, it had been suggested that NO may not only act as an inflammatory mediator but also might have protective functions in individual cell types (tendons and osteoblasts) in

arthritic joints. It was also reported that not all chondrocytes produce equivalent amounts of NO (Steven B. Abramson, 2008).

Based on an intensive literature search for the role of inflammation in OA and the associated specific inflammatory markers, four inflammatory markers (TNF-α, IL-1β, IL-6 and NO) were identified as candidate biomarkers for assessing the anti-inflammatory (AI) activity of the CS samples in our studies. (S. B. Abramson, 2004; Steven B. Abramson, 2008; Pelletier et al., 1991; Scanzello et al., 2008; Shinmei, Masuda, Kikuchi, & Shimomura, 1989; Shinmei & Nemoto, 1996; Smith, Triantafillou, Parker, Youssef, & Coleman, 1997; Westacott & Sharif, 1996). Compounds that can regulate expression of these inflammatory markers may be able to perhaps delay and/or attenuate the progression of OA.

CS is currently being used as a symptomatic slow acting drug for osteoarthritis (SYSADOA) and had been shown to be effective in management of pain and improvement in functionality of joints in several animal studies and clinical studies. Understanding the mechanisms of action of CS might provide insight into the improved efficacy if this compound as a remediation for OA. Our study was designed to examine the effect of CS on the aforementioned inflammatory biomarkers *in vitro.*

Murine macrophage cell lines (RAW 264.7) were chosen as the model for the AI activity experiments as macrophages are known to play an important role in inflammatory response (Bondeson et al., 2010; Bondeson, Wainwright, Lauder, Amos, & Hughes, 2006). When stimulated by bacterial lipopolysaccharide (LPS), RAW 264.7 cells were

reported to express various cytokines, reactive oxygen and nitrogen species, growth factors etc., thus supporting this cell line's use as an appropriate *in vitro* model for assessing AI activity of various compounds (Achoui et al., 2010; Chun et al., 2007; Gao et al., 2012; Jung & Sung, 2004; Kang et al., 2011; Kim, Cheon, Kim, Kim, & Kim, 1999; Moro et al., 2012; Oh, Cho, Jeong, et al., 2012; Oh, Cho, Oh, et al., 2012; Suh et al., 1998; Terra et al., 2007; Tuntipopipat, Muangnoi, & Failla, 2009; Yoon et al., 2009; Zong et al., 2012).

One of the objectives of this research project was to determine the AI activity of 8 different CS samples with variable MW and sulfation patterns and correlate them, to the AI activity. AI activity was quantified by measuring the levels of the respective inflammatory markers expressed in the presence of each CS sample when the cells were stimulated by the powerful pro-inflammatory mediator, bacterial LPS. Two different concentrations (low and high) of each CS sample were used to verify if there existed an AI activity dose response.

**4.2 Materials and methods:**

Briefly, RAW 264.7 cells were cultured under standard cell culture conditions. Viable cells were then plated and incubated at 37 $^{o}$C and 5% $CO_2$ prior to addition of CS samples to facilitate adhesion of cells to the wells. CS samples were then added to the wells and incubated at 37 $^{o}$C and 5% $CO_2$ one more hour before adding LPS. This one hour incubation with CS samples was done to facilitate absorption of CS by the cells before addition of LPS. Once the cell supernatant was removed after the experiment,

MTS assay was performed on the cells in the wells. This was done to ensure that the concentrations used in this protocol, neither CS samples not LPS were toxic (causing cell death) to the cells. The detailed protocol for the experiment is described below.

Culturing of RAW 264.7 cells (Eton Bioscience, Inc. San Diego, CA. Catalog # 2800080012):

1. RAW 264.7 cells were thawed rapidly (for approximately 2 minutes) in a water bath at 37 $^{o}$C by gently agitating the vial. After thawing was complete, the surface of the vial was chemically sterilized by a solution of 70% ethanol. All operations from this point were carried out under strict aseptic conditions in a laminar flow biosafety level -2 (BSL-2) hood.
2. Contents of the vial were then transferred into a centrifuge tube containing 9.0 ml complete culture medium (DMEM + 10% FBS + 2mM L-glutamine) and centrifuged at 2795 g for 5 minutes.
3. The cell pellet was then resuspended in 10 ml of complete culture medium and was dispensed into a 75 $cm^2$ cell culture flask.
4. Cells were then incubated at 37 $^{o}$C and 5% $CO_2$ until 70-80% confluency (usually attained in 48 hours) was achieved.
5. Sub culturing was done at 80% confluency by trypsinizing and resuspending the cells in new cell culture flasks containing the culture medium and incubated at 37 $^{o}$C, 5% $CO_2$.

6. Cells were cultured as described above until sufficient numbers of viable cells were obtained for each experiment. Viability of cells was determined by Trypan blue exclusion assay and a hemocytometer.

Preparation of CS samples:

As described earlier, each CS sample was prepared in two different concentrations. Final concentrations of low and high concentration samples were 5 µg $ml^{-1}$ and 15µg $ml^{-1}$ respectively. Samples were prepared by dissolving accurately weighed amounts of CS samples in complete cell culture media. All CS samples were completely soluble in the media. Prepared samples were aliquoted and stored at 2-8 $^{o}$C until use. Samples were not stored beyond 7 days.

LPS was also prepared in complete cell culture media. The stock solution was prepared to obtain a final concentration of 1 µg $ml^{-1}$ LPS when 5 µl of this stock solution was added to the wells. Prepared LPS stock solution was stored at 2-8 $^{o}$C. According to the supplier, stability of LPS stock solution was estimated to be 4 months.

Protocol for anti-inflammatory activity experiments:

1. Costar 96 well tissue culture plates were plated with $10^5$ cells/100 µl of complete cell culture media per well.

2. The plates were then placed in an incubator at 37 $^{o}$C and 5% $CO_2$ for 18-20 hours to ensure adhesion of cells to the tissue culture plate.

3. After 18 hours, the cell supernatant was aspirated and discarded. 100 µl of fresh complete cell culture media was added to the wells.

4. After adding fresh media, the cells were incubated at 37 $^{o}$C and 5% $CO_2$ for another hour before adding the CS samples (low or high concentrations respectively).

5. Forty five µl of CS samples (to sample wells) or blank media (to blank wells and LPS control wells) were added to the wells according to plate design. Each sample was plated in triplicate.

6. Following the addition of CS samples, the plates were incubated at 37 $^{o}$C and 5% $CO_2$ for one hour to facilitate uptake of CS by the RAW 264.7 cells.

7. After this time, 5 µl of LPS was added to all wells except blank wells. 5 µl of complete cell culture media was added to blank wells. Plates were returned to the incubator and left for 4 hours.

