Proteoglycans from deep sea shark cartilage: Characterization and role in apoptosis triggered anti-cancer activity and alleviation of osteoarthritic progression

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October 2017

Certificate

This is to certify that the thesis entitled **"Proteoglycans from deep sea shark cartilage: Characterization and role in apoptosis triggered anti-cancer activity and alleviation of osteoarthritic progression"** embodies the original work done by Ajeeshkumar K. K, under my guidance and supervision. He has incorporated all the relevant corrections and modifications suggested by the audience during the pre-synopsis seminar and by the doctoral committee. I further certify that no part of this thesis has previously been formed the basis of award of any degree, diploma, associateship, fellowship or any other similar titles of this or in any other university or Institution

Cochin,
January - 2018

Dr. Asha K. K
Principal Scientist
Biochemistry & Nutrition Division

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Declaration

I, Ajeeshkumar K. K do hereby declare that the thesis entitled **"Proteoglycans from deep sea shark cartilage: Characterization and role in apoptosis triggered anti-cancer activity and alleviation of osteoarthritic progression"** is a genuine record of bonafide research carried out by me under the supervision of Dr. Asha K. K, Principal Scientist, Biochemistry & Nutrition Division, ICAR-Central Institute of Fisheries Technology, Cochin and has not previously formed the basis of award of any degree, diploma, associateship, fellowship or any other similar titles of this or any other university or Institution

Ajeeshkumar K. K
(Reg. No.5138)

Cochin,
January -2018

Dedicated to

My parents, my sister and my grand mother …
The reason of what I am today
Thanks for your great support and continuous care....

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Abstract

The present study was aimed at isolation and characterization of proteoglycans (PGs) from deep sea shark *Echinorhinus brucus* cartilage, and assessment of *in-vitro* anticancer and *in-vivo* anti-osteoarthritic effects. PGs are important macromolecules of extracellular matrix involved in various cellular functions including cell growth and differentiation. PGs were isolated by chaeotropic digestion and anion-exchange chromatography with DEAE- sephacel. Chemical composition, gel electrophoresis, FTIR, H1-NMR and chromatographic methods were adopted for characterization. Following chemical composition analysis, total protein, total carbohydrate, uronic acid, hexosamine and sulfated GAGs values were observed to be 0.468 mg/mg, 0.530 mg/mg, 0.098 mg/mg, 0.20 mg/mg and 0.06 mg/mg respectively. In AGE and PAGE analysis presence of both high molecular and low molecular weight PGs and GAGs were confirmed. Structural characterization by FTIR and NMR revealed the presence of proteoglycans by obtaining the appropriate responses corresponding to functional groups present in GAGs and proteins. Trypsin-digested samples were used to characterize protein by peptide-based LC-MS/MS analysis. The acquired data was analysed in Proteome Discoverer 2.1SP1 (Thermo Scientific) on Sequest HT search engine and NCBI full protein database. GAG characterization was carried out with positive and negative ion (500-1500 m/z) full scanning of sample and standard with aid of MS/MS QTRAP 4000 model. NCBI protein data-base revealed the presence of aggrecan core protein, decorin and epiphycan peptide. Ion patterns (m/z) were of GAG isolated from sample were comparable to those of standard GAGs. Purity of PGs was thus confirmed by structural and chemical characterization.

Anti-cervical activity and anti-breast cancer activity of PGs was assessed in HeLa and MCF-7 cell line model respectively. The sample showed a significant cytotoxic activity for HeLa cells. PGs at a concentration of 250µg/ml inhibited the colony formation capacity. Apoptosis induction was confirmed by the chromatin condensation assay, annexin V-FITC (fluorescein isothiocyanate) and PI (propidium iodide) staining by flow cytometry. PGs (250 µg/ml) were found to induce apoptosis and the ratio of apoptotic cells were 30.5%. Cell cycle arrest at G1/M and G2/M phase confirmed the cell death due to cell cycle arrest. Our study determined that proteoglycans effectively induced apoptosis and caused cell cycle arrest, establishing their anti-proliferative effect against cervical cancer. The anti-proliferative activity of PGs against MCF-7 cell lines. To establish apoptosis involvement for cytotoxicity, the following assays were performed i.e., caspases 3 and 9 assay, double fluorescent staining and DNA laddering assay. To

confirm apoptosis pathway, expression of genes BAX, BCL-2 and p53 were carried out. PGs (100 μg/ml) induced a significant rise in activity of caspase 3 and 9, major apoptotic markers. A significant rise in the expression was also observed for BCL-2 and p53. Whereas, low BAX expression was observed for PGs (100 μg/ml) treated sample. Significant anti-proliferative effect was observed and the mechanism behind cell death was concluded to be apoptosis.

Anti-osteoarthritic effect of proteoglycans isolated from deep sea shark (*Echinorhinus brucus*) cartilage was evaluated in monosodium-iodoacetate (MIA) induced osteoarthritis in Wistar strain albino rat model. Biochemical parameters such as serum C-reactive protein, uric acid, anti-oxidant markers (SOD, CAT, reduced glutathione) and lipid peroxidation were analysed. Histopathology and X-ray analysis of joints were conducted to assess the structural change articular cartilage. Western blotting of TNF-α, IL-1β, MMP-13 and COX-2 and mRNA gene expression studies of inflammatory mediators like TNF-α, IL-1β, IL-10, MMP-13, COX-2, NOs II by Q-PCR using SYBR green dye have also been observed. In our study, promising decrease of osteoarthritic progression was observed in rats treated with proteoglycans (200 mg/ Kg). Obtained results indicate the vital action of proteoglycans isolated from shark cartilage against the progression of osteoarthritis and could be a promising bioactive molecule for osteoarthritic treatment.

Proteomics study by the aid of peptide-based identification and molecular level characterization of osteoarthritic joint protein was conducted to understand disease progression and anti-osteoarthritic effect of PGs. Protein identifications were obtained by searching against the *Rattus norvegicus* (Wistar strain albino rat) protein database downloaded from UniProt. Above analysis revealed the presence of important protein biomarkers in all the studied groups. Important biomarkers such as proteoglycans namely, aggrecan core protein, decorin, biglycan, lumican, fibromodulin and type II collagen showed enhanced fold expression in proteoglycans treated rat groups. Similarly anti-oxidant defence molecules, thioredoxin and SOD were up-regulated in the proteoglycans treated groups. Whereas, immunoglobulins, IL-20, cytochrome oxidase, compliment factor H were down regulated in the proteoglycans treated groups. Our results indicate that proteoglycans could be an effective anti-osteoarthritic molecule that acted by restoring cartilage structure and preventing the production of adverse osteoarthric biomarkers.

ABBREVIATIONS

Ab	:	Anti-body
AGE	:	Agarose gel electrophoresis
ANOVA	:	Analysis of variance
AO	:	Acridine orange
BCIP	:	5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt
BSA	:	Bovine serum albumin
CAT	:	Catalase
cDNA	:	Complementary DNA
CFA	:	Complete Freund's adjuvant
cm	:	Centimetre
COX	:	Cyclooxygenase
CPCSEA	:	Committee for the Purpose of Control and Supervision of Experiments on Animals
CRP	:	C-reactive protein
CS	:	Chondritin sulphate
CS1	:	Chondroitin sulfate binding region 1
CS2	:	Chondroitin sulfate binding region 2
DEAE	:	Diethyl amino ethyl
DISC	:	Death-inducing signalling complex
DMEM	:	Dulbbecos modified Eagle’s medium
DMMB	:	1, 9-dimethyl methylene blue
DMSO	:	Dimethyl sulfoxide
DNA	:	Deoxy-ribonucleotide
DS	:	Dermatan sulphate
DTNB	:	5, 5′-dithiobis 2-nitrobenzoicacid
EB	:	Ethedium bromide
ECM	:	Extra cellular matrix
EDTA	:	Ethylene diamine tetra acetic acid
EEZ	:	Exclusive Economic Zone

ESI	:	Electron spray ionization
FADD	:	Fas-associated death domain
FGFs	:	Fibroblast growth factors
FTIC	:	Fluorescein isothiocyanate
FTIR	:	Fourier transform infrared spectroscopy
g	:	Gram
GAG	:	Glycosaminoglycans
GalNAc	:	*N*-acetylgalactosamine
GC	:	Gas chromatography
Gd. HCl	:	Guanidium hydrochloride
GlcA	:	D-glucuronate
GlcNAc	:	*N*-acetyl glucosamine
GPx	:	Glutathione peroxidase
GSH	:	Reduced glutathione
H_2O_2	:	Hydrogen peroxide
HA	:	Hyaluronic acid
HCl	:	Hydrochloric acid
HeLa cells	:	Human epithelial cervix carcinoma
HGF	:	Heptocyte growth factor
hrs	:	Hours
HS	:	Heparin sulphate
HSPG	:	Heparan sulfate proteoglycans
IAEC	:	India and approved by the Institutional Animal Ethics Committee
ICMR	:	Indian Council of Medical Research
IdoA	:	L-iduronate
IGD	:	Interglobular domain
IL-10	:	Interleukin 10
IL-1β	:	Interleukin 1 beta
kDA	:	Kilo Dalton
Kg	:	Kilogram

KS	:	Keratan sulphate
LC-MS	:	Liquid chromatography-mass spectrometry
M	:	Molar
m/z	:	Mass-to-charge ratio
MALDI	:	Matrix assisted laser desorption/ionization
MCF-7	:	Michigan Cancer Foundation
mins	:	Minutes
ml	:	Millilitre
mm	:	Millimetre
MMPs	:	Metalloproteinases
mRNA	:	Messenger ribimuncleotde
MTT	:	3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide
MW	:	Molecular weight
MWCO	:	Molecular weight cut off
N	:	Normal
NBT	:	Nitro-blue tetrazolium chloride
NCBI	:	The National Center for Biotechnology Information
NCCS	:	National Centre for Cell Science
nm	:	Nanometre
NMR	:	Nuclear magnetic resonance
NO	:	Nitric oxide
NOS II	:	Nitric oxide synthase II
NSAIDs	:	Non-steroidal anti-inflammatory drugs
^{o} C	:	Degree Celsius
OA	:	Osteoarthritis
OD	:	Optical density
PBS	:	Phosphate buffer saline
PGs	:	Proteoglycans
PMSF	:	Phenylmethane sulfonyl fluoride
PSK	:	Polysaccharide-K

PSP	:	Polysaccharide-Peptide
ROS	:	Reactive oxygen species
rpm	:	Rotation per minute
RT-PCT	:	Real time-polymerase chain reaction
SD	:	Standard deviation
SDS-PAGE	:	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SLRPs	:	Small leucine rich proteoglycans
SOD	:	Super oxide dismutase
TAE	:	Tris acetate EDTA
TAP	:	Tumor angiogenesis factor
TBARS	:	Thiobarbituric acid reactive substance
TBS	:	Tris buffer saline
TBST	:	Tris buffer saline triton-100
TEMED	:	Tetramethylethylenediamine
TGF-β	:	Tissue growth factor beta
TNFR1	:	Tissue necrotic factor receptor
TNF-α	:	Tissue necrotic factor-alpha
TRADD	:	TNF receptor-associated death domain
Tris	:	Hydroxymethyl aminomethane
U/mg	:	Units per milligram
UA	:	Uric acid
UV	:	Ultra violet
UV–Vis	:	Ultraviolet-visible
VEGF	:	Vascular endothelial growth factor
WHO	:	World Health Organization
µg	:	microgram
µl	:	microliter

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Chapter 1

Review of Literature

1.1 General introduction

The advancements in life styles along with the dietary changes of late has led to the occurrence of many life style diseases. Cancer is one of the leading causes of death in developed countries and the second leading cause of death in developing countries. India is likely to have over 17.3 lakh new cases of cancer and more than 8.8 lakh deaths due to cancers with cancer of cervix, breast and lung topping the list. Similarly, Indian Council of Medical Research reports that the total number of new cases of cancer were about 14.5 lakh during 2016 and the figure is likely to reach nearly 17.3 lakh around 2020. Approximately 270,000 women have died from cervical cancer during 2015 with more than 85% of these deaths occurring in low- and middle-income countries like India (WHO, 2015). Breast cancer is the most prevalent cancer in the world after lung cancer in terms of occurrence (Parkin *et al*., 2001). This disease is a serious concern especially in developing countries like India and has caused about 75,000 mortalities out of 1,50,000 reported in the country (ICMR, 2014). Moreover, this has become the leading cause of death in females globally. Because of its high rate of incidence, especially among females, there is an urgent need to look into this issue by offering promising solutions.

Chemotherapy is widely used to treat cancer worldwide which uses chemical substances to weaken and destroy cancer cells in the body. However, chemotherapy drugs are reported to have several side effects such as hair loss and thinning, nausea and vomiting, mouth and dental problems, loss of fertility, fatigue etc. Vishnu *et al*., (2017) reported that high dose of doxorubicin, a chemotherapeutic drug causes cardiotoxicity in humans. An IC_{50} value of 1000 nM on HeLa cells shows that HeLa cells are not too sensitive to treatment with doxorubicin (Maruti *et al*., 2017; Larasati *et al*., 2011). Taking into consideration the side effects of chemotherapeutic drugs, there is a pressing need to develop a new approach of treatment. Apoptosis, also called programmed cell death, plays a key role in protection against uncontrolled proliferation of cells by enabling the body to eradicate unwanted cells and thereby maintaining proper homeostasis (Elmore, 2007). Cancer cells respond to treatment with chemotherapeutic drugs because these trigger apoptosis pathway. Hence it can be assumed that natural compounds having the ability to trigger apoptotic pathway will be beneficial to prevent cancer progression without side effects associated with chemotherapy.

Osteoarthritis (OA) is one of the most reported joint disorders mainly seen in elderly people (Busija *et al*., 2010). OA is characterized by degeneration of cartilage structure and biological function, and loss of regulation of pro-inflammatory and anti-inflammatory pathways (Goldring and Otero, 2011). Cyclooxygenase (COX) class of enzymes, are known to be responsible for arthritic related pain development. Both selective and non-selective COX inhibitors have found to possess antipyretic, anti-inflammatory and analgesic effects and are commonly employed in treating severe painful conditions of OA diseases (Kroenke *et al*., 2009). NSAIDs (Non-steroidal anti-inflammatory drugs) like naproxen, ibuprofen, celecoxib and diclofenac are most effective and widely used in counteracting the symptoms associated with OA. Owing to the pain relieving effects of NSAIDs, they are commonly used in treating OA without considering the relative side effects (Vonkeman *et al*., 2007). The most common of the side effects are the gastrointestinal (GI) side effects (Bhatt *et al*., 2008). Natural compounds such as resveratrol, an antioxidant are found to offer

protection against monosodium iodoacetate-induced osteoarthritic pain in rats (Wang *et al*., 2016). Certain marine natural compounds are also found to possess anti-arthritic effects. For instance, shark oil and collagen peptides derived from fish skin are observed to possess anti-arthritic effect (Mathew, 2010; Hema *et al*., 2016). Taking a cue from the potential of natural compounds to combat arthritis, research is being carried out globally to isolate such compounds and assess their effects using both *in vivo* and *in vitro* assays.

Proteoglycans (PGs), which are glycosylated proteins, are important macromolecules of extracellular matrix found in vertebrate and invertebrate tissues. They are involved in various cellular functions such as cell growth, adhesion, and differentiation (Kjellen and Lindahl, 1991; Iozzo, 1997). PGs are composed of a core protein onto which a variable number of glycosaminoglycan (GAG) side chains are attached. Glycosaminoglycans are long unbranched polysaccharides consisting of repeating disaccharide units composed of hexosamine (glucosamine or galactosamine) and uronic acid (glucuronic acid or iduronic acid). Depending on the disaccharide units, the GAGs are categorised into five groups, viz, keratan sulphate (KS), chondroitin sulphate (CS), dermatan sulphate (DS), heparin sulphate (HS) and hyaluronic acid (HA) (Nakano *et al*., 2002).

Proteoglycans (PGs) occur in almost all mammalian tissues and are mostly prominent in cartilages. Cartilage is a type of smooth, flexible connective tissue found in the skeletal systems of many animals, including humans. It is comprised of several types of collagens, other proteins and glycosaminoglycans. Unlike mammals, birds, reptiles, amphibians, and bony fishes, shark skeleton is entirely made up of cartilage. The major constituents in shark cartilage are proteoglycans, glycoproteins (large molecules with protein and carbohydrate components), other proteins like collagens and calcium salts. The predominant proteoglycan present in cartilage is the large chondroitin sulfate proteoglycan, 'aggrecan'. The other important types of proteoglycan present in cartilage include decorin, biglycan, epiphycan, versican etc. Decorin and biglycan are small leucine rich PGs (SLRPs) in ECM (extra cellular matrix) and possess one and two CS/DS chains (Roughley

and Lee, 1994). Decorin was originally named due to its binding on the surface of the collagen fibrils (Scott and Orford, 1981). Epiphycan, another small leucine rich CS/DS PG with a 35 kDa core protein (Iozzo, 1997), is involved in chondrocyte differentiation and osteogenesis (Kurita *et al*., 1996; Knudson and Knudson, 2001). Versican, another HA binding PG found in cartilage at low levels, has an N terminal G1 and C terminal G3 domain similar to that of aggrecan.

The present study is aimed at investigating the isolation and characterization of proteoglycans from deep sea shark *Echinorhinus brucus* cartilage and their anti-cancer (breast cancer and cervical cancer) and anti-osteoarthritic effects.

1.2 Scope of the study

The advancements in lifestyles along with dietary changes have together contributed to the occurrence of many deadly diseases. Cancer is one such deadly disease with a high rate of mortality. The occurrence of breast cancer and cervical cancer is becoming common in women which is a cause for serious concern. Treatment involving synthetic drugs is mainly adopted to resist the progression of those deadly diseases which are known to have serious side effects. Osteoarthritis (OA) one of the most common causes of pain and disability in middle-aged and older people, is increasing year by year. There is no proper treatment modalities yet discovered for OA and available synthetic drug target only the symptoms associated with it. Development of natural drugs, especially from marine sources, has received great attention in recent times. Natural drugs have the advantage of providing proper recovery with less or no side effects and this fact makes natural bioactive compounds from marine sector have an edge over available drugs. In this regard, the present study is designed to isolate, purify, characterize and analyze anti-cancer and anti-osteoarthritic effects of proteoglycans extracted from deep sea shark *Echinorhinus brucus* cartilage.

1.3 Objectives of the study

- Isolation and biochemical characterization of proteoglycans from deep sea shark (*Echinorhinus brucus*) cartilage
- Structural characterization of proteoglycans and glycosaminoglycans by using FTIR, H^1-NMR and LC-MS/MS
- Anti-breast cancer effect of proteoglycans against *in vitro*- MCF-7 human breast cancer cells and study of apoptosis involvement
- Anti-cervical cancer effect of proteoglycans against *in vitro*- HeLa human cervical cancer cells and study of apoptosis involvement
- Anti-osteoarthritis effects of proteoglycans in monosodium iodoacetate (MIA) induced rat model
- Proteomics pattern analysis of knee joint proteins in anti-osteoarthritic studies using proteoglycans in monosodium iodoacetate (MIA) induced rat model

1.4 Review of Literature

Ocean world which covers more than 70% of the earth's surface contain 36 known living phyla, 34 taxonomies with more than 300,000 unique species of flora and fauna (Yan, 2004; Jimeno *et al*., 2004; Kijjoa and Sawangwong, 2004). Bioactive molecules produced in the diverse group of marine organisms show a wide ranging role in the pharmaceutical and nutraceutical industry. Most pronounced marine-derived bioactive molecules include polysaccharides, bioactive peptides, polyunsaturated fatty acids, proteoglycans, collagens and carotenoids which are found to have many bioactivities such as anticancer, anti-inflammatory, antioxidant, anti-obesity, hypocholesteroleic, antimicrobial, prebiotic, and probiotic activities. Since natural compounds are considered safe for human consumption, marine bioactive compounds can be better option to develop nutraceutical and pharmaceutical products (Ibañez *et al*., 2012). It is evident that scientists have given much attention to marine organisms and the

unique bioactive molecules they produce because of their immense bioactivities. Studies are progressing in the right direction to identify and utilize safe and effective bioactive molecules to fight against diseases like cancer, diabetes, arthritis etc. (Faulkner, 2000). The Indian Exclusive Economic Zone (EEZ) covers approximately 2.02 million km sq. comprising rich marine flora and fauna and includes different types of commercially important species such as molluscs, finfish and crustaceans. However, EEZ remains unexploited in terms of bioprospecting of marine sources for bioactive molecules.

Echinorhiniformes

Kingdom	-	Animalia
Phylum	-	Chordata
Class	-	Chondrichthyes
Order	-	Squaliformes
Family	-	Ecinorhinidae
Scientific name	-	*Echinorhinus brucus*
Common name	-	Bramble shark, spinous shark, spiny shark

Fig. 1.1: *Echinorhinus brucus*

Bramble sharks are common deep sea sharks and are distributed mainly over continental and insular shelves and are seen in 500-900 m depth range. They are distributed mainly in Western Indian, Atlantic, Mediterranean and Pacific Ocean, and the Black Sea. The main morphological characters include grey to black coloration and scattered thorn like denticles distributed on body and fins. *Echinorhinus*, a genus of shark, is having two species, *brucus* and *cookei* (Compagno, 2001). Seldom data is available regarding its occurrence and distribution. They are mainly by-catch fishes and are rarely encountered in catch.

They are mainly caught in deep water trawls and line fishing. It has been reported that they are hardly used for edible purposes, however, shark liver oil has been used for medicinal purposes in many parts of the world, eg. South Africa (Quero and Cendrero, 1996). *Echinorhinus* species, which are non-edible and highly cartilaginous, are mainly used for extraction of oil and the remaining portion is discarded. Shark cartilage is known for high quantity of proteoglycans and GAGs (Higashi *et al*., 2015; Michelacci and Horton, 1989) and *E. brucus* cartilage has not been exploited for the same.

1.4.1 Cartilage

Cartilage is a part of skeleton system of body and is made up of specialized tissues called chondrocytes. Cartilage, which is both stiff and resilient, is an important filler material and has a bearing surface that reduces friction and stress exerted during compression. It is also responsible for lubrication mechanisms in the joints (Hall, 2005; Sophia *et al*., 2009). It makes up the abundant extracellular matrix, contributing to 90% of the dry weight of the tissue (Hardingham and Fosang, 1992). In most of the vertebrates cartilage is present in much lesser quantity, whereas in elasmobranchs (sharks and rays), the whole skeletons are made of cartilage, which therefore perform dual roles carried out by bone and cartilage (Boyan *et al*., 1996; Macesic and Summers, 2012).

Cartilage is a type of connective tissue composed of many types of proteoglycans, collagens, other proteins and sulphated and non-sulphated glycosaminoglycans (Fontenele *et al*., 1997). It contains a wide variety of proteoglycans such as aggrecan, decorin, biglycan, fibromodulin and lumican that are essential for its normal function. Each proteoglycan serves several functions that are determined by both its core protein and its glycosaminoglycan chains.

1.4.2 Shark cartilage

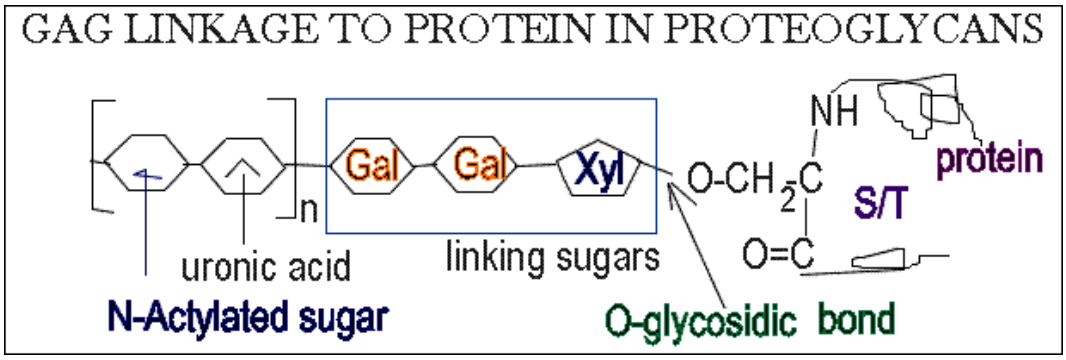
Shark cartilage is very much similar to human cartilage tissues and has been found to possess protective action against several diseases such as cancer, osteoarthritis, arthritis, and other types of diseases (Himmel and Seligman, 1999;

Loprinzi *et al*., 2005; Patra and Sandell, 2012). Biochemically, shark cartilage is composed of a mixture of glycosaminoglycans, an important nutritional source of hyaluronic acid, chondroitin sulfate, dermatan sulfate and keratin sulfate. There are more than 40 different products of shark cartilage available on the market and attract numerous consumers due to their medicinal properties (Holt, 1995). Studies have revealed that cartilage of shark origin has the ability to inhibit tumor-angiogenesis-factor (TAP), which further prevents capillary proliferation and cancer (Brem and Folkman J, 1975). Many research findings also discussed the role of shark cartilage in inhibiting neovascularization or angiogenesis thereby retarding the proliferation of tumor tissues (Lee *et al*., 1984). Shark cartilage was also effectively used for treatment of arthritis (Sculti, 1994). Numerous studies have exhibited the advantage of using cartilage formulations as an anti-inflammatory and analgesic for arthritis patients.

1.4.3 Proteoglycans

Proteoglycans are conjugated macromolecules that are made up of a protein core to which one or more GAG heteropolysaccharide chains are attached by covalent bond (Hascall and Sajdera 1969; Kuettner and Kimura 1985; Lohmander *et al*., 1980). Fig. 1.2 and 1.3 depict structure of proteoglycan. The core proteins of the known proteoglycans vary in size from 11,000 to about 220,000 Dalton (Doege *et al*., 1991). Proteoglycans are the major constituents of extracellular matrix network and are located either at the cell surface or in the extracellular matrix. They are unique molecules involved in wide varieties of biological actions such as cell to cell interactions, cell signaling, cell alignment (Iozzo, 1997; Esko and Lindahl, 2001). They are interlinked with each other and also with many other structural proteins of extracellular matrix, viz., collagen (type I and II) and elastin. They play vital role in regulating structural organization of the matrix and in important biological phenomena such as cell growth and development (Esko and Selleck , 2002 ; Hacker *et al*., 2005; Bulow and Hobert, 2006).

Proteoglycans and GAGs play different roles in diverse processes such as enzyme regulation and cellular adhesion, growth, migration and differentiation (Esko and Lindahl, 2001; Turnbull *et al*., 2001). The study of proteoglycans started at the beginning of the 20th century when investigators discovered a compound named "chondromucoid" from cartilage which was recognized as anticoagulant heparin containing proteoglycans.



Source:http://www.cryst.bbk.ac.uk/pps97/assignments/projects/emilia/Proteoglycans.HTM

Fig. 1.2: Structure of Proteoglycans (PGs)

Proteoglycans can be categorized based upon the nature of their glycosaminoglycan chains. They can also be categorised on the basis of size (kDa) (Gabius and Gabius., 2008).

1.4.3.1 Classification of proteoglycans

Table 1.1 Classification of proteoglycans (PGs)

Glycosaminoglycans	Small proteoglycans	Large proteoglycans
Chondroitin sulfate/dermatan sulphate	Decorin = 36 kDa Biglycan = 38 kDa	Versican = 260-370 kDa present in many adult tissues including blood vessels and skin
Heparan sulfate/chondroitin sulphate	Testican, kDa= 44	Perlecan = 400-470 kDa
Chondroitin sulphate		Neurocan = 136 kDa Aggrecan = 220 kDa the major proteoglycan in cartilage
Keratan sulphate	Fibromodulin = 42 kDa Lumican = 38 kDa	

1.4.3.2 Extracellular matrix proteoglycans

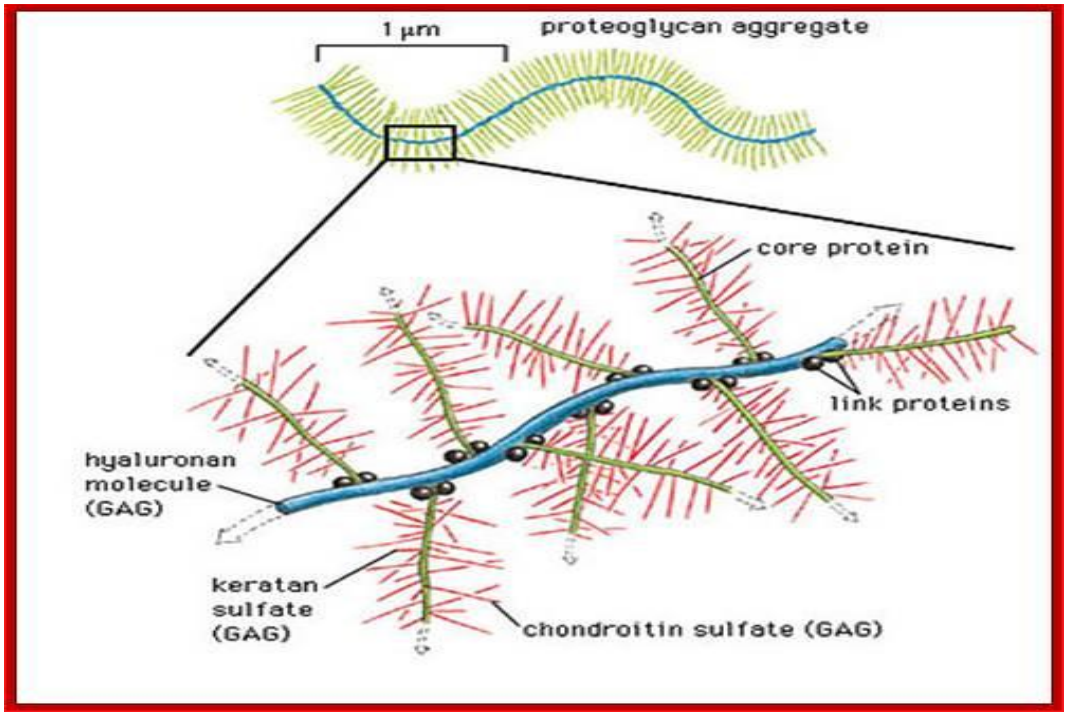


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Fig. 1.3: Structure of aggrecan

Aggrecan, is the dominant and the large extracellular PG of the cartilage and is characterized by many GAG chains attached to its core protein (Heinegård and Paulsson 1987; Carney and Muir 1988; Kiani *et al*., 2002). As far as its size is concerned, an aggrecan monomer is of 14 x 106 Da, whereas its protein backbone is of molecular size of 210-250 kDa. Aggrecan is well characterized and documented among all large aggregating PG and are predominantly seen in the articular cartilage (Watanabe *et al*., 1998; Kiani *et al*., 2002). Aggrecan is characterized by the presence of highly negatively charged GAG chains. About 90% of aggrecan contains chondroitin sulfate (CS) chains, but it also consists keratin sulfate (KS) chains and both N linked and O linked oligosaccharides (Buckwalter *et al*., 1994; Kiani *et al*., 2002). Major function of aggrecan is to distribute the load in weight bearing joints. Aggrecan is found to play a vital role in mediating both chondrocyte and matrix interactions through binding to hyaluromic acid (HA) (Hardingham and Fosag, 1992; Watanabe *et al*., 1998).

The molecular weight of aggrecan core protein is about 230 kDa and is composed of three globular regions (Watanabe *et al*., 1998; Doege *et al*., 1991), namely, G1, G2 and G3. The main GAG domain namely CS 1 and 2 of aggrecan is located between G2 and G3 domains, whereas, KS are chains situated in the starting portion of core protein between G2 and G3. A short domain called short interglobular domain (IGD) located between G1 and G2 domains helps to attach keratan sulfate chains to the core protein and anywhere on G1 and G2. Apart from this, numerous O linked and N linked oligosaccharides are also bound to the protein core (Fig. 1. 4) (Chandrasekaran and Tanzer 1992; Knudson and Knudson 2001; Kiani *et al*., 2002).

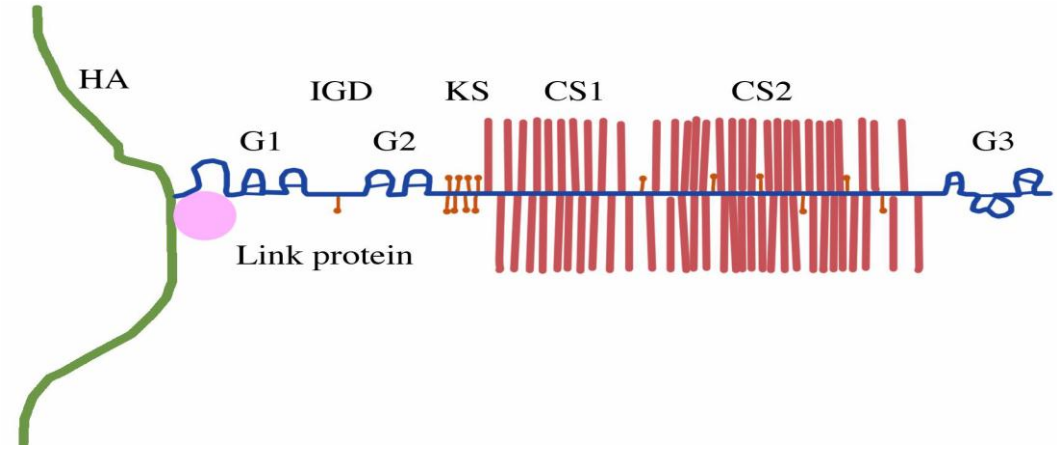


Fig. 1.4: Molecular structure of aggrecan molecule showing its characteristic structural domains (HA: hyaluronan; IGD: interglobular domain; KS: keratin sulfate binding region; CS1: chondroitin sulfate binding region 1; CS2: chondroitin sulfate binding region 2

Majority of the large, multidomain extracellular matrix proteins consists of at least one GAG binding site. For example, collagens (type I, III, V) and fibronectin which form fibrous network in extracellular matrix attach to heparan sulfate chains. In turn, they are bound to the integral membrane core proteins of cell surface proteoglycans such as fibroglycan and syndecan. Besides aggrecan, decorin and biglycan are the most important small leucine rich PGs (SLRPs) in the ECM. The name decorin was originally derived due to its binding on the surface of the collagen fibrils and arrangement in ECM (Scott and Oxford, 1981). Decorin and biglycan are characterized by the presence of one and two

CS/dermatan sulfate (DS) chains (Roughley and Lee, 1994). Molecular weight of their core proteins are approximately 40 kDa (Iozzo, 1997). Lumican and fibromodulin which are the other PGs of the SLRP family contain KSPG chains and have protein cores of molecular weights of 38 and 42kDa respectively (Plaas *et al*., 1990; Roughley and Lee, 1994; Iozzo, 1997). The O linked GAG chains are attached to the core protein in decorin and biglycan whereas, KS chains in fibromodulin are N linked oligosaccharides. Other than these major PGs, versican is another large molecular weight HA binding PG found in cartilage at lower levels. Structurally, it has an N terminal, G1 domain and C terminal G3 domain similar to that of aggrecan.

1.4.3.3 Cell surface proteoglycans

Cell surface proteoglycans well known for their role as co-receptors are associated with normal cell-surface receptor proteins. They are mainly involved in connecting cells to the ECM and forwarding the response of cells to biochemically vital extracellular signal proteins. Cell surface proteoglycans mainly help in anchoring the cell to matrix fiber proteins. One of the important small leucine rich CS/DS PG is epiphycan and has 35 kDa sized core protein (Iozzo, 1998). This is structurally similar to osteoglycin. It also plays a vital role in cell to cell interaction and signaling. It has also been found to participate in the differentiation of chondrocytes and osteogenesis (Kurita *et al*., 1996; Knudson and Knudson, 2001).

Collagens type I, III and V also called fibrous collagens and fibronectin anchor to heparan sulfate chains of proteoglycans in ECM. They are associated to the core proteins of syndecan and fibroglycan, known as high molecular weight integral membrane cell surface proteoglycans. Syndecans and betaglycan, membrane spanning CS / heparan sulfate (HS) PGs, consist of a core protein which is composed of three domain namely, a single membrane spanning domain, a short cytoplasmic domain and an extracellular domain (Bernfield and Sanderson, 1990; Hardingham and Fosang, 1992). Syndecan is another high molecular weight cell surface PGs which plays an important role in cell signalling and cell to cell interaction. Four different syndecan PGs are present, namely, syndecan1; syndecan2 (fibroglycan);

syndecan3 (Nsyndecan); and syndecan4 (amphiglycan). They have similar cytoplasmic domains and transmembrane, but altered extracellular domains (Tkachenko *et al*., 2005). Syndecan can interact with a variety of growth factors and cell signalling molecules in extracellular matrix with the aid of HS moieties and thus regulates cell behavior (Bernfield and Sanderson 1990). Other than the above mentioned PGs, another type of soluble membrane bound PGs are seen in extra cellular matrix known as betaglycans, which play an unique role in ECM by interacting with TGF-β (Andres, *et al*., 1989).

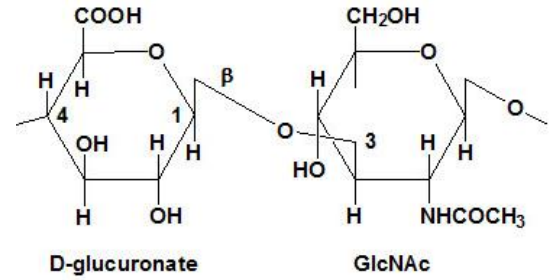
Intracellular granular PGs are another family of proteoglycans which are mainly found in storage granules of nucleus and some cellular organelles. Serglycin is one of the main intracellular granular PG, present in the storage granules of connective tissue mast cells. They help in packaging and regulation of the activity of proteases (Schick *et al*., 2001).

1.4.4 Glycosaminoglycans (GAGs)

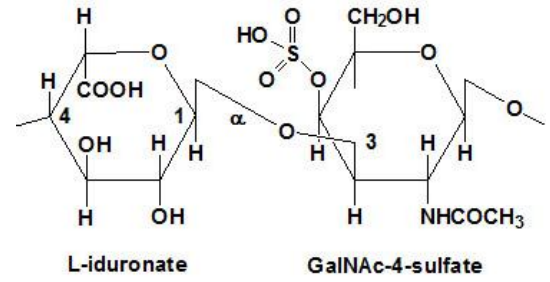
Glycosaminoglycans (GAGs) are the most abundant heteropolysaccharides in the body. These molecules characterized by long unbranched polysaccharides chains are composed of a repeating disaccharide unit. Structurally, the disaccharide units are made up of either of two modified sugars, *N*-acetylgalactosamine (GalNAc) or *N*-acetyl glucosamine (GlcNAc) and uronic acid such as glucuronate (GlcA) or iduronate. GAGs are highly negatively charged macromolecules with stretched and spreaded conformation that are responsible for high viscosity to the surroundings in which they present. GAGs are situated mainly on the cell surfaces or inside the extracellular matrix (ECM). They are also present in secretory vesicles of some category of cells (Gandhi and Mancera, 2008; Jackson *et al*., 1991; Casu and Lindahl, 2001).

The most important and common GAGs of biological significance are hyaluronic acid, dermatan sulfate, chondroitin sulfate, heparin, heparan sulfate, and keratan sulfate. Although each of these GAGs has a prominent disaccharide unit (Table 1.2), heterogeneity is evident in the sugars present in the composition of any given type of GAG.

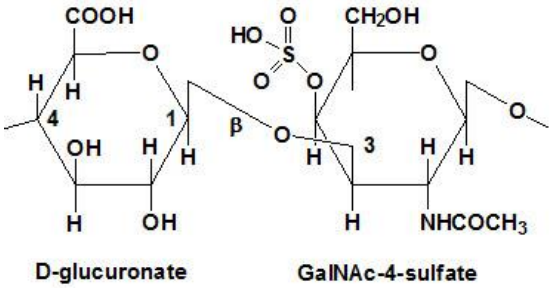
Table 1.2 Classification of glycosaminoglycans (GAGs)



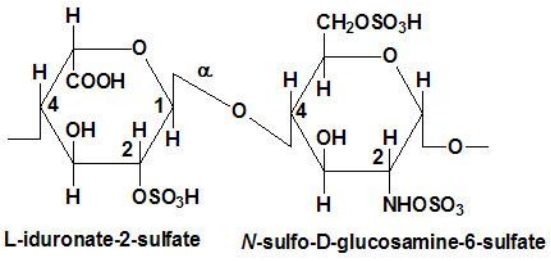
Hyaluronates:
composed of D-glucuronate (GlcA) plus GlcNAc; linkage is β(1,3)



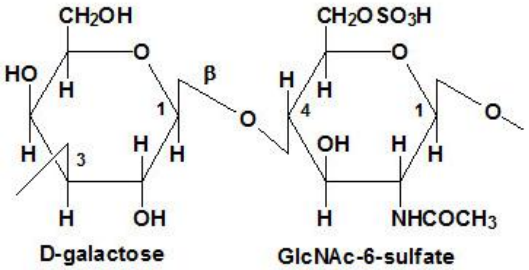
Dermatan sulfates:
composed of L-iduronate (IdoA) of D-glucuronate (GlcA) plus GalNAc-4-sulfate; GlcA and IdoA sulfated; linkages is β(1,3) if GlcA, α(1,3) if IdoA



Chondroitin 4- and 6-sulfates:
composed of D-glucuronate (GlcA) and GalNAc-4- or 6-sulfate; linkage is β(1,3) (the figure contains GalNAc 4-sulfate)



Heparin and Heparan sulfates:
composed of L-iduronate(IdoA: many with 2-sulfate) or D-glucuronate (GlcA: many with 2-sulfate) and *N*-sulfo-D-glucosamine-6-sulfate; linkage is α(1,4) if IdoA, β(1,4) if GlcA: heparans have less overall sulfate than heparins



Keratan sulfates:
composed of galactose plus GlcNAc-6-sulfate; linkage is β(1,4)

https://themedicalbiochemistrypage.org/glycans.php © 1996–2017 themedicalbiochemistrypage.org

GAGs possess high viscosity and low compressibility, which makes these heteropolysaccharides appropriate as lubricating fluid in the joints of the body. Apart from this, GAGs help to impart structural integrity to cells and act as passage ways between cells, hence cell migration. They have great biological importance since they help in intercellular communication in organisms. This unique category of linear polyanions associates with many types of proteins, such as growth factors and cytokines, which control vital physiological processes.