8. After 4 hours, the cell supernatant was carefully transferred into another 96 well plate and stored at -20 $^{o}$C until analysis for inflammatory biomarkers.

9. Once the cell supernatant was removed, the MTS assay (see below) was performed on the cells to examine cell viability.

MTS assay for cell viability:

CellTiter 96® AQueous One Solution reagent was thawed and a stock solution was made by combining the MTS reagent with cell culture medium according to the kit protocol. One hundred and twenty µl of stock solution was pipetted into each well containing the cells. The cells were then incubated at 37 $^{o}$C, 5% $CO_2$ in an incubator for 30-60 minutes to allow full color development. After incubation, the absorbance values (OD) of the samples in that 96 well plate were measured at 490 nm. The amount of colored product formed is proportional to the number of live cells in culture. Taking viability in blank wells (cells with no sample or LPS) as reference, percentage viability of cells treated with CS samples as well as cells treated with LPS was calculated using Equation 4.1.

$$\% \text{ cell viability} = 100*(OD_S / OD_B) \quad \text{.....Equation 4.1}$$

Where, $OD_S$ is mean absorption of samples and $OD_B$ is mean absorption of blank.

ELISA assays for TNF-α, IL-1β and IL-6: The protocols provided with Mouse Enzyme linked immunosorbent assay (ELISA) kits for TNF-α, IL-1β and IL-6 were followed to quantitatively determine the cytokines respectively. For detailed protocols, see Appendices 3, 4 and 5.

Protocol for nitric oxide (NO):

The level of NO production was monitored by measuring the nitrite concentration in cell culture supernatant. Griess reagent kit was used for quantification of nitrite. For this assay, the supernatant obtained in step 8 in the aforementioned procedure was used. The

assay was performed according to the protocol provided with the kit. Refer to Appendix 6 for detailed protocol.

**4.3 Results:**

Cell viability (MTS assay):

As previously noted in methods, cell viability was calculated using Equation 7. 100% viability of cells was observed in cells treated with CS samples as well as cells treated with LPS only. This indicates that the CS samples were not toxic to the cells at the concentrations used (both low and high). 100% viability of cells treated with LPS alone indicates that LPS was not toxic to the cells at the concentration used. As could be seen from the results of the cytokine expression assays, 1μg/ ml concentration of LPS was able to stimulate an inflammatory response in RAW 264.7 cells without being excessively toxic and resulting in cell death.

TNF-α:

The levels of TNF-α were determined in blank samples, CS samples (low and high conc.) and LPS only samples following the procedure outlined in the methods section of this chapter. The samples were ranked in descending order of their TNF-α concentration for low conc. and high conc. CS samples respectively. Difference between samples was determined using t-test. Table 4.3.1 presents ranking of CS, blank and LPS samples in descending order of their TNF-α concentration values for CS at 5 μg/ml (low conc.) while Table 6 presents the same for CS at 15 μg/ml (high conc.).

Table 4.1: Ranking of CS samples (conc. 5 μg/ml) according to TNF-α concentration Samples compared by Student's t-test

| **Sample** | **Mean (conc. of TNF-α in pg/ml)** |
|---|---|
| **LPS** | **232.14$^{(\alpha)}$** |
| Sample D | 186.33$^{(\alpha)(\beta)}$ |
| Sample G | 178.10$^{(\alpha)\beta)}$ |
| Sample F | 170.46$^{(\alpha)(\beta)}$ |
| Sample E | 168.98$^{(\alpha)(\beta)}$ |
| Sample C | 145.96$^{(\alpha)(\beta)}$ |
| Sample H | 139.75$^{(\beta)}$ |
| Sample B | 123.99$^{(\beta)}$ |
| Sample A | 117.96$^{(\beta)}$ |
| **Blank** | **25.45$^{(\gamma)}$** |

Note: Levels not connected by same Greek letter are significantly different. ($p<0.05$)

From Table 4.1, it was noted that the TNF-α concentrations obtained for samples A, B, and H were significantly different from that of the LPS only samples. This indicated that the samples A, B and H, at concentration of 5 μg/ml, significantly lowered expression of TNF-α by the RAW-264.7 cells used in this study. Although the rest of the samples (C, D, E, F and G) also appeared to have TNF-α concentration lower than that of LPS, the difference was not significant.

Table 4.2: Ranking of CS samples (conc. 15 μg/ml) according to TNF-α concentration

Samples compared by Student's t-test

| **Sample** | **Mean (conc. of TNF-α in pg/ml)** |
|---|---|
| **LPS** | **232.14$^{(\alpha)}$** |
| Sample H | 215.24$^{(\alpha)(\beta)}$ |
| Sample G | 166.73$^{(\alpha)(\beta)(\gamma)}$ |
| Sample D | 140.76$^{(\alpha)(\beta)(\gamma)}$ |
| Sample C | 128.50$^{(\alpha)(\beta)(\gamma)(\delta)}$ |
| Sample E | 122.51$^{(\beta)(\gamma)(\delta)}$ |
| Sample F | 114.62$^{(\beta)(\gamma)(\delta)}$ |
| Sample A | 111.08$^{(\beta)(\gamma)(\delta)}$ |
| Sample B | 84.60$^{(\gamma)(\delta)}$ |
| **Blank** | **25.45$^{(\delta)}$** |

Note: Levels not connected by same letter are significantly different. ($P < 0.05$)

At 15 μg/ml concentration, samples A, B, E and F significantly lowered expression of TNF-α by the RAW 264.7 cells used in this study. Samples C, D, G and H did not show significant lowering of TNF-α from LPS samples at this concentration.

Relationships between amount of TNF-α expressed and MW, % 6 sulfated disaccharides, % total sulfated disaccharides of CS samples:

Bivariate fits were obtained to determine any correlations between percentage reduction in TNF-α values (of CS samples relative to LPS) and molecular weight, percentage 6-sulfated fraction and, total sulfated fraction of CS in samples. '$R^2$' was used to assess the above mentioned relationships. $R^2$ between 0.5 and 1.0 was considered high.

The bivariate fits showed $R^2 < 0.5$ indicating absence of any correlation between the percentage reduction of TNF-α and the MW of CS samples at both low and high concentrations of CS. Similarly, no correlation in percentage reduction of TNF-α with percentage 6-sulfated disaccharides at both high and low concentrations of CS were observed. No correlation between TNF-α lowering and percent total sulfated disaccharides was observed at low CS sample concentrations (5 μg/ml). However, at a concentration of 15 μg/ml, there appears to be slight correlation between TNF-α lowering effect of CS and total sulfation ($R^2 = 0.58$). But, as percentage of sulfated disaccharides did not vary much between the samples (range 89%- 93%), samples with a wider range of variable sulfated disaccharide percentages are needed to verify this relationship.