1.4.4.1 Characteristics of Glycosaminoglycans

Table 1.3 Characteristics of Glycosaminoglycans (GAGs)

GAG	Localization	Description
Hyaluronate	Articular cartilage, synovial fluid, vitreous humor, skin, ECM of connective tissue	Very large polymers; molecular weight up to 1 million daltons; high shock absorbing character and low compressibility; synthesis takes place in plasma membrane by three hyaluronan synthases: HAS1, HAS2, and HAS3
Chondroitin sulfate	Cartilage, bone, heart valves	Most abundant GAG in the body; main part of the ECM; usually bound with protein to form proteoglycans; the chondroitin sulfate proteoglycans form a family of high molecular weight molecules called lecticans and include versican, aggrecan, neurcan and brevican. Degradation of chondroitin sulfate from joint cartilage is a major reason behind osteoarthritis
Heparan sulfate	Basement membranes of cells, components of many cell surface proteins	Contains maximum acetylated glucosamine than heparin; have connection with protein forming high molecular weight heparan sulfate proteoglycans (HSPG); major HSPG present are the syndecans and glypicans; HSPG anchors many growth factors ligands such as vascular endothelial growth factor (VEGF), fibroblast growth factors (FGFs), and heptocyte growth factor (HGF); HSPG synthesized from endothelial tissues function as anti-coagulant factors
Heparin	Main component of intracellular granules of mast cells, found in the of lining the blood vessels of the liver, skin and lungs	High degree of sulfation compared to heparan sulfates; widely used as an anticoagulant. Heparin is well known for its role in killing invading bacteria and foreign factors and thus provide immunity
Dermatan sulfate	Tendons, skin, heart valves, blood vessels, and lungs	Previously known as chrondroitin sulfate B ; helps in coagulation process, wound healing mechanisms, and anti-infection mechanisms
Keratan sulfate	Cornea, cartilage aggregated with chondroitin sulfates bone	Found usually in connection with core proteins of proteoglycans forming keratan sulfate proteoglycans; which include lumican, fibromodulin, keratocan, aggrecan, prolargin and osteoadherin

Characterization of GAG has gained much attention due to its unique chemical and biochemical properties. Hyaluronic acid possesses only non-sulphated GAGs and is of anionic nature. Hyaluronic acid normally form non-covalent attachment to serine residues of extracellular matrix proteins and has a varying molecular weight (MW) of 1000 Da to 10,000,000 Da (Schiraldi *et al.*, 2010). It has also been revealed that hyluronan is very much related to biochemical properties of cartilage cells. Among GAGs, heparin is known for its anticoagulant properties. They are produced by granulated mast cells and basophils which have a molecular weight of 3 to 30kDa. Heparin sulphate, sulfated heteropolysaccharide of GAG family, has its structure very much similar to heparin with size ranging from 5-70 kDa (Lindblom *et al*., 1991). HS is attached to a wide variety of polypeptide ligands and regulates diverse biological functions (Necas *et al.*, 2008). Chondroitin sulphate (CS) is another type of sulfated GAGs and is a prominent structural component of cartilage tissue (Baeurle *et al.*, 2009). It is the most abundant GAG present in the body. Molecular weight of CS ranges from 5–50 kDa and is mainly involved in many protein interactions. Dermatan sulphate is the most abundant GAG present in the skin and has a molecular weight of 15–40 kDa. Keratan sulphates, another important GAGs, is also known as keratosulphate (M.W. 4 to 19kDa) and is mainly produced in the neural tissues.

Marine organisms are a rich source of a variety of sulphated and non-sulphated glycosaminoglycans with considerable variations in sugar moieties and sulphation patterns. Marine GAGs produced by many organisms are found to possess regenerative capacity. They are known for their wide range of bioactivities by interacting with growth factors, cell signaling molecules etc. Studies have pointed out the diverse scope of these bioactive GAGs for pharmaceutical and nutraceutical activities (Senni *et al*., 2011).

1.4.5 Purification and characterization of proteoglycans

1.4.5.1 Isolation of proteoglycans (Anion exchange chromatography)

Glycosaminoglycans are highly negatively charged anions associated with different kinds of proteins (Wang *et al*., 1997) and can be very well separated by anion exchange chromatography techniques. Anion exchange chromatography is an important type of adsorption chromatography which helps to hold back a negatively charged solute due to its opposite electrostatic interaction with the positively charged ions on an ion exchange resin. DEAE-Cellulose (Sephacel) is an example of a weak anion exchanger which bears a positively charged amino head at below pH~9 and subsequently will decrease its charge at higher pH values. Thus, this technique can be employed to separate GAGs from a mixture of compounds. Proteoglycans are negatively charged because of glyosaminoglycan chains and researchers exploit this anionic nature to isolate proteoglycans from their surroundings. Weak anion exchange chromatography is used to isolate proteoglycans or glycosaminoglycans from complex extracellular matrix. Chondroitin sulphate attached proteoglycans can also be separated using DEAE sephacel anion exchange chromatography. Researchers used high molar (eg: 7M urea and Tris buffer of pH 6.5) solutions to separate chondroitin sulphate attached peptides from samples (Srichamroen *et al*., 2013). Several studies exploited the negative charge of glycosaminoglycans chains and separated GAGs after β-elimination by anion exchange chromatography. β-elimination process releases GAG chains from protein. DEAE sephacel chromatography is an efficient method to separate GAG chains and proteins of proteoglycan molecules (Lu *et al*., 2010). Nearly twenty four heparan sulfate and chondroitin sulfate motifs including a novel sialic acid-modified chondroitin sulfate linkage hexasaccharide has been separated by using the above method. Several chondroitin sulphate moieties have been separated, isolated and characterized by using anion exchange chromatography previously. Guanidium hydrochloride (Gd. HCl) a chaeotropic agent is very useful to break hydrogen and other weak bonding of proteoglycans with their surrounding extracellular proteins. This process releases GAG chains or proteoglycans to the solution after digestion process and are separated by weak anion exchange method.

Roughley and White, 1989 reported the identification and separation of proteoglycans from articular cartilage using chaeotropic digestion and anion exchange chromatography. Proteoglycans from adult bovine tendon were isolated by chaeotropic digestion and were separated using anion exchange chromatography by high molar (7 M) urea as elution reagent. This was followed by chilled ethanolic precipitation. (Vogel and Heinegard, 1985). Similar way of isolation of PGs from articular cartilage has also been reported (Tomiosso *et al*., 2005).

1.4.5.2 Electrophoretic characterization of GAGs and PGs

Electrophoresis is the process of migration of charged molecules through solutions in an applied electric filed. The high resolution ability of polyacrylamide and agarose gel electrophoresis makes them one of the reliable methods for the separation of macromolecules. Electrophoresis is proven to be an ideal method of characterization of GAGs and proteoglycans because of their macromolecular nature and negative charge. Electrophoretic characterization of GAG oligosaccharides has been employed to study this molecule. Extraction and characterization of different types of GAGs and proteoglycans from bone tissue were reported and clear identification of various GAGs and PGs were observed (Coulson-Thomas *et al*., 2015). GAGs are heteropolymeric in nature and also are present as oligosaccharides. 10% polyacrylamide gel electrophoresis was used to separate GAG oligosaccharides. Oliosaccharides can be generated by digestion with chondrotinase ABC and hyaluronidase enzymes which releases several GAG oligomers. This digestion method was employed to digest chondroitin 4- or 6-sulphate and subsequent electrophoresis revealed several ladder like pattern of GAG oligomers (Cowman *et al*., 1984).

Polyacrylamide gel electrophoresis (PAGE) was employed to characterize heparin, a type of GAG present in human plasma. Higher grade polyacrylamide, 24-30%, was used to obtain GAG band after enzymatic digestion with heparinase. Major heparin chains were identified and characterized using this method (Cavari and Vannucchi, 1996). Extracellular GAG chains were isolated with 4 M guanidinium chloride and purified by anion exchange chromatography with DEAE-sephacel. The GAGs thus obtained were electrophoretically identified and

visualized by SDS-PAGE (Tomiosso *et al*., 2005). Proteoglycans are often characterized by SDS-PAGE method due to the presence of protein core. Modified agarose/acrylamide method was found efficient to separate and identify proteoglycans such as decorin, biglycan etc (Bashey *et al*., 1993). A modified method was reported (McDevitt and Muir, 1971) to electrophoretically separate and characterize PGs of higher molecular masses. The bands were visualized by toluidine blue staining because of the presence of GAG chains.

1.4.5.3 Mass spectrometry of GAGs and PGs

Characterization of proteoglycans comprises analysis of glycosaminoglycan and protein. Gel electrophoresis, mainly agarose gel electrophoresis and polyacrylamide gel electrophoresis, is a useful method to characterize proteoglycans and GAG chains of varying molecular weight (Cavari and Vannuchi, 1996). Mass spectrometry (MS) is a sophisticated tool that identifies and quantifies molecules based on their mass-to-charge ratio (m/Q, m/q, m/Z, or m/z) and is known for high specificity, sensitivity and accuracy (McLafferty and Turecek, 1993; Bruins *et al*., 1987). GAGs and GAG oligosaccharides are effectively characterized by tandem mass spectroscopy using electron spray ionization (Crawley *et al*., 2004).

Mass spectrometry (MS) is widely used for the structural characterization of GAGs present in biological samples. Advanced ionization techniques are available to determine the molecular weight of GAGs in an accurate way. Tandem mass spectroscopy has emerged as a promising option for structural analysis of GAGs. Combination of LC/MS and tandem MS has gained great attention to characterize glycosaminoglycans recently (Staples and Zaia, 2011). Researchers have developed precise and sensitive methods with LC-MS/MS for determination of many disaccharides produced from specific GAGs such as heparan sulfate (HS), dermatan sulfate (DS), chondroitin sulfate (CS) and keratan sulfate (KS). Disaccharides produced by enzyme digestion were identified and quantified using a method which includes a solvent system of acetonitrile gradient in ammonium acetate (pH 11.0) (Tomatsu *et al*., 2014). Auray-Blais *et al*., 2012 introduced liquid chromatography–tandem mass spectrometry (LC–MS/MS) based characterization of GAGs from different types of mucopolysaccharidosis patients.

Robust method was developed in LC–MS/MS to accurately quantify GAGs of varying length from urine samples of patients.

Liquid chromatography (LC) has been employed for the quantification of nearly twenty five types of heparan and chondroitin sulphate oligomers and disaccharides. Further characterization was accomplished by LC/MS. Disaccharides were detectable even at picomolar level following derivitization procedure from minute volume of serum from human and fetal bovine (Lu *et al*., 2010).

Proteomics technology has enabled identification of targeted proteins in a given sample. The technology identifies typical proteins and is employed to develop profiles of known or unknown proteins in samples using protein database search (Hanash, 2003). Mass spectrometry can be better employed to generate and analyze complete proteomes. Matrix assisted laser desorption/ionization mass spectrometry (MALDI-MSI) of biological samples helps in comprehensive characterization of numerous unknown molecular species such as peptides and proteins (Seeley and Caprioli, 2008). LC-MS/MS protocols with electron ionization spray (ESI) ionization has also been useful for proteomics study. Proteomics based characterization of proteoglycans was employed to characterize extracellular profiling of proteoglycans and predominant proteoglycans namely, versican, decorin, perlecan, and lumican (Talusan *et al*., 2005).

UV-Vis spectroscopy is another frequently employed method used in laboratories for the quantitative determination of GAGs moieties. DMMB (1, 9-dimethyl methylene blue) staining can be used for the visualization of intact GAGs (Gandra *et al*., 2000; Barbosa *et al*., 2003). DMMB specifically binds to sulphated GAGs and produces purple to pink colour with an absorption maxima at 656 nm.

1.4.6 Cancer

Cancer is one of the most dreadful diseases which is found globally distributed among the world's population. One of the ways cancer occurs is due to excessive free radical generation which ultimately causes damage to the genetic material DNA, protein and lipids. This DNA damage leads to mutations which

causes normal cells to transform into cancer cells (Evan and Vousden, 2001). Failure of apoptosis and increased rate of cell survival occurs due to DNA damage that results in cancer development. Apoptosis is the major form of programmed cell death which takes place in all the cells to maintain homeostasis and cellular integrity. Cancer treatment target this apoptotic pathway by increasing apoptosis in cells and thus preventing cancer (Kalimuthu and Se-Kwon, 2013; Lv *et al*., 2014).

1.4.7 Apoptosis

Apoptosis, also called programmed cell death, plays a key role in protection against uncontrolled proliferation of cells and thereby enabling the body to eradicate unwanted cells and maintain proper homeostasis (Elmore, 2007). Morphological changes in the nucleus is an important mechanism in apoptosis. During apoptosis several actions occur in nucleus which include chromatin condensation and chromosomal fragmentation, rounding-up of the cell, decrease in cellular volume and nuclear shrinkage. The plasma membrane remains intact during the course of the entire mechanism. The end stage of apoptosis is characterized by some of the morphological events such as structural modification of cytoplasmic organelles, membrane blebbing and loss of membrane integrity (Kroemer *et al*., 2005; Ziegler and Groscurth, 2004).

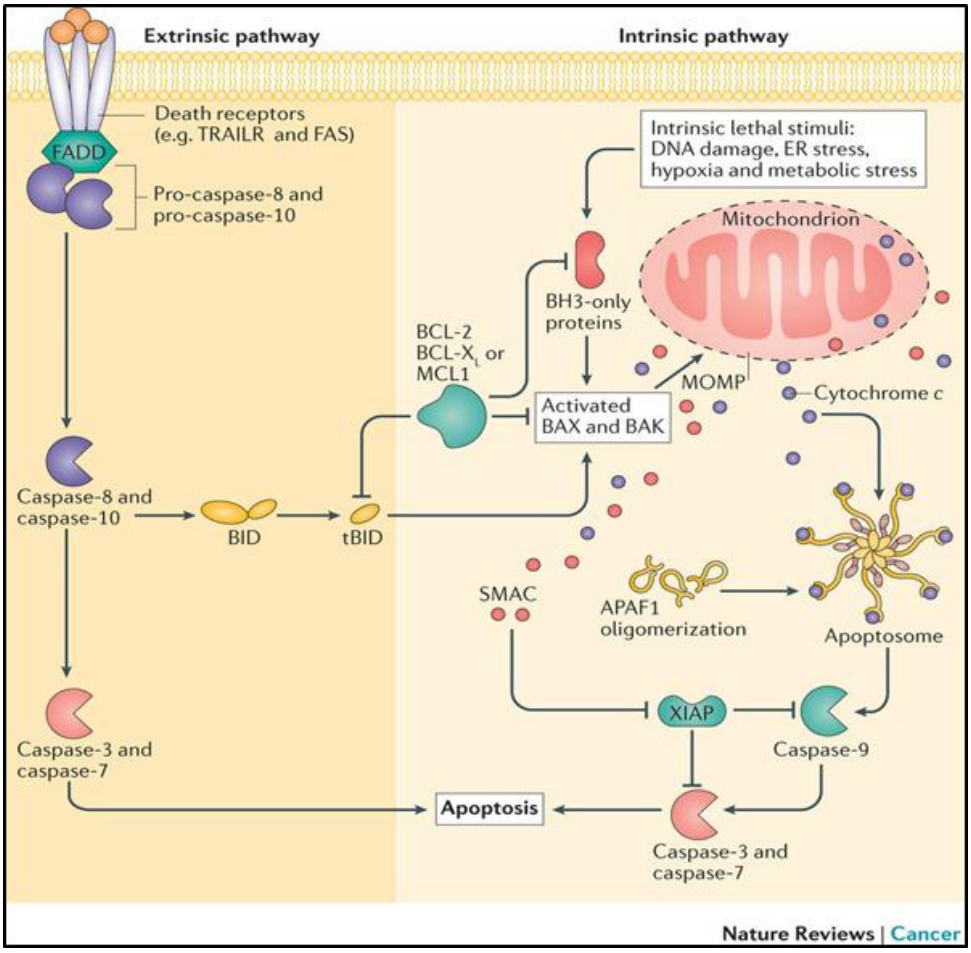
1.4.7.1 Biochemical changes in apoptosis

Changes in apoptosis can be classified broadly into following phases: 1) caspases activation, 2) breakdown of DNA and protein and 3) changes in the membrane and 4) activation of phagocytosis (Kumar *et al*., 2010). Fragmentation of DNA is the hallmark of apoptosis in which characteristic breakdown of DNA into 50 to 300 kilobase fragments occurs (Vaux and Silke, 2003). Another characteristic feature of apoptosis is the activation of a group of enzymes belonging to the cysteine protease family called caspases. The "c" of "caspase" denotes a cysteine protease, whereas the "aspase" denotes the enzyme's specific property to break protein chain after aspartic acid moieties (Kumar *et al*., 2010). Caspases, when activated, start to cleave several important cellular proteins and results in cytoskeleton breakdown. They also trigger the activation of unique

DNAase, which in turn break down nuclear DNA (Lavrik *et al*., 2005). Therefore it is evident that biochemical analyses of DNA fragmentation or caspase activation can be used to identify the involvement of apoptosis in the cells.

1.4.7.2 Mechanisms of apoptosis

Identifying the mechanisms of apoptosis is vital and this helps to monitor the status of cancer progression. This in turn, may be used in the development of medicines that specifically target apoptotic genes or pathways. Caspases are major markers in the mechanism of apoptosis as they are known as initiators and executioners of apoptosis. Caspases can be activated in three major pathways and the two commonly observed starting pathways are the intrinsic and extrinsic pathways of apoptosis (Fig. 1.5). Eventually these pathways lead to a common pathway known as execution phase of apoptosis (Elmore, 2007).



Source: (Ichim & Tait, 2016)

Fig. 1.5 Apoptosis pathways

1.4.7.3 The extrinsic death receptor pathway

Extrinsic pathway begins as death ligands bind to a death receptor which includes type 1 TNF receptor (TNFR1) and Fas (CD95) (Hengartner, 2001). Major proteins present in this pathway are TNF receptor-associated death domain (TRADD) and Fas-associated death domain (FADD), as well as a major type of cysteine proteases called caspase 8 (Schneider and Tschopp, 2000). When death ligand binds to the death receptor, binding site for an adaptor protein is formed known as death-inducing signalling complex (DISC) (O'Brien and Kirby, 2008). This complex then helps in the assembly and triggering of pro-caspase 8. This in turn gets converted into caspase 8, an initiator caspase, which is responsible for the beginning of apoptosis by breakdown of executioner caspases (Karp, 2006).

1.4.7.4 The intrinsic mitochondrial pathway

This is a major pathway in apoptosis and is initiated inside the mitochondria. Stimuli such as irreversible gene damage, low oxygen in the cells, very high concentrations of cytosolic Ca^{2+} and strong oxidative stress are known to initiate intrinsic mitochondrial pathway (Karp, 2006). Apart from these stimuli, this pathway is activated by increased mitochondrial permeability and production and release of one of the major pro-apoptotic enzyme called cytochrome-c into the cytoplasm (Danial and Korsmeyer, 2004). The pathway is mainly regulated by a group of unique proteins belonging to the Bcl-2 family (Tsujimoto *et al*., 1984). The Bcl-2 protein family comprises two major categories of highly specialised proteins, viz the pro-apoptotic proteins (e.g. Bax, Bak, Bcl-Xs, Bid, Bad, Bim, Bik, and Hrk) and the anti-apoptotic proteins (e.g. Bcl-2, Bcl-W, Bcl-XL, Mcl-1 and Bfl-1). The function of anti-apoptotic proteins is to regulate apoptosis by preventing the release of cytochrome-c into the mitochondria. At the same time the pro-apoptotic proteins act by reversing anti-apoptotic action. A balance between the pro- and anti-apoptotic proteins determines whether apoptosis needs to be activated or not (Reed, 1997).

1.4.7.5 The common pathway

The execution phase of apoptosis is the end stage which involves the activation of a cascade of caspases. Caspase 9 is the major upstream caspase for the intrinsic pathway and caspase 8 for the extrinsic pathway. The intrinsic and extrinsic pathways finally trigger the activation of caspase 3. Caspase 3 breaks down the inhibitor of the caspase-activated deoxyribonuclease, which trigger the initiation of nuclear apoptosis. Apart from this, downstream caspases accelerate the cleavage of many protein kinases, cytoskeletal proteins, DNA repair proteins family and endonucleases family. They also activate cytoskeleton, cell cycle and signalling pathways, which are all together responsible for the morphological changes in apoptosis (Kroemer *et al*., 2007; LaCasse *et al*., 2008; Ghobrial *et al*., 2005).

1.4.7.6 Apoptosis in cancer

Cancer cells respond to treatment with chemotherapeutic drugs because the latter trigger apoptosis pathway. Activation of apoptosis is characterized by the activation of the caspase cascade of enzymes belonging to aspartate protease family. These enzymes are responsible for various mechanisms involved in apoptosis such as DNA laddering, cytochrome-c activation, breakdown of different proteins and finally cell death (Fulda and Debatin, 2006; Elmore, 2007).

p53 gene is considered as one of the major regulators of cell division and is associated with mutations that eventually lead up to cancer development. It is also a potential inducer of positive and negative mediators of apoptosis such as BAX, BCL_2 etc (Brown and Attardi, 2005). This gene is a subject of extensive investigations in relation to its role in apoptosis-induced cell death. The possible ways in which these mediators control apoptosis is by either regulating mitochondrial permeability or by controlling cytochrome *c* release (Kuo *et al*., 2002; Singh *et al*., 2005; Ferlini *et al*., 2003). Therefore, a study of expression of these proteins could indicate the involvement of apoptosis during investigations into anti-cancer effect of biomolecules.

1.4.8 Cervical cancer and breast cancer

Cervix is the lower portion of the uterus and cervical cancer develops when cells in the cervix grow abnormally due to an unknown mechanism. Like other types of tumors, it takes several years for the development of cervical cancer. Early detection is the safest mode of prevention and approximately 92% of recovery cases were reported for early detection of cervical cancer. It was reported that cervical cancer cause more than 288,000 deaths every year worldwide and most frequently affects women from poor and most susceptible populations. More than 80 percent of cervical cancer mortalities are reported in developing countries, with maximum incidences from the underdeveloped regions of - South Asia, regions of Africa and areas of South American countries (International Agency for Research Cancer). Human papillomavirus which infect a woman's reproductive area such as vagina is one of the main reasons behind cervical cancer (Liu and Zhongs, 2011).

Breast cancer is the most prevalent cancer in the world after lung cancer in terms of occurrence (Parkin *et al*., 2001). This malignant tumor starts in the cells of the breast. It is found mostly in women, but men get breast cancer too. This disease is a serious concern in developing countries like India. This is the most common type of cancer among women in both developed as well as developing countries (Sen *et al*., 2002; Pandey and Chandravati *et al*., 2013). The incidence of breast cancer cases has been increasing among Indian women and it has been estimated that by the end of 2030, there will be about 200,000 new breast cancer cases (Datta *et al*., 2012; Sharma *et al*., 2013). In India, about 1 out of 28 women has breast cancer during her life time. This incidence is 1 out of 22 in rural areas and 1 out of 60 women in urban areas. Because of its high rate of incidence, especially among females, there is urgent need of offering promising solutions. The exact reason behind breast cancer remains uncertain owing to its heterogenous nature, both histopathologically and genetically (Hedenfalk *et al*., 2002). *In vitro* cell line studies are an excellent option to study about cancer, evaluate the effect of different drugs elucidate their possible mechanisms of action. MCF-7 cell line is the most widely used breast cancer cell line in the

world, which was introduced in 1973 at the Michigan Cancer Foundation. This has become the ideal model for studying breast cancer *in vitro* (Soule *et al*., 1973).