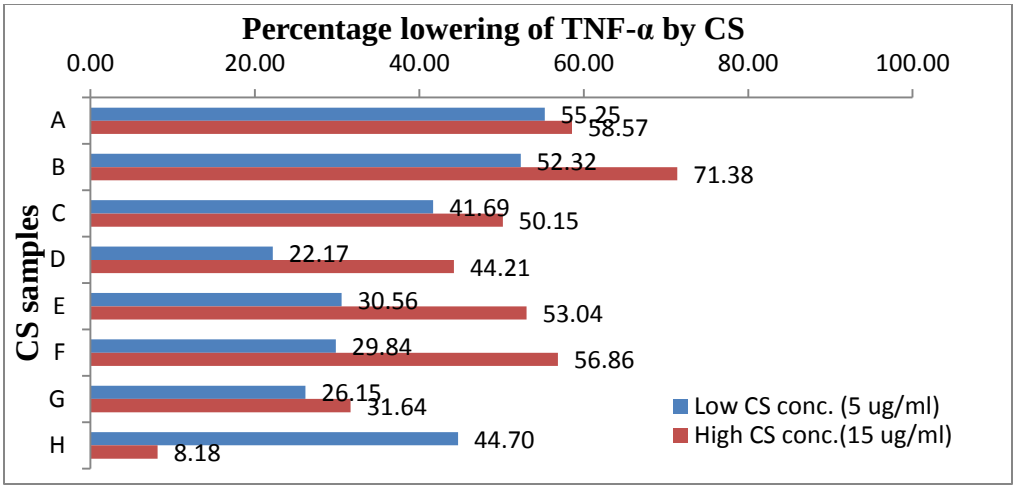


Figure 4.1: Percentage lowering of TNF-α by CS samples at low and high concentrations with respect to LPS only samples.

Figure 4.1 summarizes the percentage lowering of TNF-α by CS samples of low and high concentrations with respect to TNF-α expressed by cells stimulated with LPS in the absence of CS samples. From this figure, it can be seen that all of the CS samples were more effective at 15 µg/ml concentration than at 5 µg/ml concentration in reducing the TNF-α, except sample H. Sample B, at 15 µg/ml concentration appeared to have the highest anti-inflammatory (AI) activity in terms of its ability to lower expression of TNF-α and sample D appeared to have lowest AI activity.

IL-1β:

The levels of IL-1β were determined in blank samples, CS samples (low and high conc.) and LPS samples following the procedure outlined in the methods section. Samples were ranked in descending order of their IL-1β concentration for 5 µg/ml and 15 µg/ml CS samples respectively. Ranking was obtained by means comparison of each pair of

samples using student's t-test. Table 4.3 presents ranking of CS, blank and LPS samples in descending order of their IL-1β concentration values for CS at 5 μg/ml (low conc.) while Table 4.4 presents the same for CS at 15 μg/ml (high conc.). As seen from Tables 4.3 and 4.4, the IL-1β produced by the macrophage cells in blank and LPS stimulated conditions was not significantly different from each other. Because of this, under the cell culture conditions and methods used in current experiments, IL-1β was not useful in estimating the anti-inflammatory activity of the CS samples.

Table 4.3: Ranking of CS samples (conc. 5 μg/ml) according to IL-1β concentration

Samples compared by Student's t-test

| **Level** | **Mean (conc. of IL-1β in pg/ml)** |
|---|---|
| Sample B | $76.57^{(\alpha)}$ |
| **LPS** | $\mathbf{73.18^{(\alpha)(\beta)}}$ |
| **Blank** | $\mathbf{56.46^{(\alpha)(\beta)(\gamma)}}$ |
| Sample C | $53.84^{(\alpha)(\beta)(\gamma)}$ |
| Sample H | $51.93^{(\alpha)(\beta)(\gamma)}$ |
| Sample F | $48.29^{(\alpha)(\beta)(\gamma)}$ |
| Sample E | $38.13^{(\alpha)(\beta)(\gamma)}$ |
| Sample A | $37.90^{(\alpha)(\beta)(\gamma)}$ |
| Sample G | $26.91^{(\beta)(\gamma)}$ |
| Sample D | $25.01^{(\gamma)}$ |

Note: Levels not connected by same Greek letter are significantly different. ($P < 0.05$)

Table 4.4: Ranking of CS samples (conc. 15 μg/ml) according to IL-1β concentration

Samples compared by Student's t-test

| Level | Mean (conc. of IL-1β in pg/ml) |
|---|---|
| Sample C | $116.39^{(\alpha)}$ |
| **LPS** | $\mathbf{73.18^{(\alpha)(\beta)}}$ |
| Sample H | $61.74^{(\alpha)(\beta)}$ |
| **Blank** | $\mathbf{56.46^{(\alpha)(\beta)}}$ |
| Sample F | $54.75^{(\alpha)(\beta)}$ |
| Sample B | $53.50^{(\alpha)(\beta)}$ |
| Sample A | $37.38^{(\beta)}$ |
| Sample G | $35.90^{(\beta)}$ |
| Sample E | $31.71^{(\beta)}$ |
| Sample D | $29.85^{(\beta)}$ |

.

Note: Levels not connected by same letter are significantly different. ($P < 0.05$)

IL-6:

The levels of IL-6 were determined in blank samples, CS samples (low and high conc.) and LPS samples following the procedure outlined in the methods section. The high concentration CS group and low concentration CS group were ranked separately in order of IL-6 production by the macrophages in the presence and absence of those samples. Ranking was obtained by means comparison of each pair using student's t-test. Table 4.5 presents ranking of CS, blank and LPS samples in descending order of their IL-6

concentration values for CS at 5 μg/ml and Table 4.6 presents the same for CS at15 μg/ml.

Table 4.5: Ranking of CS samples (conc. 5 μg/ml) according to IL-6 concentration Samples compared by Student's t-test

| **Level** | **Mean (conc. of IL-6 pg/ml)** |
|---|---|
| Sample F | $445.41^{(\alpha)}$ |
| Sample D | $378.97^{(\alpha)(\beta)}$ |
| Sample A | $354.11^{(\beta)(\gamma)}$ |
| Sample C | $325.17^{(\beta)(\gamma)(\delta)}$ |
| **LPS** | $\mathbf{314.11^{(\beta)(\gamma)(\delta)}}$ |
| Sample B | $300.66^{(\gamma)(\delta)(\varepsilon)}$ |
| Sample E | $264.94^{(\delta)(\varepsilon)(\phi)}$ |
| Sample H | $233.44^{(\varepsilon)(\phi)}$ |
| Sample G | $223.86^{(\phi)}$ |
| **Blank** | $\mathbf{35.10^{(\chi)}}$ |

Note: Levels not connected by same Greek letter are significantly different. ($P < 0.05$)

As seen from Table 4.5, at 5 μg/ml CS concentration, baseline IL-6 produced by macrophages (from blank wells) is significantly less than that produced by the cells stimulated by LPS alone. However, cells incubated with some of the CS samples seem to have produced as much IL-6 as the LPS only cells. Samples, A, C, D and F, did not show

the IL-6 lowering effect. But, samples, B, E, G and H showed IL-6 levels less than that of LPS alone. Samples G and H especially were effective in lowering LPS stimulated IL-6.