1.4.9 Proteoglycans in cancer treatment

Studies have reported anti-cancer activities of mushroom species in animal models due to the presence of special proteoglycans. Some mushrooms contain proteoglycans or peptide bound polysaccharides which are chemically formed by the covalent attachment of protein and glycans such as β-glucans. Two mushroom derived proteoglycans namely, PSK (Polysaccharide-K) and PSP (Polysaccharide-Peptide) isolated from *Coriolus versicolor* (formerly *Trametes versicolor, Polyporus versicolor*) have been extensively studied in human carcinoma experiments such as those of stomach, colon, lung, esophagus and breast cancer. In clinical trials, PSK was found to control the spreading of cancer growth and extended the survival of the affected people for longer periods compare to the untreated. PSP in particular is found to be effective against oesophagus cancer. PSP was found to significantly enhance the quality of life of affected people, to alleviate the pain generated during the disease, and to improve immune response in 70-97% of patients affected with cancers (Fritz *et al*., 2015).

Phellinus linteus (PL), is a type of mushroom that is widely seen in mulberry trees and one of the major ingredients of traditional medicine in Korea, Japan, China and other Asian countries. It is used for treatment of many types of diseases such as digestive tract ulcer, gastroenteric problems, lymphatic diseases and some kind of tumors. Studies reported a special type of proteoglycan, Proteoglycan (P1) isolated from *Phellinus linteus* mushroom is found to exhibit anti-cancer activities. The novel proteoglycan purified from *Phellinus linteus* found was to be a potential anti-cancer biomolecules. The possible mechanism of action of P1 could be by protecting T cells from prostaglandin attack, triggering IgA anti-body activity and inhibiting the cancer activating cell signalling pathways (Li *et al*., 2011). These results suggest the important role of proteoglycans in controlling the cancer progression and its potential to prevent cancer growth.

Small leucine-rich proteoglycan (SLRP) family comprises low molecular weight proteoglycans with repeating leucine residues in their protein chain. The major types of SLRP include decorin, biglycan, lumican, fibromodulin etc. The role of lumican has been documented in many tumor studies such as pancreatic cancer and melanoma. For example, increased lumican mRNA expression is reported in pancreatic cancer cells (Li *et al*., 2014). Lumican is found to trigger the apoptotic mechanism in cancer and thus controls tumor progression. This was found effective against melanoma cancer by controlling cellular mechanism involved in cancer (Vuillermoz *et al*., 2004). Decorin is an important type of SLRP which function as a cellular alignment protein especially in collagen arrangement. Decorin acts as a key cell signalling proteoglycan which controls normal cellular homeostasis and any alteration may lead to cancer. Studies have reported that it has the ability to inhibit multiplication and growth of different types of cancer (Nash *et al*., 1999) by down regulating the EGF receptor-dependent kinases and stimulating p21CIP1/WAF1 (Iozzo *et al*., 1999).

1.4.10 Osteoarthritis (OA)

Osteoarthritis (OA) is the most reported joint disorder mainly seen in elderly people (Busija *et al*, 2010). The common characteristics of OA include joint injury, degeneration of cartilage structure and biological function and loss of regulation of pro-inflammatory and anti-inflammatory pathways (Goldring and Otero, 2011). The articular cartilage and subchondral bone of synovial joints are affected at the beginning leading to gradual joint cartilage erosion and causing pain upon weight-bearing such as walking and standing (Krasnokutsky *et al*., 2008). Although cartilage degradation is the most common observation in OA, the progressive degradation of collagen is the basic incident that is responsible for the irreversible progression of OA. Studies have shown that pathogenesis of OA is due to inflammation at the site of synovium joints. Synovial inflammation is characterized by an infiltration of neutrophils, T lymphocytes and monocytes as well as degeneration of synovium due to hypoplasia (Smith *et al*., 1997; Fiorito *et al*., 2004).

Till date, there is no proper cure for OA since it is almost impossible to restore the cartilage once it is damaged or destroyed (Sgaglione, 2005). Treatments are mainly used to alleviate symptoms viz., pain, maintain or improve joint mobility, make the joints stronger and minimize the side effects as less as possible. Major treatment options of OA target inflammatory mediators and protection of chondrocytes in order to relieve pain and maintain matrix integrity in the joints of OA patients. Therefore, alleviated inflammation will play a key role in OA treatment. Studies have showed control of arthritic progression using herbal extracts and the mechanism was found to be by regulating pro-inflammatory and inflammatory mediators.

1.4.10.1 Articular cartilage

Fig. 1.6 depicts structure of articular cartilage. Articular cartilage is made up of highly specialized cells that plays vital role in joint mobility and to withstand mechanical pressure at the joints (Buckwalter and Mankin, 1998; Bhosale and Richardson, 2008). The ECM of cartilage has a definite composition of macromolecules to meet the characteristics features of the tissue (Mow *et al*., 1992). Cartilage ECM is made up of mainly collagens and heavily negatively charged proteoglycans. Apart from this other non-collagenous matrix proteins also add to the complexity and is vital for ECM arrangement and tissue homeostasis. (Schaefer and Schaefer, 2010).

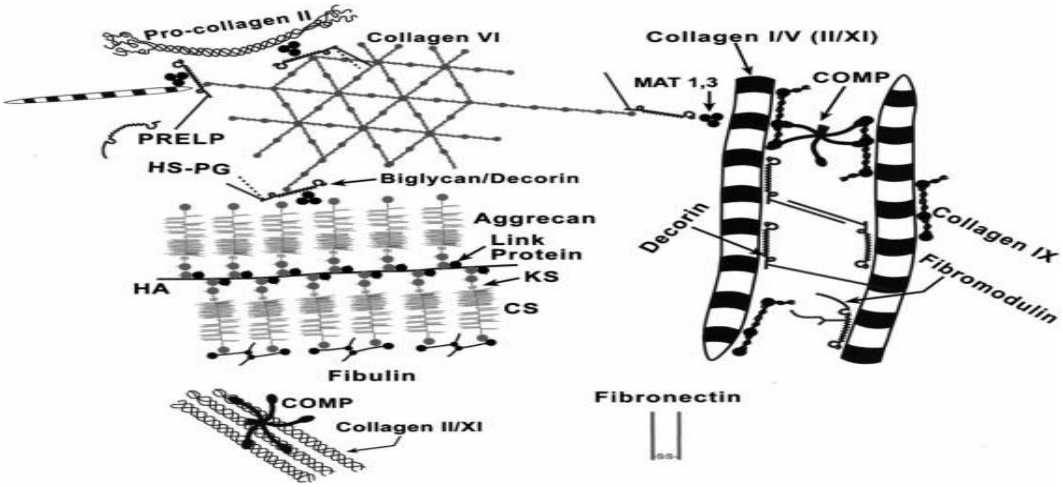


Fig 1.6: Cartilage extracellular matrix. Schematic representation of cartilage illustrates the complex alignment of variety macromolecules such as

collagens, glycoproteins, proteoglycans and glycosaminoglycan in the ECM. Cartilage is composed of these molecules and well decorated in the extracellular matrix by unique integration which is the reason behind the specific characteristics of the cartilage tissue (Feng *et al.* 2006).

1.4.10.2 Enzymes in osteoarthritis

Osteoarthritis is a joint degenerative disorder often associated with degradation of extracellular matrix at joints. Articular cartilage is the major affected area in OA due to degeneration of cartilage protein mainly, proteoglycans and type II collagen. Proteolytic enzymes produced during OA condition specifically cleave aggrecan, a major proteoglycan of the joint cartilage and type II collagen by collagenase (Mankin and Lippiello, 1970). Proteolytic cleavage of articular cartilage results in the erosion of cartilage at the joints and creates severe physiological symptoms by losing structural integrity (Jubb and Fell, 1980). The loss of aggrecan due to proteolysis is due to proteolytic breakdown within the interglobulin domain (IGD) between the G1 and G2 globular domains exclusively at two sites, Asn341-Phe342 and Glu373-Ala374. Many types of MMPs (metalloproteinases) have been found to cut at the Asn341- Phe342 (Flannery *et al*, 1992; Fosang *et al*., 1993) whereas the proteolysis of the Glu373- Ala374 is by the action of ‘‘aggrecanase’’ enzymatic activity (Sandy *et al*., 1992; Lohmander *et al*., 1993; Arner *et al*., 1998). ADAMTS-4 and ADAMTS-5 were the first reported aggrecanases and belongs to Disintegrin A and Metalloproteinase gene family (Tortorella *et al*., 1999; Abbaszade *et al*., 1999). Recently, a third ADAMTS (ADAMTS-1) has been discovered to cleave proteoglycan aggrecan at different sites and has been included in ‘‘aggrecanase’’ enzyme group (Rodriguez-Manzaneque *et al*, 2002; Somerville *et al*, 2003). Type II collagen is the most important collagen in articular cartilage responsible for structural integrity. It was discovered that many types of MMPs (MMPs 1, 8, 13, 14 and 18) are able to cleave intact collagen between residues Gly775 and Leu776. Based on the enzymatic action, neo-epitope antibodies that specifically identify the carboxy terminal neo-epitope of the cleavage site, antibodies to denatured collagen have been developed (Billinghurst *et al*, 1997). Metallo proteinases and aggrecanase

are mainly responsible for the prognosis of inflammation during osteoarthritis. This was observed in iodo acetamide induced animal studies using Wistar rats.

1.4.10.3 Inflammatory mediators in osteoarthritis

OA is reported to be associated with activation of several pro-inflammatory cytokines which in turn create severe inflammatory symptoms. The major cytokines produced are, interleukin (IL-1β) and tumor necrosis factor (TNF-α). These cytokines trigger degradation of articular cartilage and synovium which enhances the severity of OA symptoms (Goldring and Goldring, 2004; Kobayashi *et al*, 2005; Goldring and Otero, 2011; Zwerina *et al*., 2007). TNF-α, a pro-inflammatory cytokine produced by macrophage, is responsible for cartilage damage, recruitment of other inflammatory cells in the synovium and accelerated production of other pro-inflammatory cytokines such as IL-1β. IL-1β triggers bone degradation and collagen destruction through activation of many metalloproteinase, MMPs (Arend and Dayer, 1995). Studies showed that production of TNF-α and IL-1β was controlled by another pro-inflammatory cytokine called, IL-17 (Jovanovic *et al*., 1998). CINC-1, a pro-inflammatory cytokine, activates the inflammatory symptoms by attracting the neutrophils to the site of affected joints (Takano and Nakagawa, 2001).

IL-1β and TNF-α are elevated in the areas such as synovial membrane, synovial fluid, and articular cartilage in OA affected persons. (Kapoor *et al*., 2011). In succession to the activation of IL-1β and TNF-α, a series of proinflammatory cytokines, chemotaxis agents, several metalloproteinases (MMPs), and nitric oxide (NO) production are also triggered (Kapoor *et al*., 2011; Daheshia and Yao, 2008). Thereafter, cytokines are produced to inhibit proliferation of chondrocyte and induce apoptosis mediated cell lysis (Kapoor et al, 2011; Caramés *et al*, 2008; Wang *et al*, 2015). Many studies have shown that IL-1β and TNF-α inhibit the natural production of cartilage matrix components (collagen type II and aggrecans) (Séguin and Bernier, 2003; Stabellini *et al*, 2003). Studies suggests that inhibiting IL-1β or TNF-α could be an ideal strategy

to control the degradative mechanisms associated with OA prognosis (Chevalier *et al*, 2013; Martel-Pelletier *et al*, 2012; Jotanovic *et al*, 2012). The use of IL-1β inhibitors in *in vitro* and *in vivo* animal models of OA have shown that IL-1β can be considered a vital new target for OA treatment. Hence, controlling IL-1β production during development of OA could be effective to inhibit a series of pathways associated with production of cartilage degrading enzymes, interleukins and chemotaxis agents.

1.4.10.4 Monosodium iodoacetate induced animal model

Monosodium iodoacetate induced animal model is an ideal model to study osteoarthritis. Monosodium iodoacetate is a protein inhibitor which blocks biosynthesis of glycosaminoglycans and proteoglycans by inhibiting certain enzymes. Kalbhen and Blum, 1997 were the pioneers who described the iodoacetate-induced model of degenerative arthritis in chickens and later the model was experimented in many other animals (Kalbhen and Blum, 1997; Williams and Brandt, 1984; Buchmann and Kalbhen, 1985; Van der Kraan *et al*., 1989) Monosodium iodoacetate impairs the synthesis of cartilage and causes degeneration of cartilage. The severity of cartilage degradation, reduced mobility and inhibition of synthesis of proteoglycans is very much related to monosodium iodoacetate in a dose dependent manner (Guingamp *et al*., 1997).

1.4.11 GAGs and PGs in arthritis

Various compounds, including GAG (chondroitin sulfate, D-glucosamine sulfate and hyaluronic acid), piroxicam, ademetionin, oxaceprol and heparinoids (Steinmeyer, 2017) have been found to produce not only an anabolic effect on chondrocytes but also an inhibitory effect on free catabolic metabolites under *in vitro* and *in vivo* conditions (Towheed, 2002).

Aggrecans are glycosylated proteoglycan molecules present in cartilage as large aggregates bound with hyaluronan and link proteins, and they are highly hydrated due to the negatively charged glycosaminoglycan heteropolymer chains attached to the core proteins. This is responsible for the ability of cartilage to

resist compressive loads. Type II collagen present in cartilage forms a fibrillar meshwork that helps the tissue with tensile strength. These macromolecules play a vital role in regulating homeostasis as well as the structural integrity of cartilage. In all cartilage types, proteoglycans and collagen fibrillar network help to maintain the mechanical functioning of joints and cartilage integrity (Han *et al*., 2011). Hence, treatment to restore the mechanical and structural functioning of joints through regeneration of aggrecan and type II collagen can be the best option of OA treatment. In OA, there is an elevated production of proteolytic enzymes called aggrecanases and matrix metalloproteinases (MMPs), which are responsible for the degradation of cartilage tissues at joints (Murphy and Nagase, 2008).

Studies have reported the importance of glycosaminoglycans against cartilage regeneration in arthritic condition. Anti-inflammatory effects of glycosaminoglycan (GAG) isolated from (*Gryllus bimaculatus*) were reported in a chronic arthritic rat model induced by complete Freund's adjuvant (CFA)-treatment. GAG administration significantly reduced symptoms of arthritis by inhibiting C-reactive protein (CRP), inflammatory cytokines such as IL-1βeta, tumor necrosis factor (TNF)-α, interleukin-6 etc in a dose dependent manner (Ahn *et al*., 2014). Glycosaminoglycans such as hyaluronic acid and chondroitin sulfates are found to reduce arthritic inflammation in Lewis rats by multiple intradermal injections of 250 µl emulsion containing bovine type II collagen in complete Freund's adjuvant. Biochemical and immunological parameters and histological assays in blood, synovial tissue and articular cartilage showed significant anti-arthritic effects. Several positive responses were observed after treatment with GAG mainly, reduced articular cartilage and paw erosion, decreased cellular damage at inflammation site, rejunivated anti-oxidant defence system, decreased pro-inflammatory mediators and neutrophil infiltration (Campo *et al*, 2003). Chondroitin sulfate is a vital component of articular cartilage. Administration of chondroitin sulfate in patients with osteoarthritis can cause the stimulation of the synthesis of proteoglycans, decrease in degradation of chondrocytes by inhibiting the production of metalloproteinases and other pro-inflammatory mediators (Martel-Pelletier *et al*., 2010).

1.4.12 Treatment and side effects of OA

The main focus of rheumatology treatment is to provide the best possible care for patients and alleviation of disease-related pain through pain relievers. It is also important to create an awareness among patients. The major symptom of OA is the development of inflammation and associated symptoms thereafter. Studies has shed light on the mechanism of action behind inflammatory symptoms such as pain and on possible preventive action to be taken. Several pain-modifying analgesic drugs have been invented which are efficient in alleviating pain associated with OA prognosis. (Perrot, 2009). The following drugs are the main types of pharmacological compounds used for to control pain in OA patients.

1.4.12.1 Non-steroidal anti-inflammatory drugs (NSAIDs)

Cyclooxygenase (COX) class of enzymes are responsible for arthritic related pain development. Both selective and nonselective cyclooxygenase (COX) inhibitors are found to possess anti-pyretic, anti-inflammatory and analgesic effects and are commonly employed in treating severe painful conditions of OA diseases (Kroenke *et al*., 2009). NSAIDs are most effective and widely used in counteracting the symptoms associated with OA. Most commonly used NSAIDs are naproxen, ibuprofen, celecoxib and diclofenac. NSAIDs are commonly used without considering the relative side effects as they are found to be strong pain relievers (Vonkeman *et al*., 2007). Side effects are quite common with conventional NSAIDs and are known to cause gastrointestinal (GI) side effects (Bhatt *et al*., 2008). Major concern with the usage of NSAID is occurrence of number of deaths associated with NSAID-related gastrointestinal bleeding. Approximately 3500 to 16, 500 deaths per year are reported in relation to NSAID-related gastrointestinal bleeding (Food and Drug Adminstration, 2002). Apart from NSAID-related gastrointestinal bleeding, conventional NSAIDs and COX-2 inhibitors are found to increase cardiovascular risk (Antman *et al*., 2007;

Hippisley-Cox and Coupland, 2005; Laine *et al*., 2008; Trelle *et al*., 2011) and elevate blood pressure (Friedewald *et al*., 2010), particularly in patients with hypertention (Gaziano, 2006). Among NSAIDs, naproxen observed to cause myocardial infarction (Hippisley-Cox and Coupland., 2005).

1.4.12.2 Natural compounds against osteoarthritis

Resveratrol, a natural antioxidant, protects monosodium iodoacetate-induced osteoarthritic pain in rats. Resveratrol has shown the potential to improve MIA-induced cartilage damage by inhibiting the levels and expressions of inflammatory mediators suggesting that it may be a potential therapeutic agent for OA (Wang *et al*., 2016). Shark liver oil, a marine product, has also been reported to show anti-arthritic effect in animal model and it indicates the effectiveness of marine natural products against arthritis (Mathew, 2010). *In vivo* animal studies revealed that collagen peptides from fish skin can exert potent anti-arthritic activity by altering the pathogenesis during arthritis without exerting any side effect during chronic treatment (Hema *et al*., 2016).

1.4.13 Proteomics and metabolomics in arthritis

Proteomics technology has become a popular technology to understand the pathological and biochemical changes during disease. It is used to identify typical proteins and employed to develop new drugs for treatment. (Hanash *et al*., 2003). This technique has gained attention in the profiling of proteins in arthritis. Complete proteome analysis was used to differentiate rheumatoid arthritis (RA), spondyloarthropathy (SpA) and osteoarthritis (OA). For example, alpha-enolase and fructose bisphosphate aldolase A were the protein biomarkers used to identify SpA and OA condition. Whereas, Calgranulin Amyeloid related protein 8 was observed higher in RA but trace level in SpA patients and OA patients (Tilleman *et al*., 2005).

Mass spectroscopy can be better employed to generate and analyse complete proteomes. Matrix assisted laser desorption/ionization mass spectrometry study (MALDI-MSI) of biological samples is a sophisticated and useful technique that helps in comprehensive and proper characterization of

numerous unknown molecular species such as peptides, proteins or other metabolites (Seeley and Caprioli, 2008). Matrix assisted laser desorption ionization mass spectrometry imaging (MALDI-MSI) tool was found effective in protein identification and profiling. MALDI-MSI is a novel tool to study cartilage degradation in osteoarthritis and peptide based protein characterization and is recommended to develop drugs against target molecule (Peffers *et al*., 2014).

Mass spectrometric based proteomics data generation and biochip when used for analyses of plasma and synovial fluid proteins revealed status of RA and OA patients (Sinz *et al*., 2002; Uchida *et al*., 2002). Studies of the pattern and profile proteins of cartilage (Müller *et al*., 2014) are essential to understand age related disorders such as osteoarthritis. Studies have shown peptide profiles during aging and hence can be used to demonstrate the mechanisms of osteorarthritic development.

Cancer prevalence is becoming a big health issue around the world and a proper remedy is yet to be introduced. This disease has maximum mortality rate and cervical and breast cancer together leading the list of cancer occurrence in women. Similarly the incidence of osteoarthritis, a degenerative disease of joint are increasing tremendously and remain a big threat to the health of women. Chemical drugs which are extensively being used to treat the cancer and arthritis of any form is reported to adversely affect the patients due to their several side effects. Recently, drugs from natural sources are gaining attention due to their ability to cure diseases with little side effects. Natural drug from marine sector is gaining attention as it carry several therapeutic and nutraceutical biomolecules. Natural compounds which trigger apoptosis mechanism to control cancer progression and control osteoarthritis by down regulating inflammatory mediators have great importance in treatment. Proteoglycans are unique biomolecules which are reported to have several bioactivities. Their activities are mainly due to the presence of glycosaminoglycans and protein moieties. Cartilage is a rich source of proteoglycans and shark has fully cartilaginous skeleton. Shark cartilage was used as a folk medicine for cancer and arthritic treatment but no reports

substantiate the exact mechanism of action and molecules responsible for its bioactivity. No previous reports are available regarding the anti-cancer (breast cancer & cervical cancer) and anti-osteoarthritic effects of proteoglycans from shark cartilage. In this context, an attempt was taken to evaluate anti-cancer and anti-osteoarthritic effects of proteoglycans isolated from shark cartilage.

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Chapter 2

Extraction, isolation, purification and characterization of proteoglycans from the deep sea shark, *Echinorhinus brucus* cartilage

2.1 Introduction

Proteoglycans (PGs) are heavy glycosylated protein macromolecules of extracellular matrix found in vertebrate and invertebrate tissues, involved in various cellular functions including cell growth, adhesion and differentiation (Kjellen and Lindahl *et al*., 1991; Iozzo, 1998). They are composed of a core protein onto which a variable number of glycosaminoglycans (GAG) side chains are attached. There are two main types of GAGs. Non-sulfated GAGs include hyaluronic acid (HA), whereas sulfated GAGs include chondroitin sulphate (CS), dermatan sulphate (DS), keratan sulphate (KS), heparin and heparin sulphate (HS). There are over twenty types of proteoglycans reported in mammalian and avian species, among which there are more than ten types carrying CS or CS/DS (Nakano *et al*., 2002).

Proteoglycans (PGs) occur in virtually almost all mammalian tissues and are especially prominent in cartilage. Cartilage is a type of smooth, flexible connective tissue comprised of several types of collagens, other proteins, and glycosaminoglycans and is found in the skeletal systems of animals, including humans. Unlike birds, reptiles, mammals, amphibians, and most other fish, sharks have skeletons made up entirely of cartilage. The major compounds in shark

cartilage are proteoglycans and glycoproteins (large molecules with protein and carbohydrate components), as well as protein and calcium salts (Michelacci & Horton, 1989). The predominant proteoglycan present in cartilage is the large chondroitin sulfate proteoglycan, aggrecan. The other important types of proteoglycan present in cartilage are decorin, biglycan, epiphycan, versican etc. Decorin and biglycan are the major small leucine rich PGs (SLRPs) in the ECM, and decorin was originally named due to its binding on the surface of the collagen fibrils (Scott and Oxford, 1981). They usually contain one and two CS/DS chains (Roughley and Lee, 1994).

Epiphycan is a type of small leucine rich PG of CS/DS GAG with a 35 kDa core protein (Iozzo, 1997) and may participate in the chondrocyte differentiation and osteogenesis (Kurita *et al*., 1996; Knudson and Knudson, 2001). Versican is a large molecular weight an HA binding PG found in cartilage at low levels. Glycosaminoglycans are long unbranched heterogeneous polysaccharides consisting of repeating disaccharide units composed of hexosamine (glucosamine or galactosamine) and an uronic acid (glucuronic acid or iduronic acid) that are involved in numerous biological functions, including organogenesis, growth control, cell adhesion, signalling, inflammation and interactions with pathogens. The analysis of isolated GAGs includes both the determination of macroscopic properties and the study of the GAG composition. This is done firstly by the evaluation of the purity of isolated polysaccharides and second by their characterization: Chromatographic, electrophoretic and spectroscopic methods are commonly used for these two purposes (de Lima *et al*., 2007; Higashi *et al*., 2015). So the current unit was aimed at extraction, isolation, purification and characterization of proteoglycans from the deep sea shark *Echinorhinus brucus* cartilage.

2.2 Materials and methods

All chemicals were used of analytical grade and procured from Sigma Aldrich.

2.2.1 Extraction of PGs

2.2.1.1 Shark cartilage collection

Two coastal sharks of weight 3.5 kg, were collected from Kollam Landing centre, Kerala and transported to lab in chilled condition. Cartilage was separated and weighed (500g) and kept at -20^{0}C (Fig 2.1).

Fig 2.1 Shark cartilage

2.2.1.2 Sample preparation

Proteoglycans extraction, isolation and purification was carried out by standard protocol (Vogel and Heinegård, 1985). Cartilage was first cleaned and washed using distilled water in order to remove flesh and debris. The shark cartilage was ground or pulverized to increase the efficiency of extraction (fig. 2.2). A chaotropic agent, guanidine hydrochloride (Gd. HCl) was added to the extraction medium to facilitate extraction by breaking (Hydrogen) H-bonding between adjacent molecules. The extraction was done with sodium acetate buffer at pH 5.8. Complexing agent such as 0.05 N EDTA (ethylene diamine tetraacetic acid), protease inhibitors such as 0.005 N PMSF (phenylmethane sulfonyl fluoride) and other additives like iodoacetamide were also added. Homogenised the mixture and incubated at 40C for 24 h.

Fig 2.2: Shark cartilage pulverized for extraction

2.2.2 Dialysis

50-100 ml of sample was loaded into nitrocellulose dialyzing membrane tubing. Sample was placed into an external chamber of dialyzing buffer containing a little amount of guanidine hydrochloride, sodium acetate and EDTA (Ethylene diamine tetra acetic acid) with gentle stirring of the buffer. Sample mixture was dialyzed for 10-12 h at 4^{o}C temperature with continuous stirring (Fig 2.3). The volume of sample and dialysate determine the final equilibrium concentration of the small molecules on both sides of the membrane.

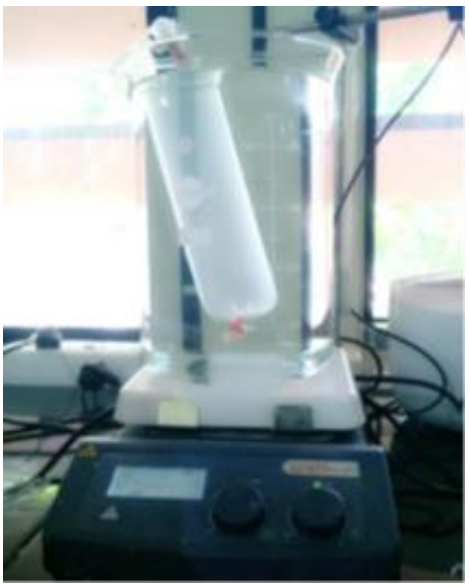


Fig 2.3: Dialysis of proteoglycan extract

2.2.3 Purification

2.2.3.1 Chromatographic method

A) DEAE (Diethyl aminoethyl) cellulose anion exchange chromatography

Purification of PGs was carried out by DEAE-cellulose anion exchange chromatography (Fig 2.4) with 7 M urea and 0.1, 0.2 and 0.4 M NaCl as elution agents. Column was packed in 1:5 DEAE sephacel: solvent ratio and equilibrated in 7 M urea for 12 h at 37^{o}C. 10 ml of dialyzed extract was added on the column and eluted with elution solution and collected.

Fig 2.4: DEAE sephacel anion exchange chromatography for isolation of proteoglycans

2.2.4 Freeze drying

100 ml of the dialyzed sample was subjected to direct freeze drying to obtain proteoglycan powder and stored in refrigerated condition. This material was used for most of the biochemical analysis.

2.2.5 Characterization

Qualitative, quantitative, wavelength scan at 656 nm and electrophoretic methods were adopted for the analysis of isolated PGs to evaluate their degree of purification.

2.2.5.1 Qualitative analysis

A) Biuret method

Sodium Potassium tartarate, sodium hydroxide, copper sulphate, bovine serum albumin (BSA), biuret reagent**,** standard protein solution: 2.5 mg/ml standard bovine serum albumin (BSA) solution, biuret reagent: 1.5 g of copper sulphate and 6 g sodium potassium tartarate are dissolved in 500 ml water and 300 ml 10% NaOH and made up to 1 L with distilled water.

Protein in the sample was estimated according to biuret method using bovine serum albumin (BSA) as standard. Pipetted out 0.5 ml standard BSA solution in one test tube as well as sample is taken in another test tube and the volume was made up to 1.0 ml in each tube. To all test tubes 2.5 ml biuret reagent was added and kept for 20 min at room temperature. After incubation development of bluish colour was observed to indicate the presence of proteins.

B) DMMB assay

Dimethyl methylene blue (DMMB), ethanol, guanidine HCl (Gd. HCl), sodium formate, formic acid, sodium acetate, propanol, chondroitin sulphate, complexation solution: 16 mg of dimethyl methylene blue (DMMB) were dissolved in 25 ml ethanol and filtered (pH-3). 100 ml of 1 M Gd. HCl and 1g sodium formate and 1 ml of 98% formic acid added to the DMMB ethanol solution. Finally, the solution was made upto 500 ml; diluted to 1:1 with formate solution instantly and stored in darkness. Decomplexation solution: 4 M Gd. HCl was prepared with solution 50 mM sodium acetate solution buffer (pH 6.8) containing 10% propanol.

Sulfated GAGs were determined according to the 1, 9-dimethyl methylene blue (DMMB) dye-binding assay of Barbosa *et.al* (2003). Three fresh test tubes were taken. To the test tubes sample and chondroitin sulphate standards were added. Third tube acted as negative control with reagent only. 1.0 ml DMMB complexation solution was added to the sample. Chondroitin sulphate standards

were also subjected to DMMB complexation and decomplexation and the colour change was observed.

C) Wave length scan of GAGs

Purity and presence of GAGs was determined by analysing the spectrum chart of the Blyscan Dye reagent (contains DMMB reagent) in the dissociation reagent which has a peak maximum of 656 nm when binds with GAGs.

Presence of sulfated GAGs was determined according to the absorbance range of Blyscan dissociation reagent containing 1, 9-dimethyl methylene blue (DMMB). 1.0 ml Blyscan dissociation reagent was added to chondroitin suphate standards and sample. Chondroitin sulphate standards and sample were subjected to DMMB complexation and decomplexation and the absorbance was measured at 656 nm. Absorption curve of 1, 9-dimethyl-methylene blue dye in Blyscan dissociation reagent has a scan range from 400 to 700 nm. GAGs and 1, 9-dimethyl-methylene blue dye complex show an absorption maxima at 656 nm. Sulfated GAG reference standard curve was compared the GAG sample curve obtained, with the curve formed by DMMB in Blyscan dissociation reagent.

2.2.5.2 Quantitative analysis

The content of protein, carbohydrate, hexuronic acid (uronic acid), hexosamines and sulfated GAG were estimated using the standardized procedures. All OD (optical density) measurements were taken using a Hitachi (Model No. U UV/VIS) spectrophotomer. The details of various methods adopted are described below:-

A) Estimation of protein

Sodium citrate, sodium hydroxide, copper sulphate, bovine serum albumin (BSA), sodium carbonate, Folins Phenol reagent, standard protein solution: 25 mg of bovine serum albumin was made up to 10 ml using distilled water, working standard: 1 ml of standard protein solution is made up to 10 ml, Reagent A: 0.5 g copper sulphate and 1g sodium citrate was dissolved in 100 ml distilled water, Reagent B: 20 g of sodium carbonate, 4 g of NaOH was dissolved in 1 L of

distilled water, Reagent C: 50 ml of Reagent B and 1ml of Reagent A was mixed to obtain reagent C, Reagent D: Folins phenol reagent and distilled water were mixed in 1:1 ratio.

Protein in the sample was estimated according to Lowry method (1951) using bovine serum albumin (BSA) as standard. 0.1 to 0.5 ml of standard BSA solution as well as samples were taken into the test tubes and the volume was made up to 0.5 ml in each tube. To all test tubes 2.5 ml Reagent C was added and kept for 10 minutes incubation at room temperature. To all the test tubes 0.25 ml of Reagent D was added. After incubation absorbance was measured at 640 nm.

B) Estimation of total carbohydrate

Glucose, conc. sulphuric acid, standard solution: 100 mg of glucose is made up to 100 mL using distilled water, working solution: 10 ml of stock is made up to 100 ml using distilled water, anthrone reagent: Dissolve 200 mg anthrone reagent in 100 mL conc. sulphuric acid.

Carbohydrate in the sample was estimated by the anthrone method (Hedge & Hofreiter, 1962) using glucose as standard. 0.1ml to 1.0 ml of standard glucose solution as well as samples were taken into test tubes and the volume was made up to 1.0 ml with distilled water in each test tube. To all the test tubes 4 ml anthrone reagent was added and mixed well. The tubes were incubated for 10 min in boiling water bath and cooled rapidly. After incubation absorbance was measured at 620 nm.

C) Estimation of uronic acid

Glucuronolactone, conc. sulphuric acid, sodium tetra borate, carbazole reagent, ethanol, Borax solution: 0.025% sodium tetra borate was dissolved in 100 ml conc. sulphuric acid, Carbazole reagent: 0.125 g carbazole was dissolved in 200 ml ethanol solution and stored in darkness, stock standard: 10 mg of glucoronolactone was dissolved in 10 ml distilled water, working standard: 1ml of standard solution was made up to 10 ml using distilled water.

The estimation of hexuronic acid in the samples was carried out by the carbazole reaction (Bitter & Muir, 1962)) using glucuronolactone as standard 0.1ml to 1 ml of standard solution was taken and the volume was made up to 1 ml in each tube using distilled water. To the tubes placed in an ice bath 5ml of borax reagent was added. Tubes were cooled at 0^{o}C in glass stopper tubes. After 10 min incubation, the tubes were shaken gently, then vigorously. It was then brought to room temperature and 0.2 ml of carbazole reagent was added to each tube. Contents were mixed well in a vortexer and tubes were then placed in boiling water at 100^{o}C for 15 min and cooled to room temperature. Later absorbance was measured at 530 nm.

D) Estimation of hexosamines

Glucosamine, hydrochloric acid, acetyl acetone, distilled ethanol, Ehrlich's Reagent, ethanol, stock standard solution: 10 mg of glucosamine was made upto10 ml using distilled water, working standard solution: 0.5 ml of the stock standard was made up to 10 ml using distilled water.

Hexosamines were determined after hydrolysis of the samples with 6M HCl at 100^{0}C for 4 h according to the method of Rondle and Morgan (1955). To 0.2 ml of the hydrolysed samples 1ml acetyl acetone reagent was added. Contents were washed down the tubes with 1ml distilled water, capped using glass stoppers and kept in a boiling water bath for 20 min. Contents were cooled to room temperature and 5.0 ml of redistilled ethanol was added. Finally 1.0 ml Ehrlich's reagent was added and the volume made up to 10.0 ml with ethanol. The tubes were warmed in water bath at 65 to 70^{o}C for 10 min. Tubes were cooled and absobance were measured at 530 nm. Glucosamine/Galactosamine standard were also assayed for calculation.

E) Measurement of Total sulfated GAGs by kit test (Blyscan Kit)

The test for glycosaminoglycan assay is a quantitative dye-binding method for the analysis of sulfated glycosaminoglycans.

Dye reagent: contains 1,9-dimethyl-methylene blue in an inorganic buffer which contains surfactants, dissociation reagent: contains the sodium salt of an anionic surfactant. This reagent has been formulated to dissociate the sulfated GAG-dye complex and enhance the spectrophotometric adsorption profile of the free dye, working standard: a sterile solution of bovine tracheal chondroitin-4-sulphate of concentration 100 µg/ml, N- and O- sulfated GAG differentiation reagents, sodium nitrite: 5% (w/v) sodium nitrite, sterile solution acetic acid solution: 33% (v/v) acetic acid, ammonium sulfamate: 12.5% (w/v) sterile solution.

A set of 1.5 ml micro centrifuge tubes were labelled. Glycosaminoglycan standards were added to each tube, from 1.0 to 5.0 µg. A volume between 10 and 100 micro litre of test sample was used. Each tube was made up to 100 µl using reagent blank. 1.0 ml of dye reagent was added to each tube. The contents were mixed by inverting the contents and mechanical shaking for 30 minutes (a sulfated GAG dye precipitate). Contents were spinned in a micro centrifuge at 12000 rpm for 10 minutes. non-sulfated GAGs were drained. 0.5 ml of dissociation reagent was added. Mixture was vortexed for 10 minutes, centrifuged at 12000 rpm for 5 minutes to remove foam. Tubes were capped until measuring absorbance. 200 µl of each sample was transferred to individual wells of a 96 micro well plate. The micro plate reader was set to 656 nm. Absorbance was measured against water for the reagent blank, standards and test samples.

2.2.5.3 Electrophoresis

A) Agarose/polyacrylamide minislab gel electrophoresis of intact cartilage proteoglycans

A modified method (McDevitt and Muir, 1971) was used to electrophoretically separate PGs. This method was used to separate PGs of wide molecular weight distribution. 1g of agarose and 1.5 g polyacrylamide (1:1.5 ratio) were weighed and transfered into a conical flask and was dissolved in 100ml of 1X TAE buffer. The agarose was melted completely into a clear solution in a microwave oven. The molten agarose was cooled to 50^{o}C. The molten

agarose was poured into a preset template with well -forming comb. It was placed on a leveling table. The gel was allowed to set for 30-45 mins. The comb was removed carefully without any damage to the well. The template was mounted into an appropriate electrophoresis tank and the tank was filled with 1X TAE buffer just to immerse the gel. The PGs sample was loaded into the wells without disturbing the wells and proteoglycan and standard aggrecan purchased from Sigma Aldrich was used to track the PGs presence. The electrophoresis apparatus is connected to a power supply and electrophoresis was performed at 50V using TAE as running buffer. After completion of run the bands were visualized by toluidine blue staining.

a) GAGs separation for AGE and PAGE analysis

GAGs were separated and digested to produce fragments of GAGs by following processes namely proteolysis, β-elimination and chondroitinase ABC digestion

b) Proteolysis

Proteoglycans were isolated and purified by DEAE-sephacel and anion exchange chromatography as mentioned previously. In order to separate GAGs from proteoglycans proteolytic digestion with proteinase K enzyme was used. 50 mM Tris-HCl buffer of pH-8.0 was used as buffer and 5 mM $CaCl_2$ was added to activate proteinase K enzyme and 0.01 % Triton-X 100 was also added. 500 mg PGs were taken for digestion and incubated at 37 ^{o}C for 16 h to complete proteolysis.

c) β-elimination

After proteolysis peptide bound GAGs were separated by the process called β-elimination. In this process, O-linked peptides can be removed to release free GAGs. The method involved in this procedure was as follows. Sample was dissolved in 1M sodium borohydribe ($NaBH_4$) in 0.5 M NaOH solution and incubated for 24 h at room temperature in an open tube since H_2 gas is produced during this process. 2 M HCl at room temperature was added till the pH becomes

zero to completely remove H_2. After complete removal of H_2 1 M NaOH was added until the pH was 6-8. After this, separated GAGs were collected by chilled ethanolic precipitation.

d) Chondroitinase ABC digestion

Chondroitinase enzyme was used to breakdowm GAG polymers to shorter fragments such as di, tri, tetrasaccharides etc. 50 mM Tris-HCl buffer of pH-8.0 was used as buffer and 50 mIU chondroitinase was used for enzyme digestion. Incubation was done at 37^0C for 16-18 h. After incubation enzyme activity was arrested by keeping the sample in 80-90^0C heat for 3-5 min. Thus produced GAGs fragments were used for AGE and PAGE analysis.

B) Agarose/polyacrylamide minislab gel electrophoresis of GAGs from cartilage

Isolated GAG precipitates were electrophoresed on an agarose/polyacrylamide (1% agarose and 1.2% acrylamide) gel electrophoresis using 10 mM Tris /acetate buffer, pH 8.3. Chondroitin 6 sulphate, chondroitin 4 sulphate and hyaluronic acid were used as standards. The gels were run on a horizontal electrophoretic system at 120V for 1.5 h. After electrophoresis the macromolecules were stained with 0.02% (w/v) toludine blue for about 20 minutes. Gels were destained by using 3% acetic acid and the results were documented.

C) Polyacrylamide gel electrophoresis of chondroitinase ABC digested GAGs from cartlage

Polyacrylamide gel electrophoretic (PAGE) patterns of the different GAGs chains were analysed according to the method of Laemmli (1970) and modified GAGs separation procedure (Riley *et al*., 1994). GAGs samples were prepared by chondroitinase ABC digestion and samples were mixed (1:1 v/v) with the sample buffer (0.5 M Tris-HCl, pH 6.8 containing 40 g/l SDS, 200 ml/l glycerol in the presence or absence of 100 ml/l b mercaptoethanol). The sample were loaded onto a polyacrylamide gel having a 14% (10% SDS: 100 µl, acrylamide: 4.62 ml,

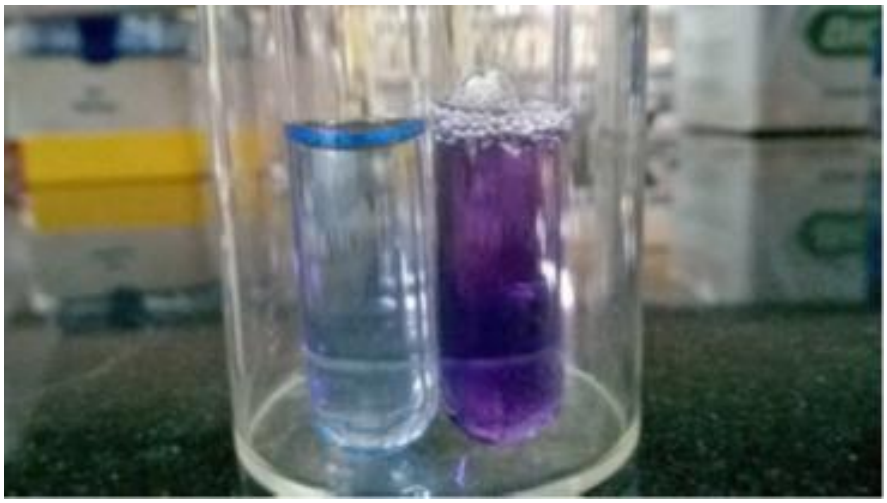
distilled water: 2.62 ml, Tris-HCl, 1.5 M: 2.5 ml, Ammonium per sulphate 50 µl, TEMED: 10 µl) and 16 % resolving gel (10% SDS: 100 µl, acrylamide: 5.28 ml, distilled water: 2.07 ml, Tris-HCl, 1.5 M: 2.5 ml, Tris-HCl, 1.5 M: 2.5 ml, Ammonium per sulphate 50 µl, TEMED: 10 µl) respectively. The electrophoresis was done at a constant current of 20 mA per gel using a Bio-Rad Tetra Mini Protean II unit (Bio-Rad Laboratories, Inc., USA). After electrophoresis, gels were separated, followed by staining with 0.02% Toludene blue solution in 0.1 M acetic acid for 45 min. Finally, they were destained with 3 % 100 ml/l acetic acid for 2-4 h and destained again with the same solution for 1 h.