Table 4.6: Ranking of CS samples (conc. 15 μg/ml) according to IL-6 concentration Samples compared by Student's t-test

| **Level** | **Mean (conc. of IL-6 in pg/ml)** |
|---|---|
| Sample H | $450.52^{(\alpha)}$ |
| Sample G | $444.44^{(\alpha)}$ |
| **LPS** | $\mathbf{314.11^{(\alpha)(\beta)}}$ |
| Sample F | $223.40^{(\beta)(\gamma)}$ |
| Sample A | $188.06^{(\beta)(\gamma)(\delta)}$ |
| Sample E | $185.07^{(\beta)(\gamma)(\delta)(\varepsilon)}$ |
| Sample C | $124.53^{(\gamma)(\delta)(\varepsilon)}$ |
| Sample D | $49.82^{(\delta)(\varepsilon)}$ |
| Sample B | $46.17^{(\delta)(\varepsilon)}$ |
| **Blank** | $\mathbf{35.10^{(\varepsilon)}}$ |

Note: Levels not connected by same Greek letter are significantly different. ($P < 0.05$)

At 15 μg/ml concentration, IL-6 produced by untreated cells was significantly lower than the levels produced by LPS only cells. As shown in Table 4.6, samples B, D and C significantly inhibited the amount of IL-6 produced by the LPS stimulated macrophage cells. Although cells treated with samples A, E and F produced lower IL-6 than LPS only

cells, the difference was not significant at P value of 0.05. Samples G and H did not show any IL-6 inhibiting effect.

Relationship between amount of IL-6 expressed and MW, % 6 sulfated disaccharides, % total sulfated disaccharides of CS samples:

Bivariate fits were obtained to determine any correlations between percentage reduction in IL-6 values (of CS samples relative to LPS) and molecular weight, percentage 6-sulfated fraction and, total sulfated fraction of CS in samples. Correlation coefficients ($R^2$) 0.5 to 1.0 were considered high.

No correlation was observed between the IL-6 inhibiting effect and molecular weight of the CS samples ($R^2$ = 0.115, P= 0.4) at 5 μg/ml concentration, although, at 15 μg/ml concentration, there appears to be a slight correlation with $R^2$ of 0.52 (P= 0.042). No significant correlation seems to exist between % 6-sulfated disaccharides and inhibition of IL-6 of the CS samples at either 5 μg/ml ($R^2$= 0.49, P= 0.05) or 15 μg/ml ($R^2$= 0.22, P= 0.23) concentrations. Similarly, no correlations were observed between IL-6 inhibiting effect and percentage of total sulfated disaccharides ($R^2$= 0.34, 0.42 and, P= 0.12, 0.08, respectively for low concentration and high concentration CS samples).

Figure 4.2 summarizes the percentage lowering of Il-6 by CS samples of low and high concentrations with respect to IL-6 expressed by cells stimulated with LPS in the absence of CS samples. From this figure, it can be seen that all of the CS samples were more effective at 15 μg/ml concentration than at 5 μg/ml concentration in reducing IL-6, except samples G and H. Sample B, at 15 μg/ml concentration appeared to have the highest anti-

inflammatory (AI) activity in terms of its ability to lower expression of IL-6. Although at 5 µg/ml concentrations, samples A, C, D and F appear to increase Il-6 production compared to LPS stimulated cells, from table 4.5, it can be seen that the IL-6 values of those samples were not significantly different from that of LPS stimulated cells in the absence of CS samples.

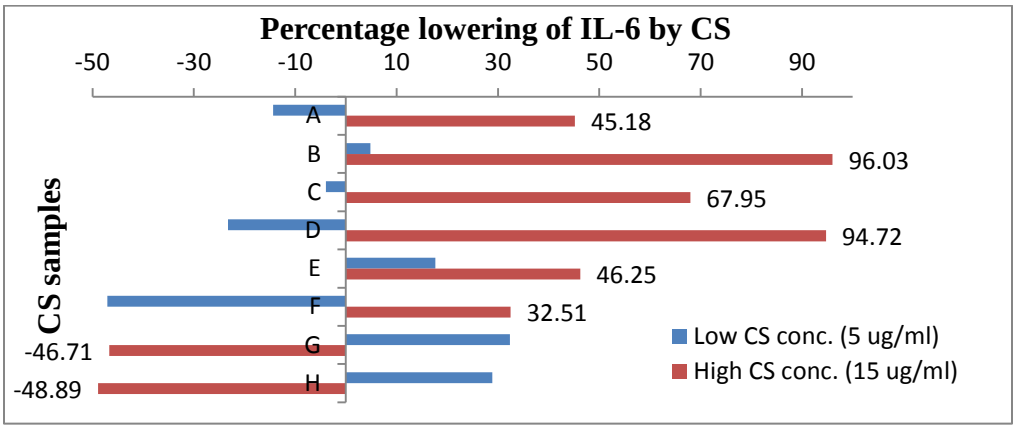


Figure 4.2: Percentage lowering of Il-6 by CS samples at low and high concentrations with respect to LPS only samples.

NO:

NO was measured as the amount of nitrite in the samples. The levels of nitrite were determined in blank samples, CS samples (low and high conc.) and LPS samples following the procedure outlined in materials and methods section. The samples were ranked in descending order of their nitrite concentration for low concentration and high concentration CS samples separately. Ranking was obtained by means comparison of each pair using student's t-test. Table 4.7 presents ranking of CS, blank and LPS samples

in descending order of their nitrite concentration values for CS at 5 µg/ml (low conc.) while Table 4.8 presents the same for CS at 15 µg/ml (high conc.).