2.3 Results and discussion

The yield of PGs After extraction and isolation was around 3.75 g which is account for 25 % recovery and was found significant since cartilage consists of 15 % proteoglycans on dry weight basis (Sophia *et al*., 2009).

2.3.1 Qualitative analysis

2.3.1.1 Biuret method

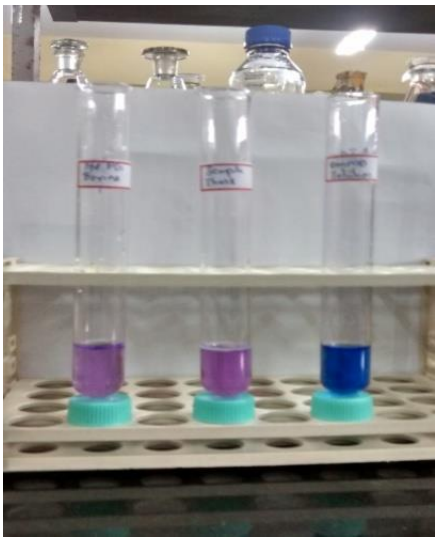


(a)Sample (b) Control

Fig 2.5: Qualitative biuret test for purified proteoglycans (A) Violet-purple colour produced shows the presence of proteoglycans in the sample (B) Control shows the absence of proteoglycans

Qualitative Biuret result is depicted in figure 2.5. Proteoglycans are proteins that are covalently bonded at multiple sites along the protein chain to a class of polysaccharides, known as glycosaminoglycans. So in biuret test a violet purple colour was observed which indicates the presence of protein. Hence protein response in sample could be due to the presence of protein part of isolated PG sample.

2.3.1.2 DMMB assay



a b c

Fig 2.6: Qualitative DMMB test for purified proteoglycans (A) GAG standard and DMMB mixture (B) Sample and DMMB mixture (C) DMMB reagent only

The 1, 9 –DMMB dye binding assay proposed by Barbosa *et al.*, 2003, is a versatile method to detect the relative amount of sulfated GAGs within a preparation. Negatively charged GAG react with DMMB to give purple pink complex which indicates the presence of GAG. Figure 2.6 depicted the qualitative GAG detection by DMMB method. Purple pink colour was observed in both purified PGs and standard GAG (Chondroitin-4-sulfate) added tubes and blue colour specific to DMMB reagent was observed in blank tube with no standard and PGs. This results indicated the presence of GAG in isolated PGs sample.

The dimethylmethylene blue (DMMB) dye binding assay is widely used to quantify sulfated glycosaminoglycan (sGAG) contents of tissues and tissue-

engineered constructs, particularly in cartilage tissue engineering (Zheng and Levenston, 2015). The dye 1, 9-dimethylmethylene blue (DMMB) is a thiazine chromotrope agent that presents a change in the absorption spectrum due to the induction of metachromasia when bound to sulfated GAGs (Whitley *et al*., 1989; Chandrasekhar *et al*., 1987; Farndale *et al*., 1986). The DMMB assay can be very efficient when performing chromatography to rapidly assay for fractions containing GAGs (Burton-Wurster *et al*., 2003). Proteoglycans are proteins that are covalently bonded at multiple sites along the protein chain with glycosaminoglycans. So this is a quantitative analysis to confirm that our sample contains sulfated GAGs. The colour change in the standards and in GAG sample shows that our sample is of chondroitin type, it is pure that and it reveals the presence of sulfated GAGs. Biuret reagent readily reacts with peptide nitrogen of proteins to form blue coloured complex and this method is effective in identification and quantification of proteins. Protein part of proteoglycan can react with biuret reagent and gives blue coloured complex. In qualitative analysis we observed blue coloured complex in the PGs sample tube similar to BSA standard taken tube. This results confirm the presence of protein nature in the sample. Both GAG and protein nature are confirmed in purified PGs sample and hence confirming purity of proteoglycans isolated from deep sea shark cartilage.

2.3.2 Wavelength scan of GAGs

The spectrum chart of the dye reagent in the dissociation reagent has a peak maximum of 656 nm. To confirm the purity of PGs isolated from deep sea shark cartilage absorption peak maximum at 656 nm was analysed by comparing with the sulfated GAG reference standard curve obtained using micro plate and GAG sample curve (Fig. 2.7). Absoptiom maxima at 656 nm was observed for PGs sample purified from deep sea shark cartilage and similar response was observed for standard GAG (chondroitin-4-sulfate).

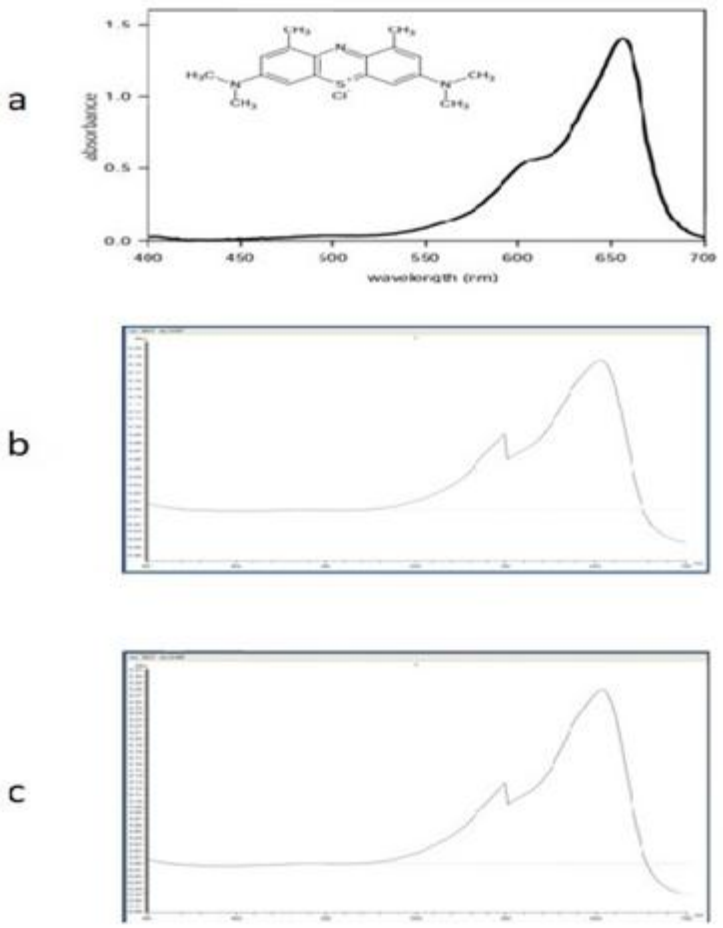


Fig 2.7 : Wavelength scan of glycosaminoglycans of purified PGs at 656 nm (A) The structural formula of 1,9-dimethyl-methylene blue and the absorption spectra (max 656 nm) of the dye in Blyscan dissociation reagent (B) spectra of sulfated GAG reference standard curve obtained (C) spectra of GAG sample curve obtained

By pre-incubating samples with specific enzymes, different types of sGAG can be measured with wave length scanning (Goldberg and Kolibas, 1989). As a rapid technique for measuring tissue sGAG contents, the DMMB assay has become a standard tool for analyzing in vitro and in vivo studies (Zheng and Levenston, 2015). For unknown samples or the study of analytical conditions, the wavelength scan of samples is performed to determine the wavelength range. As a rapid, straight forward technique for measuring tissue sGAG contents, the DMMB assay has become a standard tool for analyzing in vitro and in vivo tissue

engineering studies (Zheng & Levenston., 2015). DMMB reacts with the sulfate group of the GAG chain and therefore does not detect unsulfated GAGs such as hyaluronic acid. Absorption maxima at 656 nm indicates the presence of sulfated GAG (Barbosa *et al*., 2003). Significant absorption maxima at 656 nm was observed for our sample which was very much similar to chondroitin sulphate standard. So we concluded that our sample is pure and it contains sGAG as it showed a max absorption range of 656 nm. UV-visible techniques are fairly rapid and simple to carry out, and are sensitive to low concentrations of proteoglycans. UV-VIS spectroscopy can be used to quantify levels of this element for both safety and effectiveness, and also provide an easy, non-destructive, chemical-free, and effective method of detection.

2.3.3 Quantitative analysis

2.3.3.1 Chemical composition analysis

Chemical composition of proteoglycans is depicted in figure 2.8. Proteoglycans purified by chromatography method were analyzed for carbohydrate, protein, uronic acid, hexosamine and sulfated GAG content through UV/ visible spectroscopy.

2.3.3.1.1 Estimation of protein

Clear quantity of protein was observed in estimation. Linear relation was revealed between BSA concentrations and absorbance values at 640 nm. The standard curve was used to determine amount of proteins in extracted proteoglycan sample from cartilages. Total amount of protein present in 1 mg of proteoglycan extract was about 0.530 mg.

2.3.3.1.2 Estimation of carbohydrate

Good quantity of carbohydrate was present in cartilage sample. Calibration curve was plotted between known concentrations of standard glucose and respective absorbance values at 620 nm which showed a linear relationship. Total amount of carbohydrate present in 1 mg of proteoglycan extract was about 0.468 mg.

2.3.3.1.3 Estimation of uronic acid

Uronic acid is a component of proteoglycans. In proteoglycans extract, uronic acid is a major component in the form of hyaluronic acid. Hyaluronic acid is unique among the GAGs because it does not contain any sulfate and is not found covalently attached to proteins. Carbazole reaction revealed good quantity of uronic acid in cartilage sample. Calibration curve plotted between known concentrations glucuronolactone as standard and respective absorbance values at 530 nm showed linear relationship. Total amount of uronic acid present in 1 mg of proteoglycan extract was about 0.098 mg.

2.3.3.1.4 Estimation of hexosamines

Clear response for hexosamine was observed in cartilages sample. Linear relation was revealed between glucosamine concentrations. The standard curve was used to determine amount of hexosamine in extracted proteoglycan sample. Total amount of hexosamine present in 1 mg of shark cartilage proteoglycan extract was about 0.20 mg.

2.3.3.1.5 Estimation of sulfated glycosaminoglycans

Microplate method estimation of sulfated GAG is described in figure 2.9. Measurement of total sulfated GAGs by kit test (Blyscan Kit) was performed. Purified proteoglycans sample contained a good quantity of sulfated GAG. Total amount of sulfated GAG present in 1mg of proteoglycan extract was about 0.06 mg.

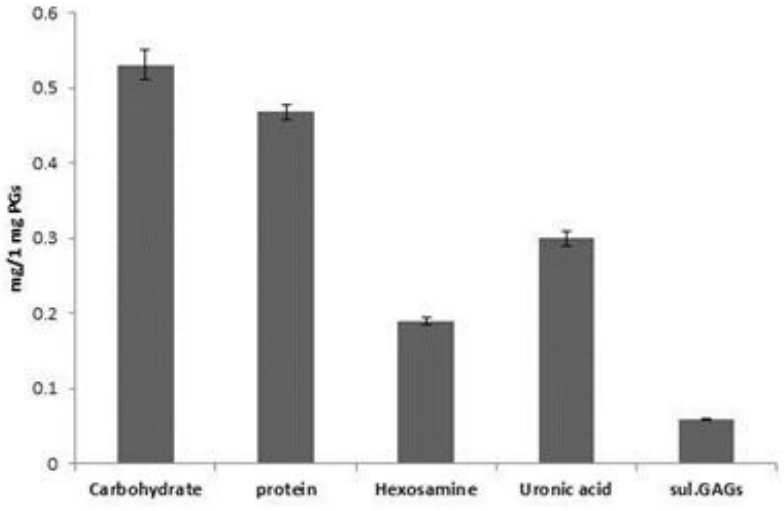


Fig. 2.8 Chemical composition of proteoglycans

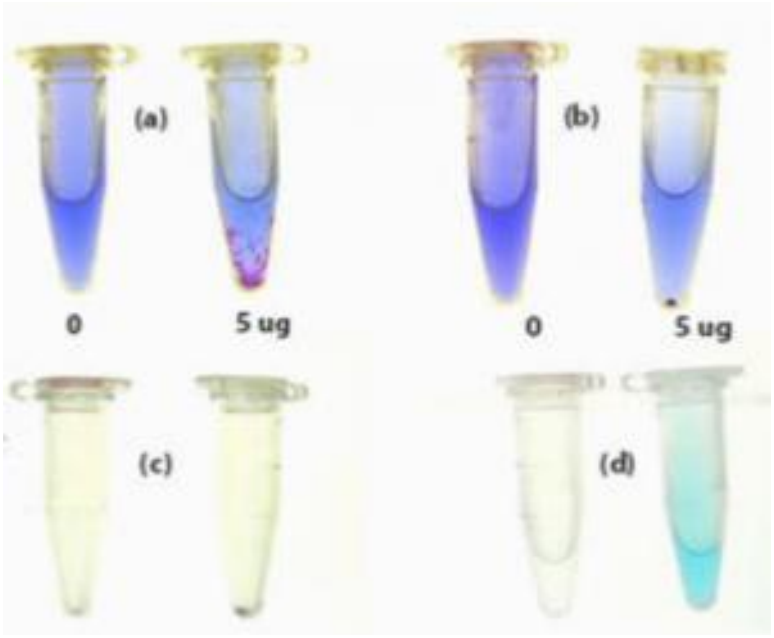


Fig 2.9 Microplate kit test for the measurement of total sulfated GAGs: (a) 0 and 5 microgram of sulfated glycosaminoglycans and Blyscan dye (b) sGAG pellets formed after mixing and centrifugation (c) The non-sGAG Dye was drained from tubes with pellet retained (d) Dye released from sGAG using dye dissociation reagent

Significant quantity of protein was noticed in the sample and indicates the presence of proteoglycan. Since qualitative confirmation of GAG and protein could be due to the proteoglycan nature of the sample it is proven quantitavely too. Anthrone method is a rapid and convenient method for the determination of carbohydrates either in free form or when present in polysaccharides. A significant carbohydrate content was observed in isolated PG sample which could be due to the presence of GAG in the sample. Majority of proteoglycans were extracted from shark fin cartilage consist a good quantity of uronic acid (84% of uronic acid) (Michelacci and Horton, 1989). Studies proved that the protein/uronic acid ratio is high in cartilage proteoglycans (Kosiagin, 1986). Hexosamine is a prominent derivative sugar moiety seen in carbohydrate portion of proteoglycans. Significant hexosamine and uronic acid content was detected in the isolated PG sample. Earlier Manjusha *et al.*, 2011 reported hexosamine content in GAG isolated from squid and in this study a slightly higher hexosamine content was

observed. In the process of characterization of PGs isolated from *Echinorhinus brucus*, it was observed that carbohydrate content was more than the protein content which is typical of chemical composition of proteoglycans. Thus determined chemical composition substantiates the presence and purity of proteoglycans isolated from deep sea shark cartilage (*E. brucus*) (Higashi *et al*., 2015).

In this study, chemical composition analysis of the proteoglycans provided data about the relative amounts of GAG present in the cartilage. GAG content in extracted samples from cartilages were determined by DMMB assay. Marine organisms are a rich source of a variety of structurally novel and biologically active metabolites (Senni *et al*., 2011). And they possess pronounced pharmacological activities and other properties that are applied in biomedical fields. Some of these pharmacological activities are attributed to the presence of sulfated polysaccharides, particularly GAGs (Valcarcel *et al*., 2017; Cesaretti *et al*., 2004; Pandian and Thirugnanasanbandan, 2008; Saravanan and Shanmugam, 2010; Köwitsch *et al*., 2017). Sulfated GAGs are major components in extracellular matrix of various mammalian organs such as cartilages, blood vessels, cell surfaces, intracellular granules and plasma in the form of proteoglycans or other forms (Esko *et al*., 2009). Clinically, sGAGs are used as chondroprotective drugs in humans. Sulfated polysaccharides such as chondroitin sulfate (FuCS) and sulfated fucan, have shown anticoagulant and antithrombotic activities (Mourão *et al*., 2001). Heparin and related GAGs are used primarily as anticoagulant and antithrombotic agents in treatment. Chondroitin sulphate plays critical roles in various physiological events such as the regulation of signalling molecules, cell–cell and cell–matrix interactions, neuron growth, and cell division and differentiation. GAGs are apparently required for the regulation of signalling molecules responsible for morphogenesis and organogenesis (Gu *et al*., 2009). In the brain, CS chains play important roles in the regulation of axonal guidance and path finding of various neurons (Purushothaman *et al*., 2007) KS containing molecules have been identified in numerous epithelial and neural tissues in which KS expression responds to embryonic development, physiological variations, and to wound healing. Dermatan sulphate was first isolated from pig skin

by Meyer and Chaffee in 1941. Dermatan sulphate helps in prevention of deep vein thrombosis (DVT) in patients with hip fracture (Agnelli *et al*., 1992). These reports highlight the vital role of GAGs as health promoting agents. Sulfated GAG content in the mantle, tentacle, fin and skin of cuttle fish is 6.11, 1.91, 8.49 and 1.47 mg/g respectivety (Manjusha *et al*., 2011) whereas, the sulfated GAG content in the cartilage tissues of *Echinorhinus brucus* observed to be 60 mg/g. It shows that *Echinorhinus brucus* cartilage is a better source of sulfated GAG and it can be better exploited for pharmaceutical purposes. In this study, significant amount of sulfated GAG (chondrotin-6-sulfate, dermatan sulfate and heparan sulfate) and non-sulfated GAG (hyaluronic acid) was observed. Overall chemical composition of our study has good accordance with the work done by Higashi *et al*., 2015. He worked on glycosaminoglycan composition of several deep sea elasmobrachs. Since GAG has great therapeutic, nutraceutical and cosmetic value, shark cartilage can be a better alternative source (Saravanan and Shanmugam, 2010).

2.3.4 Electrophoresis

2.3.4.1 Agarose gel electrophoresis of proteoglycans

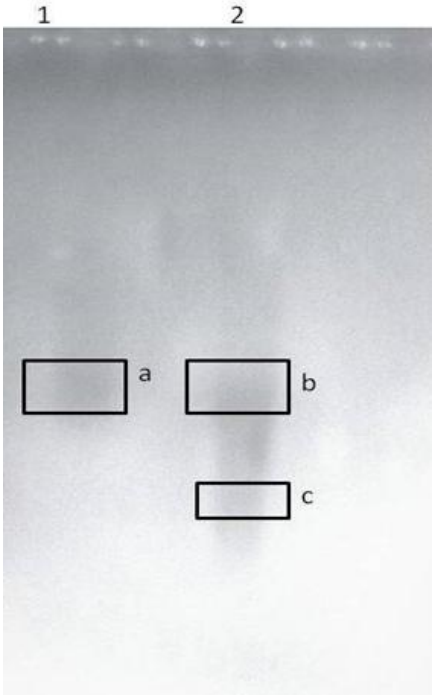


Fig 2.10 Agarose gel electrophoreis pattern of proteoglycans. Lane 1 (a) denotes the larger molecular weight cartilage proteoglycan aggrecan and similar pattern observed in lane 2 of (b) PGs sample. Band formed at bottom (c) of lane 2 shall be indicating smaller molecular weight PGs such as decorin, fibromodulin, byglycans, lumican etc.

In agarose gel electrophoresis proteoglycans stretched bands are commonly seen due to negatively charged GAG polymer and macromolecular structure (Fig. 2.10) (McDevitt and Muir, 1971). According to the results it was clear that both high molecular and low molecular weight PGs are present in the isolated sample. Bands seen with respect to aggrecan standard band could be the high molecular weight PG such as aggrecan isolated from deep sea shark cartilage. Whereas band formed well below the aggrecan band could be low molecular weight PGs namely, decorin, biglycan etc.

2.3.4.2 Agarose/polyacrylamide minislab gel electrophoresis of glycosaminoglycans

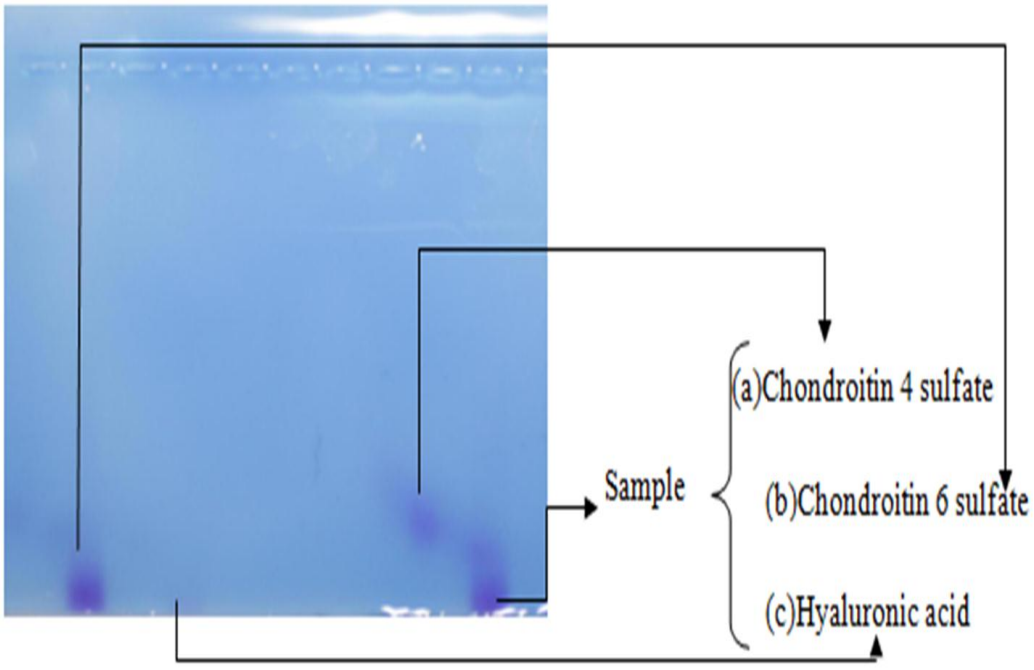


Fig 2.11: Agarose/polyacrylamide minislab gel electrophoresis lane (a) Chondroitin 4 sulphate lane (b) Chondroitin 6 sulphate and lane (c) Hyaluronic acid

Agarose/polyacrylamide mini-slab gel electrophoresis of glycosaminoglycans in purified PGs from deep sea shark cartilage is depicted in fig. 2. 11. Hyaluronic acid is not clearly seen in the gel because of its high molecular weight. Techniques

employing horizontal agarose gel electrophoresis for the characterization of acidic polysaccharides were described by many authors (Bjornsson, 1993; Pavao *et al*., 1998; Theocharis *et al*., 1999; Pandian & Thirugnanasambandan, 2008). Figure showed the toludine blue stained agarose gel pattern of the GAGs extracted from the tissues of *Echinorhinus brucus*. The relative molecular weight (MW) of the standard chondroitin 4 sulphate and chondroitin 6 sulphate were 40,000 Da and 60,000 Da respectively. According to the electrophoretic mobility of GAGs, this experiment reveals the presence of GAGs having a molecular weight similar to standard GAGs and thus confirmed the purity of our sample**.**

2.3.4.3 Polyacrylamide electrophoresis of chondroitinase ABC digested GAGs

PAGE analysis of chondritinase ABC digested GAGs fragments was done to identify the presence and purity of GAGs present in the sample. Fig. 2.12 is illustrated 14% PAGE (polyacrylamide gel electrophoresis) pattern of glycosaminoglycans fragments after digestion with chondroitinase ABC and fig. 2.13 illustrated 16% PAGE (polyacrylamide gel electrophoresis) pattern of GAGs (glycosaminoglycans) fragments after digestion with chondroitinase ABC. In both the 14% and 16% PAGE analysis PGs purified from deep sea shark cartilage showed similar band pattern of standard GAG mixture consisted chondroitin-4-sulafte, chondroitin-6-sulfate and hyaluronic acid.