Table 4.7: Ranking of CS samples (conc. 5 µg/ml) according to nitrite concentration Samples compared by Student's t-test

| **Level** | **Mean (conc. of nitrite in µM)** |
|---|---|
| Sample C | $8.29^{(\alpha)}$ |
| Sample A | $8.23^{(\alpha)}$ |
| Sample B | $7.89^{(\alpha)(\beta)}$ |
| Sample G | $7.78^{(\alpha)(\beta)(\gamma)}$ |
| Sample H | $7.78^{(\alpha)(\beta)(\gamma)}$ |
| Sample E | $7.65^{(\alpha)(\beta)(\gamma)}$ |
| Sample F | $7.46^{(\alpha)(\beta)(\gamma)}$ |
| **Blank** | $\mathbf{6.69^{(\beta)(\gamma)}}$ |
| Sample D | $6.59^{(\beta)(\gamma)}$ |
| **LPS** | $\mathbf{6.33^{(\gamma)}}$ |

Note: Levels not connected by same Greek letter are significantly different. ($P < 0.05$)

Table 4.8: Ranking of CS samples (conc.15 µg/ml) according to nitrite concentration

Samples compared by Student's t-test

| **Level** | **Mean (conc. of nitrite in µM)** |
|---|---|
| Sample C | $17.038^{(\alpha)}$ |
| Sample G | $12.80^{(\beta)}$ |
| Sample D | $12.70^{(\beta)}$ |
| Sample H | $12.36^{(\beta)}$ |
| Sample A | $10.12^{(\beta)(\gamma)}$ |
| Sample B | $10.09^{(\beta)(\gamma)}$ |
| Sample E | $10.03^{(\beta)(\gamma)}$ |
| Sample F | $9.29^{(\beta)(\gamma)(\delta)}$ |
| **Blank** | $\mathbf{6.69^{(\gamma)(\delta)}}$ |
| **LPS** | $\mathbf{6.33^{(\delta)}}$ |

Note: Levels not connected by same letter are significantly different. ($P < 0.05$)

As seen from Tables 4.7 and 4.8, the base line nitrite values did not differ from the nitrite produced by the cells upon stimulation with LPS. This makes NO (measured as nitrite) ineligible to be considered a marker for anti-inflammatory activity; in RAW 264.7 cell line under the cell culture conditions used in this particular study.

# CHAPTER 5

# DISCUSSION

CS is used extensively as a treatment for OA in not just humans but also in animals such as horses and dogs in the United States and Europe (Luo et al., 2002; McAlindon et al., 2000). It is currently recommended by EULAR as a SYSADOA in the treatment of knee and hand osteoarthritis based on research evidence and meta-analyses of numerous clinical studies (N. Volpi, 2009). The role of CS as part of the proteoglycan molecules is extended to crucial biological functions e.g., growth factor signaling, axonal growth and regeneration, neuritogenesis during CNS development, morphogenesis and cell division (Sugahara et al., 2003). It was also reported to be effective in lowering systemic inflammation associated with chronic inflammatory diseases such as cardiovascular diseases and rheumatoid arthritis (Herrero-Beaumont et al., 2008). As reported by several *in vitro* studies (G.M. Campo et al., 2008; G. M. Campo et al., 2009; Jomphe et al., 2008; F. Ronca, Palmieri, Panicucci, & Ronca, 1998), animal studies (Bauerova et al., 2011; Giuseppe M. Campo et al., 2003; Cho et al., 2004; Chou et al., 2005; Herrero-Beaumont et al., 2008; Taniguchi et al., 2012; Uebelhart, Thonar, Zhang, et al., 1998) and clinical trials (Bucsi & Poor, 1998) CS appears to play an important role in regulating cellular inflammatory events.

The heterogeneity of CS was reported to play a role in its bioavailability and bioactivity (Nicola Volpi, 2011). In spite of its high molecular weight (MW 8- 44 kDa), absorption of CS had been reported in animal as well as in humans. Studies also reported absorption

of CS in its intact form (A. Adebowale et al., 2002; Barthe et al., 2004; Palmieri et al., 1990). Several in vitro studies, animal studies, and clinical trials provided evidence that low molecular weight CS was more bio-available compared to its higher molecular weight counterparts (A. O. Adebowale et al., 2000; Conte et al., 1991; G. Ronca & Conte, 1993; N. Volpi, 2002, 2003).

In this study, the effects of heterogeneity of CS on its absorption and anti-inflammatory activities were evaluated *in vitro*. As described in the earlier chapters in detail, 8 CS samples were compared in terms of their absorbability in Caco-2 monolayer and anti-inflammatory activity in monocyte derived murine macrophages. Size exclusion chromatography (SEC) was used to determine the molecular weight of CS samples used in this study. CS samples of known molecular weight were used as the standards. As presented in Table 2.2, the molecular weights of sample A and B were calculated to be 7 kDa and 8 kDa respectively. But, due to limited ability/sensitivity of SEC in separating samples very close to each other in size, it was difficult to obtain the absolute molecular weights of these samples (Trathnigg, 2006). Hence, samples A and B were evaluated based on quite similar, "measured" molecular weights. Similarly, the sample pairs C-D, E-F, and G-H also had molecular weights that were comparable.

Disaccharide composition of the CS sample was determined by the enzyme hydrolysis and HPLC-UV method. From the results listed in Table 2.3, it can be seen that although the samples differed from each other in % ΔDi-6S, they do not differ much in total sulfation (total % ΔDi). Samples B, G and H had higher % ΔDi-6S compared to the other

CS samples used in this study. Marine sources (shark) of CS have higher % ΔDi-6S compared to terrestrial sources (bovine, porcine) as outlined in table 1.1.

The *in vitro* absorbability of the CS samples was measured in the Caco-2 cell line. Given its morphological and functional similarities to the enterocytes of small intestine, Caco-2 has been used as a well-established model for conducting *in vitro* absorption studies by several pharma industries over the years. But, care needs to be taken in drawing conclusions regarding bioavailability of a compound in humans based on its absorbability in Caco-2 cells. This is because the correlation of the permeabilities of the slowly absorbed drugs in the Caco-2 monolayers and human intestine was qualitative rather than quantitative. Hence, Caco-2 proves to be a valuable resource when a series of compounds need to be ranked in order of their possible absorbability rather than calculate their absolute absorbability. Also, one should be cautious in comparing the permeability coefficients of compounds in Caco-2 lines between two different labs because of variation in cell line as well as cell culturing conditions. Even within the same lab using a standard protocol, variations were observed in absolute permeabilities of the same sample. This problem can be solved to an extent by including a standard with known absorbability (high or low) along with the samples being tested. Then, comparing the permeability coefficients of that standard with the same from other labs might provide an insight into how the cell layers compare between the labs.

In the current absorbability study conducted with the CS samples, propranolol hydrochloride and sodium fluorescein were used as positive and negative controls

respectively. The $P_{eff}$ value of propranolol was 86.43 x $10^{-6}$ cm $s^{-1.}$ The $P_{eff}$ values of the same compound reported by different labs was about 30 x $10^{-6}$ cm $s^{-1}$ (Pade & Stavchansky, 1998; Yee, 1997). Compounds with $P_{eff}$ values over $10^{-6}$ were considered to be of high absorption with over 90% absorption in vivo and the ones with $P_{eff} < 10^{-7}$ were considered to have a very low absorption *in vivo* (Artursson et al., 2001). Positive and negative controls used in our experiments fulfill these requirements thus providing validity to the $P_{eff}$ values of CS samples.