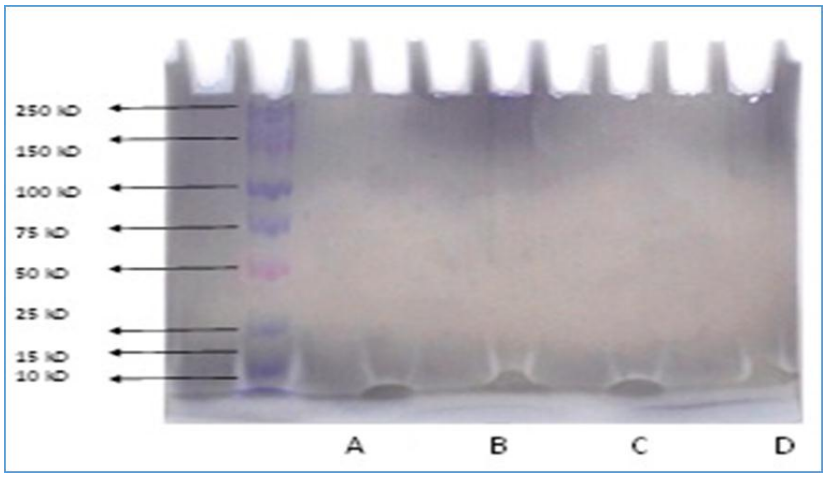


Fig 2.12 14% PAGE (polyacrylamide gel electrophoresis) pattern of glycosaminoglycans fragments after digestion with chondroitinase ABC. A & C are enzyme digested (chondroitinase ABC)

glycosaminoglycan (GAG) standard (i.e., chondroitin-4 sulfate, chondroitin-- sulfate and hyaluronate) and B & D are isolated GAG from PGs.

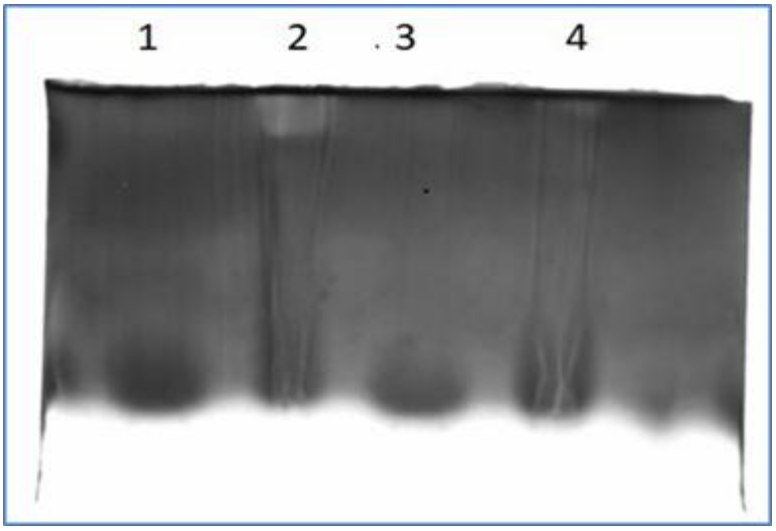


Fig 2.13 16% PAGE (polyacrylamide gel electrophoresis) pattern of GAGs (glycosaminoglycans) fragments after digestion with chondroitinase ABC. 1 & 3 are enzyme digested (chondroitinase ABC) glycosaminoglycan (GAG) standard (i.e., chondroitin-4 sulfate, chondroitin-- sulfate and hyaluronate) and 2 & 4 are isolated GAG from PGs.

Proteoglycans vary in their molecular weight and are better separated by agarose gel electrophoresis. Generally proteoglycans form stretched bands in gel due to its large size and highly negative GAG chains. Riley *et al*., (1994) described the electrophoretic pattern of PGs isolated from tendon and similar results were obtained in this work which substantiates the use of gel electrophoresis to identify and characterise proteoglycans of any source. McDevitt and Muir, 1971) described the modified agarose/polyacrylamide mini slab gel electrophoresis of intact cartilage proteoglycans. This method is very useful to separate and identify high molecular weight proteoglycans. We observed definite proteoglycan pattern and similar band pattern was observed for aggrecan standard, a high molecular weight cartilage proteoglycans. Our observation agrees with the above mentioned obsevations and presence of proteoglycans was confirmed by modified agarose gel electrophoresis method. High molecular weight, highly

linear structure and negative charge make GAG separation by electrophoresis tedious. Agarose gel electrophoresis makes separation and identification of GAGs easy than other electrophoretic methods since AGE is highly sustainable for separately highly negative and high molecular weight biomolecules like DNA. Toluidine blue staining after agarose gel electrophoresis of isolated GAGs revealed the presence of chondroitin sulfate A & B, hyaluronic acid as compared to respective standards. Previous studies confirm the role of AGE to separate high molecular weight native GAGs from any source (Coulson-Thomas *et al*., 2015). They reported the characterization of GAGs from human teeth by agarose gel electrophoresis and polyacrylamide gel electrophoresis.

Polyacrylamide gel electrophoresis is routinely used to identify and characterize GAGs chains. Highly linear and high molecular weight structure of GAGs makes separation by PAGE quite difficult. But enzymatic breakdown of GAGs by chondroitinase ABC digestion, isolation of GAG fragments by anion exchange chromatography followed by PAGE certainly helps to separate and characterize GAGs (Cowman *et al*., 1984). Chondroitinase ABC enzymes specifically cleave GAG chains to form fragments as small as di-saccharide GAGs to oligomer GAGs. We observed GAG bands at bottom of both 14 and 16% PAGE gels after enzymatic digestion. We assume that chondroitinase ABC digestion produced GAG fragment of low molecular weight, less than 10 kD and this is the reason for formation of bands below 10 kD molecular weight. Goulas *et al*, 2000 reported the role of PAGE in separation of glycosaminoglycans chain and our results are in good accordance with the study conducted.

Therefore it is concluded that the isolated sample contained pure proteoglycans which was confirmed by agarose gel and polyacrylamide gel electrophoresis.

2.4 Conclusion

Proteoglycans and glycosaminoglycans are known for their diverse bioactive potential and have gained much attention in the biomedical field. Studying the typical functionality of proteoglycans is crucial in order to identify

the unique nature of the compound. Isolation and characterization of proteoglycans is required for determination of their chemical identity and hence purity. Typical chemical composition analysis and electrophoretic separation methods following anion-exchange chromatography are considered as fundamental ways to characterize proteoglycans. In this unit, we employed spectrophotometric methods to determine protein, carbohydrate, amino sugar, sulfated and non-sulfated GAG content present in purified proteoglycans sample. Electrophoretic methods were used to separate and identify the presence of different proteoglycans and glycosaminoglycans according to their molecular weight. We observed typical chemical composition content which is specific to proteoglycans and specific electrophoretic band pattern confirmed the nature of proteoglycans and glycosaminoglycans present. Above findings substantiate the reliability of the methods used to characterize proteoglycans and reveals the purity of isolated proteoglycans. So the methods can be employed for purification and characterization of proteoglycans from shark cartilage.

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Chapter 3

NMR, FTIR and LC-MS/MS based structural characterization of proteoglycans purified from deep sea shark *Echinorhinus brucus* cartilage

3.1 Introduction

PGs are complex macromolecules made up of a protein core to which glycosaminoglycan (GAG) heteropolysaccharide chains are covalently attached. Aggrecan is the prominent PG present in the cartilage and its main function is to impart compressive strength to the articular cartilage. The major kinds of PGs in cartilage are large aggregating PG or aggrecans and small molecular weight PGs such asbiglycan, decorin and fibromodulin (Cs-Szabo *et al*, 1995). They are produced by chondrocytes and secreted into extracellular matrix where they are involved in wide varieties of biological functions (Glant *et al*., 1980).

GAGs are negatively charged heteropolymers with sulphation or no sulphation that are involved in wide variety of biological functions, such as cell multiplication, signal transmission, cell to cell interaction and haemostasis (Tumova *et al*., 2000; Volpi, 2006). Studies have reported the importance of glycosaminoglycans with respect to cartilage regeneration in arthritic condition, neural regeneration etc. Anti-inflammatory effects of GAGs were reported in a chronic arthritic rat model induced by complete Freund's adjuvant (CFA)-treatment. GAG administration significantly reduced symptoms of arthritis by

inhibiting C-reactive protein (CRP), inflammatory cytokines such as IL-1beta, tumor necrosis factor (TNF)-α, interleukin-6 etc. in a dose dependent manner (Ahn *et al*, 2014).

Characterization of proteoglycans comprises analysis of glycosaminoglycan and protein. Mass spectrometry (MS) is a sophisticated tool that identifies and quantifies molecules based on their mass-to-charge ratio (m/Q, m/q, m/Z, or m/z) and known for high specificity, sensitivity and accuracy (McLafferty, 1981; Thomson, 1987).GAGs and GAG oligosaccharides are effectively characterized by tandem mass spectroscopy using electron spray ionisation (Crawley *et al*., 2004).

Proteomics technology has enabled identification of targeted proteins in a given sample. This technology identifies typical proteins and is employed to develop profiles of known or unknown proteins in samples using protein database search (Hanash, 2003). Mass spectrometry can be better employed to generate and analyze complete proteomes. Matrix assisted laser desorption/ionization mass spectrometry (MALDI-MSI) of biological samples is a better tool in comprehensive characterization of numerous unknown molecular species such as peptides and proteins (Seeley and Caprioli, 2008) and LC-MS/MS protocols with electron ionization spray (ESI) ionization have also been useful for proteomics study.

Biochemically shark cartilage is composed of a mixture of proteoglycans with glycosaminoglycans, an important source of hyaluronic acid, chondroitin sulfate, dermatan sulfate and keratin sulfate are known to enhance human health. *Echinorhinus brucus* is a non-edible by catch shark obtained during deep sea fishing and with good quantity of cartilaginous body. They are often used for oil purpose and its body thrown as a waste. As mentioned proteoglycans are very much gaining interest in pharamaceutical and nutraceutical sector due to its great applications in cancer (Ajeeshkumar *et al*., 2017) and arthritic treatments. Even though marine sector is a huge source of proteoglycans and glycosaminoglycans it is hardly exploited till date. In this context, the current study is to isolate and characterize proteoglycans isolated from deep sea shark *Echinorhinus brucus* cartilage.

3.2 Materials and Methods

3.2.1 Sample preparation

Deep sea shark *E. brucus* (bramble shark) was brought to the laboratory in chilled condition. PGs were extracted from the separated cartilage by chaeotropic treatment with 4 M guanidine hydrochloride, 0.05 M EDTA, 0.1 M sodium acetate buffer and protein inhibitor iodoacetamide and 0.05 M benzamidine hydrochloride, pH- 5.8 at 4^{o}C for 12 h. After extraction, isolation was carried out by DEAE- sephacel anion exchange chromatography using gradient elution with 7M urea and 0.1, 0.2 and 0.4 M NaCl. The eluent was dialyzed with 12,000 MWCO (molecular weight cut off) against water for 42 h with continuous stirring at 4^{o}C to remove urea, NaCl and remaining traces of contaminants. After dialysis, proteoglycans in the eluent were precipitated with ice-cold ethanol and centrifuged. The pellet obtained presumably contained pure proteoglycans and was used for characterization and anti-cancer studies.

3.2.2 Structrural analysis

3.2.2.1 FTIR Analysis

The Functional groups of PGs were recorded by Fourier Transform Infra Red (FTIR) spectroscopy using Shimadzu FT-IR spectrophotometer (IR Prestige-21, USA). The sample pellets were prepared by mixing the fine freeze dried PGs (1 mg) with 100 mg KBr and it was scanned from 500 to 4000 cm^{-1}.

3.2.2.2 One dimensional ^{1}H nuclear magnetic resonance (NMR) spectroscopy

One-dimensional ^{1}H (1D)-NMR spectra of the PGs solution was recorded at room temperature using a Bruker Avance II-500 spectrometer. 25 mg of purified PGs sample was dissolved in 1ml of 99.96% D_2O and analyzed. For ^{1}H NMR spectra the sample was submitted to a delay (D1) and acquisition time (AQ) of 1.00 s and 3.17 s. Chemicals shifts were expressed in parts per million (ppm).

3.2.2.3 LC-MS/MS analysis of Enzyme digested GAG

Chondroitinase ABC digested GAGs were separated by anion exchange chromatography using DEAE-sephacel and eluted with 4M urea with 0.2-0.4M NaCl gradient. GAG is precipitated with chilled ethanol and used for characterization. GAG characterization was done with positive and negative ion (500-1500 m/z) manual tuning and full scanning of sample and standard with aid of MS/MS QTRAP 4000 model.

3.2.2.4 Proteomics analysis of PGs by LC-MS/MS

Table 3.1 and 3.2 depict LC solvent programme and MS/MS programme respectively. About 25 µg of protein was trypsin-digested for LC-MS/MS analysis. Samples were dissolved in 0.1% formic acid in water so that the final concentration was 0.5 µg /ul. Instrument used was Thermo Easy nLC 1200, the analytical column used was PepMap RSLC C18 (Easy nano Column) with 2µ particle size, $100A^0$ pore size and 75 µ ID × 25 cm length (P/N no. E802). Pre-column used was Acclaim PepMap 100 nano viper, with 3µ particle size, $100A^0$ pore size and 75µ ID × 2cm Length (P/N no. 164946). Data acquisition was carried out as follows. Samples were injected to Q Exactive Plus (Thermo Scientific) Bench top Orbitrap instrument coupled to Thermo Easy nLC 1200 (Thermo Scientific). For data analysis, the acquired data was analysed in Proteome Discoverer 2.1SP1 (Thermo Scientific) on Sequest HT search engine and NCBI full protein database.

3.2.2.4.1 LC Method

Thermo Easy nLC 1200 machine was employed for peptide separation in liquid chromatography part. Analytical column used was PepMap RSLC C18 (Easy nano Column); 2 µ particle size; $100A^o$ pore size; 75 µ ID × 25 cm length (P/N no. E802) and pre-colum was Acclaim PepMap 100 nano viper; 3µ particle size, $100A^o$ pore size, 75 µ ID × 2 cm Length (P/N no. 164946). Gradient programme was used ie., Solvent A: 0.1% Formic Acid (Sigma, Mass Spec grade) in water (JT Baker, LCMS grade), Solvent B: 0.1% Formic Acid (Sigma, Mass

Spec grade) in Acetonitrile (JT Baker LCMS grade). Injection volume was 10 µl and pre column equillibration was done at a flow rate of 4 µl/min with maximum pressure of 1000 bar. Analytical Column Equilibration comprised injection volume of 5 µl at a flow rate of 300 nL/min and maximum pressure was adjusted to 1000 bar.

Table 3.1: LC solvent programme of proteomic analysis

Time (min)	Flow Rate (nL/min)	%A	%B
0.0	250	95	5
50.0	250	80	20
53.0	250	60	40
55.0	250	20	80
56.0	250	20	80
57.0	250	95	5
60.0	250	95	5

3.2.2.4.2 Mass spectroscopy

MS was done with Q Exactive Plus instrument and experiment type was full MS/ddMS2 (TopN) [Full MS followed by data dependent MS/MS for Top N number of ions]

Table 3.2: Tandem mass spectroscopy MS/MS programme for protein analysis

Conditions for MS	FullMS Settings:	ddMS2 Setting:	dd Settings:
• Ionisation Settings (Easy Nano Spray): • Polarity: Positive • Spray Voltage: 2300eV • Sheath Gas: 0.0 • Auxiliary Gas: 0.0 • Capillary Temperature: 300^{o}C	• Resolution: 70000 • AGC Target: 3 × e^{6} • Maximum IT: 50msec • Scan Range: 350 – 2000 m/z • Microscans: 1 • Spectrum Data type: Profile	• Resolution: 17500 • AGC Target: 1 × e^{5} • Maximum IT: 100msec • First Mass: 100 m/z • Microscans: 1 • TopN: 15 • Isolation Window: 2.0 m/z • NCE: 27 [Normalised Collision Energy] • Spectrum Data Type: Centroid	• Intensity Threshold: 1 × e^{4} • Charge Exclusion: unassigned, +1, >+8. • Exclude Isotopes: ON • Dynamic Exclusion: 30 secs

3.3 Results and Discussion

3.3.1 FTIR spectra of proteoglycans

Table 3.3: FTIR spectra of proteoglycans extracted from deep sea shark (*Echinorhinus brucus*) cartilage

Wave number (cm^{-1})	Appearance	Bond	Possible assessment of functional group
3201.98	Broad	N-H stretch	Amide
1632.69	Strong	C=C stretch	Alkene
1333.34	Weak	C-O stretch	Carboxylic acids
1254.23	Weak	C-O-C stretch	Ether
1161.27	Medium	C-O stretch	Alcohol
1078.38	Strong to broad	S-O stretch	Sulfonate

Table 3.3 and figure 3.1 depict the FTIR spectra of proteoglycans isolated from deep sea shark cartilage. Proteoglycans molecules are characterized by the presence of N-H bonds amide stretching (≈3201.98 cm^{-1}) due to its proteineous nature. At the same time C-O and C-OH, as well C-C ring vibrations are specific for carbohydrate region (≈1333.34 cm^{-1}, 1161.27 cm^{-1} and 1161.27 cm^{-1} respectively) (Camacho et al., 2001, Kohler *et al*., 2007). Interestingly strong S-O streaching was observed 1078.38 cm^{-1} which confirm the presence of sulphated polysaccharide. It could be possibly glycosaminoglycan chains which is integral component of proteoglycans. Above results confirm the both glycosaminoglycans and protein nature of the molecule hence, proteoglycans.

FTIR is a vital analytical tool to identify the functional groups thus elucidate the nature of the purified molecules. This is widely used to characterize biological molecules to confirm its purity (Harrigan *et al*, 2004). In FTIR microspectroscopy, absorption of infrared light is detected within a microscopic tissue section and infrared absorption spectrum is recorded. Different molecules possess different absorption pattern and biological molecules have typical absorption characteristics that can be expolited to track and quantify the molecule of interest, e.g. glycosaminoglycans, proteoglycan and collagen in the case of cartilage. Thus, by employing FTIR spectroscopy it is feasible to identify the cartilage macromolecules such as proteoglycans (Saarakkala *et al*., 2010).

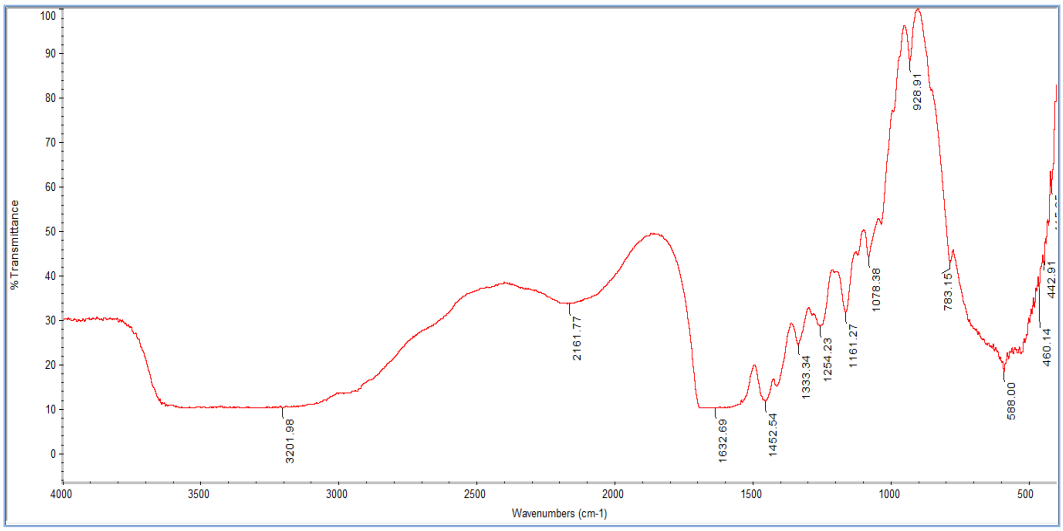

Fig. 3.1: FTIR spectra of purified proteoglycans extracted from deep sea shark (*Echinorhinus brucus*) cartilage

3.3.2 H1-NMR spectrum of purified Proteoglycans from deep sea shark cartilage

Table 3.4 and figure 3.2 depict H1-NMR spectrum of purified proteoglycans from deep sea shark cartilage. The δ range was limited to 7.5 to -.02 ppm which are ideal to recognize peaks for both protein and carbohydrate molecules. Peaks at 1.128-1.144 ppm, 1.254-1.427 ppm, 1.557-1.602 ppm, 2.151-2.272 ppm, 4.117-4.730 ppm, 5.581ppm and 7.239-7.265 ppm are specific for alcohol group, tertiary alcoholic protons, carboxylic acid protons, secondary amine protons, sulfates protons and aromatic protons respectively. All these functional groups are integral part of both carbohydrate and protein molecules, indicates the presence of proteoglycans in the purified sample (Vliegenthart, 1983).