As seen from ranking of $P_{eff}$ of CS samples, Sample B has the highest absorption among the samples. As mentioned earlier, samples A and B were of similar molecular weights and hence a similar absorption profile was expected. Sample B, whose % ΔDi- 6S is higher than that of sample A showed higher permeability as well. In spite of their comparatively higher molecular weights, samples G and H show higher absorption than the lower molecular weight samples A, C, D and E and whose % ΔDi- 6S was lower than that of samples G and H. No correlation seems to exist between molecular weight and absorption in vitro within the molecular weights used in this study. The bivariate fit of $P_{eff}$ and % ΔDi- 6S in CS sample resulted in the *R*-square value of 0.5, P = 0.0477 which revealed that these two parameters were correlated, albeit weakly. This explains the absorption of shark chondroitin sulfate in humans in spite of its high molecular weight of 44 kDa (N. Volpi, 2003). Shark CS has a % ΔDi- 6S of about 50% (Mucci et al., 2000). Also, because of the lack of CS samples with pure ΔDi-4S in our sample set, no correlation data could be obtained for $P_{eff}$ and % ΔDi-4S. However, given the significant effect of % ΔDi-6S on permeability and the lack of correlation with % total sulfated ΔDi,

it appears that presence of 4S has a tendency to negate positive effect of ΔDi-6S in CS absorbability. Further studies are needed to confirm this with samples of similar molecular weights and composed purely of either ΔDi-4S or ΔDi-6S.

From the results of the AI study conducted using the same CS samples, it can be seen that all of the CS samples exhibited AI activity by inhibiting inflammatory cytokine production by the RAW 264.7 cells. Our results are in agreement with cytokine lowering effects of CS as reported by several researchers in *in vitro* studies (G. M. Campo et al., 2009), and animal (Bauerova et al., 2011; Giuseppe M. Campo et al., 2003) studies. Although no correlations had been observed between the molecular weights, % ΔDi-6S or total % sulfated ΔDi and the anti-inflammatory activity, the ranking of samples according to their TNF-α and IL-6 levels provided us with samples with high AI activity among the set of CS samples used in this study.

In humans, the physiological concentrations of 5 μg/ml and 15 μg/ml translate to a dosage of 400 mg of CS per day at approximately 8% and 20% absorption respectively, considering an average blood volume of 5 liters. At 5 μg/ml concentration, sample B ranked high among the samples in inhibiting the production of LPS stimulated TNF-α in RAW 264.7 cells. However, at the same concentration, the inhibition of IL-6 by sample B was not significant compared to LPS positive. At 15 μg/ml concentration, sample B stands out as the best among the CS samples by inhibiting production of both TNF-α and IL-6. The TNF-α lowering effect of CS samples appear to be dose dependent (higher percentage lowering of TNF-α at a higher concentration) except in case of sample H. The

IL-6 lowering effect of CS samples also appears to be better at 15 µg/ml concentration except in case of samples G and H.

Although no clear correlation was identified between the molecular weights and percentage 6-sulfated disaccharides with absorption of the CS samples, our results show that sample B, which is a low molecular weight CS sample, with high (over 90%) 6-sulfated disaccharide percentage had highest absorption and AI activity. A higher absorption than conventionally available CS would mean lower dosage would be sufficient in achieving plasma CS concentration with effective AI activity. Synthetic CS samples with predetermined molecular weights and disaccharide composition are needed to verify correlations between absorption and molecular weight as well as disaccharide composition. This could be an objective for future studies.

In summary, this study employed, CS, a highly heterogenous molecule, to evaluate and verify the usage of in vitro models of absorption and AI activity as screening tools for ranking CS samples based on their permeability and cytokine inhibitory effect. Our results suggest that the aforementioned models can indeed be used as screening tools to identify prospective candidates for future clinical trials.

Another important outcome of this study was development of a screening tool that could be employed prior to clinical trials for selecting potential compounds to be included in the trial. It is common knowledge that clinical trials can be extremely expensive. This cost proportionately increases with an increase in the number of samples to be evaluated. The combination of *in vitro* studies employed in this project may be used as a guiding

tool in identifying the compounds that are most likely to be effective in clinical studies. Such an *in vitro* screening tool lowers the expense incurred by the clinical trials by several fold by reducing the number of samples to be evaluated *in vivo*. This is of particular importance for nutrition supplement manufacturers where different formulations of same nutrients may need to be evaluated based on their bioavailability and functionality.

# APPENDICES

## Appendix 1

### Materials and methods for the determination of molecular weights of CS samples

Preparation of standards and samples:

1. Known molecular weight CS standards were prepared by dissolving 20 mg of CS standard in 10 ml of HPLC grade water.
2. An internal standard was also prepared by dissolving 20 mg of inosine (Sigma, Catalog # I4125) in 10 ml of HPLC grade water.
3. Working standards were prepared in a HPLC vial by adding 200 µl of internal standard solution to 800 µl of CS standard.
4. To prepare working samples, about 20 mg of each sample was weighed into a 10 ml volumetric flask and 250 µl of internal standard (2 mg/ml inosine solution from step 3) was added to it. The solution was then made up to a volume of 10 ml with HPLC grade water. These sample solutions were filtered through 0.45 micron filters.

HPSEC conditions:

1. High performance liquid chromatography (HPLC) instrument- Waters, Part #
2. Columns- Two Agilent Bio SEC-3 (150 x 7.8 mm) columns packed with 3 µm silica particles coated with a proprietary neutral, hydrophilic layer with pore sizes 150 Å and 100 Å (Agilent, Part # 5190-2507 and # 5190- 2502) were connected in tandem.
3. Mobile phase – 80% buffer and 20% ethanol was used as a mobile phase.

The buffer was prepared by making a solution of 30mM Disodium hydrogen phosphate ($Na_2HPO_4$, Fisher Scientific, Catalog # S374) and 30 mM Sodium sulfate ($Na_2SO_4$, JT Baker, Catalog # 3898-01). The pH of this solution was adjusted to 7 using 0.1 N phosphoric acid($H_3PO_4$).

4. Injection volume of 5 µl was set for both standards and samples.
5. Column temperature was set at 35 $^oC$, flow rate was set at 1.0 ml/min and run time was set for 15 minutes.
6. UV signal was detected at 205 nm using a photo diode array detector (Waters,)

Protocol for determination of standard curve:

1. Each working standard was injected once and chromatograms were recorded.
2. For each chromatogram, retention volumes were calculated for CS standard as well as internal standard according to equation A.1.1:

$$RV = RT \; X \; flow \; rate \quad \text{..... Equation A.1.1}$$

Where, RV = retention volume of CS standard or internal standard, RT = retention time of the component (CS standard or internal standard), and flow rate = 1.0 ml/min

3. The standard curve was obtained by plotting a graph with the ratio of RV of internal standard ($RV_{is}$) and RV of standard ( $RV_{std}$ ) as abscissa (x-axis) and log MW as ordinate (y-axis). A third degree polynomial equation was calculated (in the form of Equation A.2) from the data.

$$log\ MW = a(RV_{is} / RV_{std})^3 + b(RV_{is} / RV_{std})^2 + c(RV_{is} / RV_{std}) + d \quad \text{...Equation A.1.2}$$

Calculation of molecular weights of CS samples (of unknown MW):

1. CS samples were also run under the same chromatographic conditions as standards and RTs of the CS samples and internal standard were obtained from their chromatograms.
2. RVs of CS samples and internal standards were calculated using their respective RTs in equation A.1.
3. RVs obtained in the above step were substituted in the standard curve (Equation 2) to calculate log MW of CS samples.
4. MW of CS samples were calculated using Equation A.3

$$MW \text{ (in kilo Daltons)} = 10^{\log MW} / 1000 \quad \text{.....Equation A.1.3}$$

**Appendix 2**

**Materials and methods for the determination of disaccharide composition of CS samples**

Preparation of reagents:

1. Mobile phase A- 340 mg of tetrabutylammonium bisulfate (Sigma, Catalog # 86868) was dissolved in 1000 ml of water (HPLC grade).
2. Mobile phase B- 340 mg of tetrabutylammonium bisulfate was dissolved in 330 ml water and acetonitrile (HPLC grade) was added to make up the volume to 1000 ml.
3. Tris- (hydroxymethyl) aminomethane (TRIS) buffer solution- 3g of TRIS (Sigma, Catalog # T1503), 2.4 g of anhydrous sodium acetate (Sigma, Catalog # S8750), 1.46 g of sodium chloride (ACS reagent grade) and 50 mg crystalline bovine serum albumin (Sigma, Catalog # A4378) were accurately weighed and dissolved in 100 ml of 0.12 M HCl. pH of this solution was adjusted to 7.3 with 6M HCl (HPLC grade).
4. Chondroitinase AC II enzyme solution- 5 units of chondroitinase AC II enzyme (Seikagaku America, Catalog # 100335-1A) was dissolved in 0.5 ml water. Enzyme was stored at $< 0\ ^{o}C$ when not in use.

Preparation of standards and samples:

1. Chondroitin sulfate disaccharide reference standards (non-sulfated or ΔDi-0S, 4-sulfated or ΔDi-4S and 6- sulfated or ΔDi-6S) were purchased from Sigma

(Catalog # C3920, C4045 and C4170 respectively). Stock solution of these disaccharide standards were prepared by dissolving 2mg of ΔDi-0S and 10 mg each of ΔDi-4S and ΔDi-6S in 50 ml of water.

2. Serial dilution of disaccharide stock solution was done to obtain standard disaccharide solutions with concentrations of ΔDi-4S and ΔDi-6S between 2 - 100 μg/ml and ΔDi-0S between 0.4 - 20 μg/ml.
3. One hundred mg of CS samples were accurately weighed and dissolved in 50 ml of water in separate volumetric flasks.

Enzymatic hydrolysis of CS samples:

1. Twenty μl of TRIS buffer solution, 30 μl of enzyme solution and 20 μl of CS sample were pipetted into 0.5 ml eppendorf tubes.
2. The Eppendorf tubes were then incubated in a water bath at 37 $^{o}$C for 3 hours.
3. Once removed from water bath, the samples in eppendorf tubes were allowed to cool to room temperature. 230 μl of mobile phase A was added to the tubes and the hydrolyzed CS solution was transferred into 2 ml HPLC vials. The eppendorf tubes were rinsed with another 200 μl of mobile phase A which was transferred into the HPLC vial with hydrolyzed CS solution. Another 500 μl of mobile phase A was added to those vials and mixed well.

HPLC analysis of hydrolyzed CS samples:

1. Waters HPLC machine connected to a PDA detector (Waters, Part # 2996) was used to analyze the CS disaccharide standards and CS samples.

2. Phenomenex Synergi$^{TM}$ Polar-RP, 4.6 x 150 mm, 4 µm column was equilibrated with 80 % mobile phase A and 20% B. The following mobile phase gradient was followed to run the standards/samples (Table A.2.1).

   Table A.2.1: Mobile phase gradient for CS disaccharide analysis

| Time, min | % mobile phase A | % mobile phase B |
|---|---|---|
| 0 | 80 | 20 |
| 0 – 7.0 | 35 | 65 |
| 7.0 – 12.0 | 35 | 65 |
| 12.0 – 12.5 | 80 | 20 |

3. Standards and samples were injected at an injection volume of 20 µl and a runtime of 20 minutes. Detection was done at 240 nm.
4. Empower 3 software was used to calculate concentrations of individual disaccharides using concentrations of disaccharide standards.
5. For each CS sample, total CS was measured as a sum of ΔDi-0S, ΔDi-4S and ΔDi-6S.
6. Percentage of ΔDi-0S in each CS sample was calculated using Equation A.2.1.

   % ΔDi-0S = 100 x (Conc. of ΔDi-0S / Total CS conc.) …Equation A.2.1

7. Percentages of 4-sulfated and 6-sulfated disaccharides were calculated by substituting conc. of ΔDi-0S with conc. of ΔDi-4S and ΔDi-6S respectively in Equation A.2.1.

## Appendix 3

### Protocol for TNF-α ELISA

Steps for determination of TNF-alpha in cell supernatant samples:

1. The supernatant samples from anti-inflammatory activity were used in this assay.
2. All the kit contents were allowed to reach room temperature before performing the assay.
3. TNF-α standards, high/low controls, streptavidin-horse radish peroxidase (Strp-HRP) and wash solution were prepared according to the manufacturer instruction manual.
4. For the standard curve, 100 µl of standards were added to the appropriate microtiter wells. 100 µl of standard diluent buffer was added to the zero standard well. Chromogen blank sample wells were left empty.
5. Controls and cell supernatant samples needed to be diluted two fold in standard diluent buffer. 50 µl of samples and controls were added to the appropriate wells followed by 50 ul of standard diluent buffer. The plate was tapped gently on the side to mix.
6. Fifty µl of biotinylated mouse TNF-α- biotin conjugate solution was pipetted into each well except chromogen blank wells and mixed gently.
7. The plate was then covered and incubated at room temperature for 90 minutes.

8. After 90 minutes, the solution was aspirated thoroughly, decanted and discarded. The wells were washed 4 times and placed upside down on an absorbent paper towel for few minutes.
9. One hundred µl of Strp-HRP working solution was added to each well except chromogen blank wells. The plate was then covered and incubated for 30 minutes at room temperature.
10. After 30 minutes, the solution was aspirated and discarded. The plate was washed four times and placed on an absorbent paper for drying.
11. One hundred µl of stabilized chromogen was added to each well. The liquid in the wells started to turn blue. The plate was covered and incubated in the dark at room temperature for 30 minutes.
12. One hundred µl of stop solution was added to each well after 30 minutes. The side of the plate was tapped gently to mix. The solution in the wells turned from blue to yellow.
13. The absorbance of each well was read at 450 nm having blanked the plate reader against the chromogen blank wells.
14. A standard curve was plotted with the OD values of TNF-α standards (0 pg/ml-1000 pg/ml) and their concentrations. The equation of standard curve was then used to determine concentrations of TNF-α in the cell supernatant samples.
15. The calculated concentrations were then multiplied with a dilution factor two to obtain the original concentrations of TNF-α in the cell supernatant samples.

## Appendix 4

### Protocol for IL-1β ELISA

Steps for determination of IL-1β in cell supernatant samples:

1. The supernatant samples from anti-inflammatory activity were used in this assay.
2. All the kit contents were allowed to reach room temperature before performing the assay.
3. IL-1β standards, high/low controls, streptavidin-horse radish peroxidase (Strp-HRP) and wash solution were prepared according to the manufacturer instruction manual.
4. Fifty µl of the incubation buffer was added to all wells except the chromogen blank wells.
5. For the standard curve, 50 µl of standards were added to the appropriate microtiter wells. Fifty µl of tissue culture media was added to the zero standard well. Chromogen blank sample wells were left empty.
6. Controls and cell supernatant samples needed to be diluted two fold in standard diluent buffer. 50 µl of samples and controls were added to the appropriate wells followed by 50 µl of tissue culture media. The plate was tapped gently on the side to mix.
7. Fifty µl of biotinylated mouse IL-1β- biotin conjugate solution was pipetted into each well except chromogen blank wells and mixed gently.
8. The plate was then covered and incubated at 37 $^{o}$C for 90 minutes.

9. After 90 minutes, the solution was aspirated thoroughly, decanted and discarded. The wells were washed 4 times and placed upside down on an absorbent paper towel for few minutes.
10. One hundred µl of Strp-HRP working solution was added to each well except chromogen blank wells. The plate was then covered and incubated for 30 minutes at room temperature.
11. After 30 minutes, the solution was aspirated and discarded. The plate was washed four times and placed on an absorbent paper for drying.
12. One hundred µl of stabilized chromogen was added to each well. The liquid in the wells started to turn blue. The plate was covered and incubated in the dark at room temperature for 30 minutes.
13. One hundred µl of stop solution was added to each well after 30 minutes. The side of the plate was tapped gently to mix. The solution in the wells turned from blue to yellow.
14. The absorbance of each well was read at 450 nm having blanked the plate reader against the chromogen blank wells.
15. A standard curve was plotted with the OD values of IL-1β (0 pg/ml- 1000 pg/ml) and their concentrations. The equation of standard curve was then used to determine concentrations of IL-1β in the cell supernatant samples.
16. The calculated concentrations were then multiplied with a dilution factor two to obtain the original concentrations of IL-1β in the cell supernatant samples.

## Appendix 5

## Protocol for IL-6 ELISA

Steps for determination of IL-6 in cell supernatant samples:

1. The supernatant samples from anti-inflammatory activity were used in this assay.
2. All the kit contents were allowed to reach room temperature before performing the assay.
3. IL-1β standards, high/low controls, streptavidin-horse radish peroxidase (Strp-HRP) and wash solution were prepared according to the manufacturer instruction manual.
4. For the standard curve, 100 µl of standards were added to the appropriate microtiter wells. 100 µl of standard diluent buffer was added to the zero standard well. Chromogen blank sample wells were left empty.
5. Controls and cell supernatant samples needed to be diluted two fold in standard diluent buffer. 50 µl of samples and controls were added to the appropriate wells followed by 50 µl of standard diluent buffer. The plate was tapped gently on the side to mix.
6. The plate was then covered and incubated at room temperature for 2 hours.
7. After 2 hours, the solution was aspirated thoroughly, decanted and discarded. The wells were washed 4 times and placed upside down on an absorbent paper towel for few minutes.

8. One hundred µl of biotinylated mouse IL-6- biotin conjugate solution was pipetted into each well except chromogen blank wells and mixed gently.
9. The plate was then covered and incubated at room temperature for 30 minutes.
10. After 30 minutes, the solution was aspirated thoroughly, decanted and discarded. The wells were washed 4 times and placed upside down on an absorbent paper towel for few minutes.
11. One hundred µl of Strp-HRP working solution was added to each well except chromogen blank wells. The plate was then covered and incubated for 30 minutes at room temperature.
12. After 30 minutes, the solution was aspirated and discarded. The plate was washed four times and placed on an absorbent paper for drying.
13. One hundred µl of stabilized chromogen was added to each well. The liquid in the wells started to turn blue. The plate was covered and incubated in the dark at room temperature for 30 minutes.
14. One hundred µl of stop solution was added to each well after 30 minutes. The side of the plate was tapped gently to mix. The solution in the wells turned from blue to yellow.
15. The absorbance of each well was read at 450 nm having blanked the plate reader against the chromogen blank wells.
16. A standard curve was plotted with the OD values of IL-6 (0 pg/ml- 500 pg/ml) and their concentrations. The equation of standard curve was then used to determine concentrations of IL-6 in the cell supernatant samples.

17. The calculated concentrations were then multiplied with a dilution factor two to obtain the original concentrations of IL-6 in the cell supernatant samples.

## Appendix 6

### Protocol for nitrite estimation

1. Standard solutions of sodium nitrite ($NaNO_2$) with concentrations ranging from 0 µM to 100 µM were prepared in DI water.
2. In a 96 well plate, 20 µl of Griess reagent, 150 µl of the supernatant (or $NaNO_2$ standards), and 130 µL of deionized water were added.
3. In photometric reference wells, 20 µL of Griess Reagent and 280 µL of deionized water were added.
4. After mixing the above reagents, the plate was covered and incubated for 30 minutes at room temperature.
5. After 30 minutes, the absorbance (OD) of the azo dye formed in the reaction as measured using a plate reader at 520 nm.
6. A standard curve was obtained using the concentrations and OD values of the $NaNO_2$ standards.
7. The standard curve was used to calculate nitrite concentrations of samples from cell supernatants.

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