NMR has been proved as vital to study polysaccharides either sulphated or non-sulphated polysaccharides based on their proton shift (Guerrini *et al*. 2001; Rudd *et al*. 2008) to obtain clear structural information and to determine their origin. NMR spectroscopy is the most acceptable method to elucidate the structure of a particular molecule based on proton chemical shifts. Through the help of NMR, localization and identication of chemical groups such as methyl, acetyl, sulfate, carboxylic, alcohotic, amines etc, is possible. H1-NMR result of the

sample depicts the chemical nature by assessing chemical shifting of proton molecules that corresponds to particular chemical structure which again confirm the PG presence.

Table 3.4: H1-NMR spectra of proteoglycans extracted from deep sea shark (*Echinorhinus brucus*) cartilage

Chemical shift range (ppm)	Possible type of group of protons	Possible structure
0.827 – 0.959	Tertiary Aliphatic Protons	$R_3C\mathbf{H}$
1.075	Secondary Aliphatic Protons	$R_2C\mathbf{H}_2$
1.128 – 1.144	Alcohol	$-CH_2O\mathbf{H}$
1.254 – 1.427	Tertiary Alcoholic Protons	$R_3C\mathbf{OH}$
1.557 – 1.602	Carboxylic Acid Protons	-COO**H**
2.151 – 2.272	Secondary Amine Protons	$R_2N\mathbf{H}$
4.117 – 4.730	Sulfates	$\mathbf{SO_3^-}$
5.581	Aliphatic methoxy linkage Protons	$(C\mathbf{H_3}O)_2C\mathbf{H_2}$
7.239 – 7.265 7.543 – 7.609	Aromatic Protons	Ar-**H**

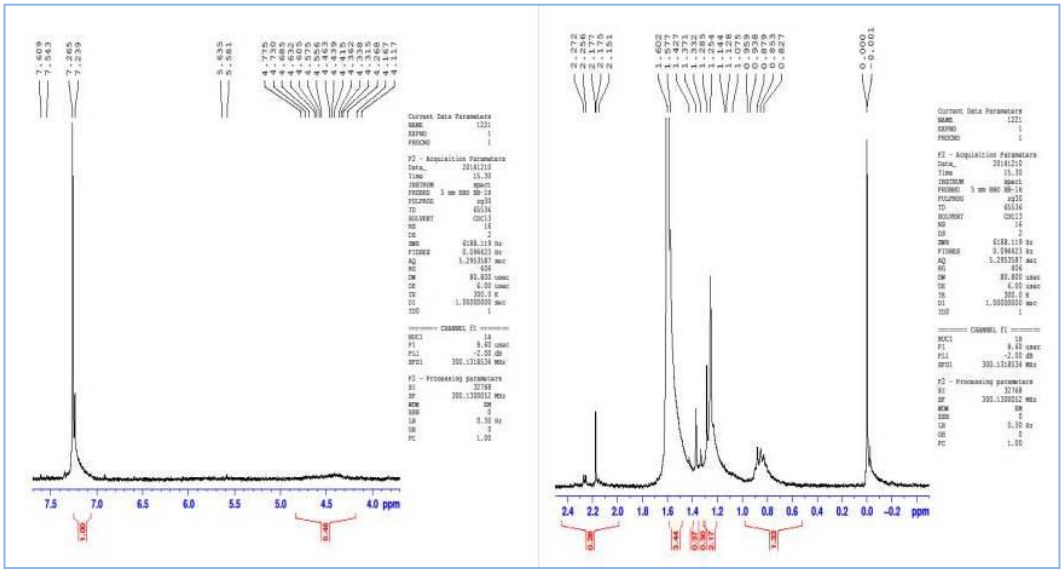

Fig. 3.2: H1-NMR spectra of extracted from deep sea shark (*Echinorhinus brucus*) cartilage 3.3.3 LC-MS/MS analysis of GAG

Figure 3.3, 3.4. 3.5 and 3.6 show the positive and negative ion scanning spectrum (200-1500 m/z) of GAGs standard and sample GAGs respectively. Mass spectrometry (MS) is a sophisticated tool that identifies and quantifies molecules based on their mass-to-charge ratio (m/Q, m/q, m/Z, or m/z). Mass spectrometry has emerged as the most ideal analytical technique because to its high specificity, sensitivity and accuracy (McLafferty, 1981;Thomson, 1987). Direct injection into MS/ MS and ion tuning in negative and positive ion mode of 200-1500 m/z had generated numerous ions in sample and standard GAGs, similar ion patterns were evident in the sample and standard GAG sample. These results suggest the presence of GAGs in the sample and hence confirms its proteoglycan nature.

GAGs are most authentically characterized by tandem mass spectroscopy using electron spray ionisation. And this technique is well established to characterize GAG chains with short chain length predominantly for oligosaccharides (Crawley *et al*., 2004). Many methods using different ionization tools such as ESI, MALDI etc are developed to identify and quantify GAGs using MS/MS. (Kiselova *et al*., 2014). Studies reported efficiency of ESI mass spectrometry methods for most accurate identification and quantification of GAG chains in samples (Oguma *et al*., 2001; Oguma *et al*., 2001). Similarly efficient separation and structural analysis of glycosaminoglycan oligosaccharides,was reported previously (Bodet *et al*., 2017)

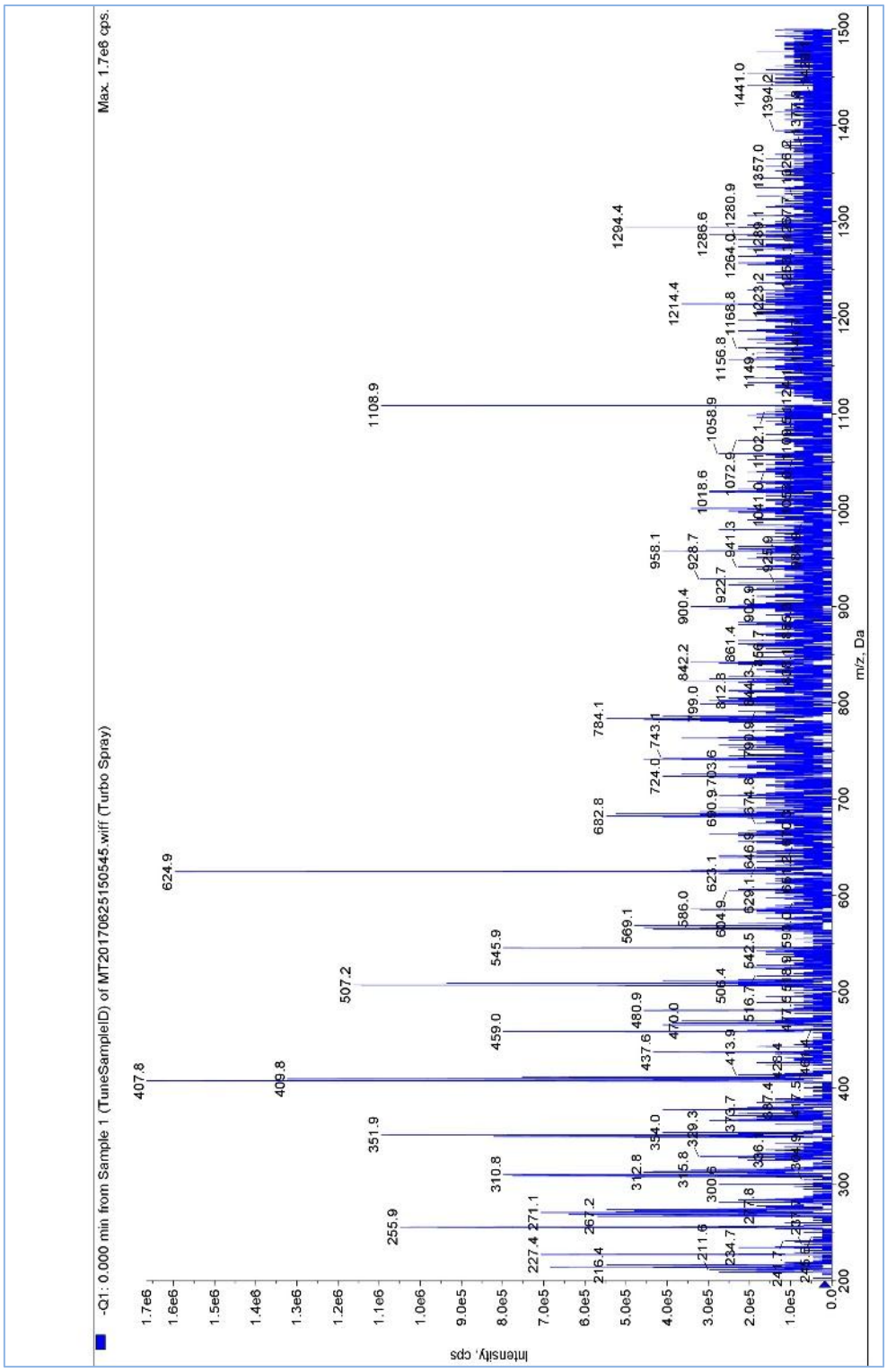

Fig. 3.3. Negative ion manual full scanning of GAG standard (200-1500 m/z)

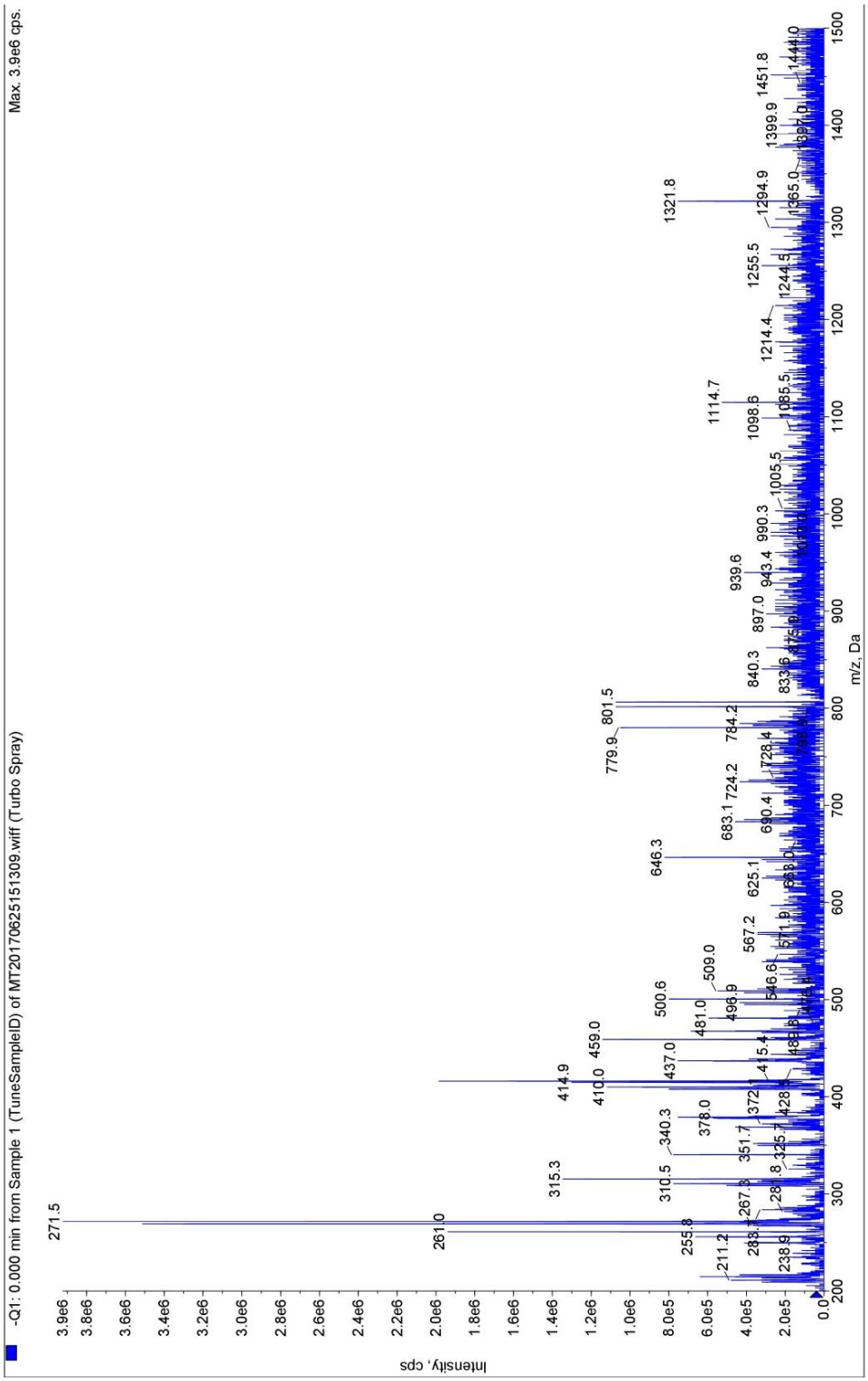

Fig. 3.4 Negative ion manual full scanning of GAG sample (200-1500 m/z)

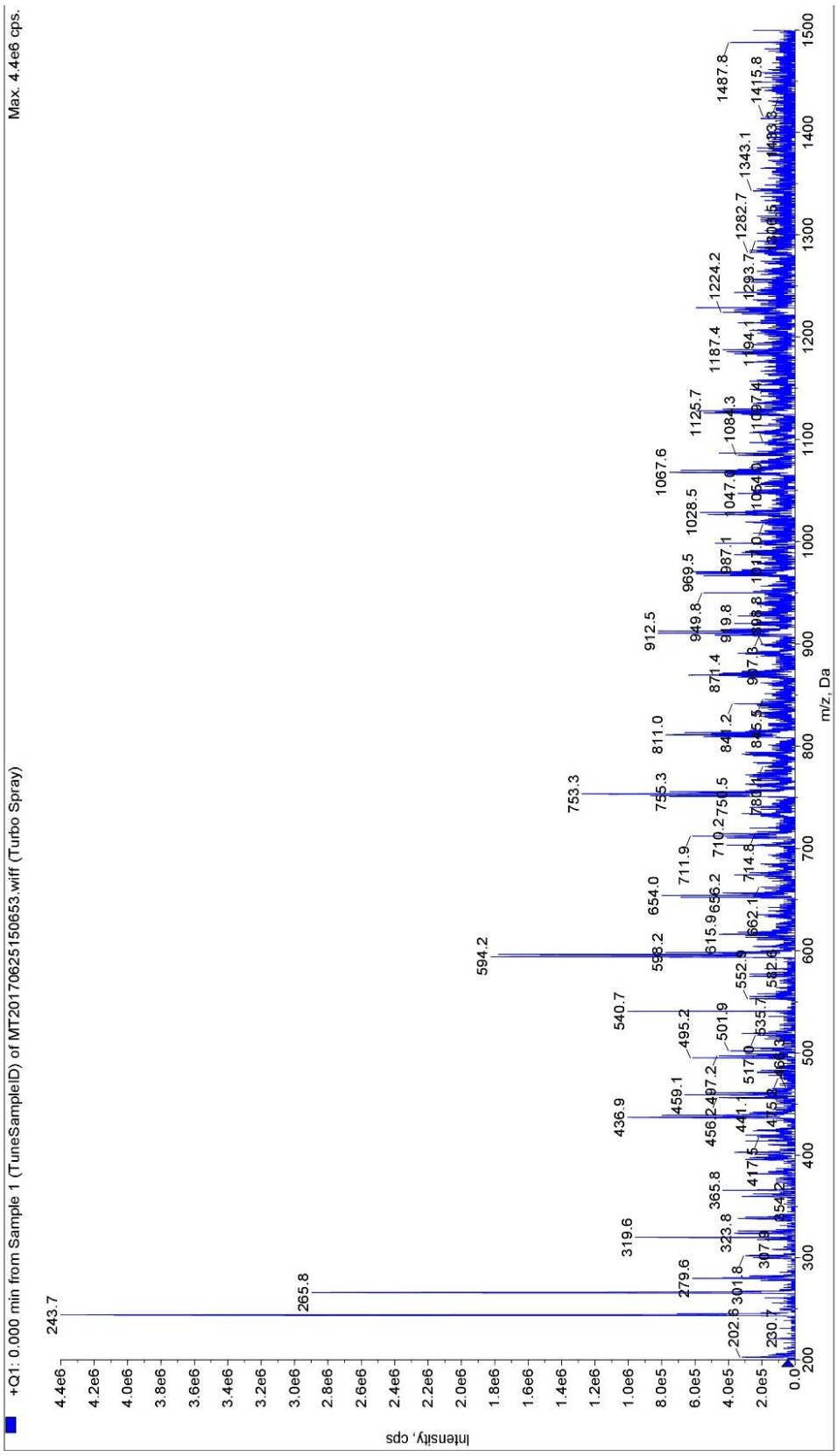

Fig. 3.5 Positive ion manual full scanning of GAG standard (200-1500 m/z)

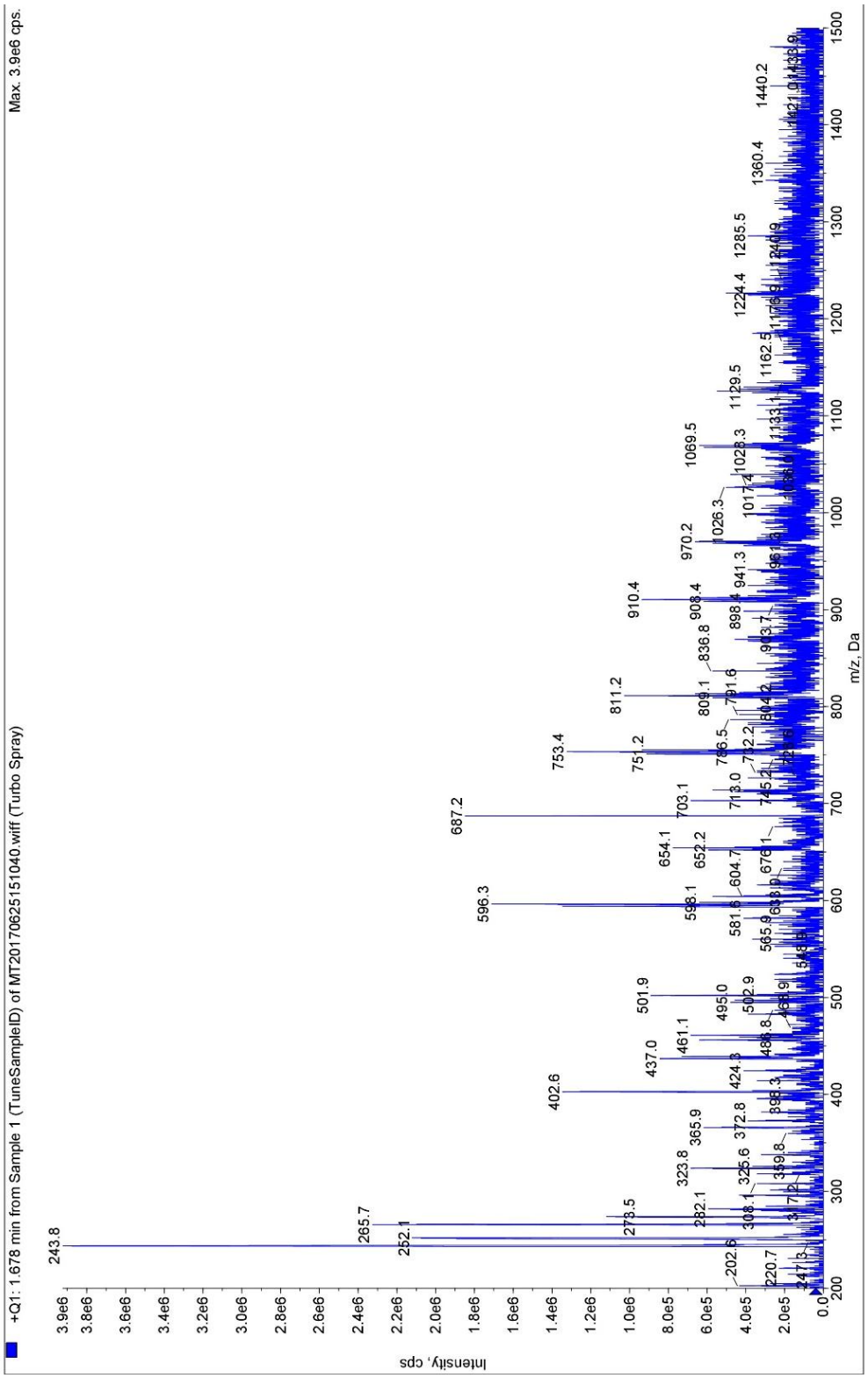

Fig. 3.6 Positive ion manual full scanning of GAG sample (200-1500 m/z)

3.3.4 Proteomics analysis of PGs

Figure 3.7 & 3.15 depicts the LC-MS/MS spectrum of PGs peptide and individual aggrecan, decorin and epiphycan peptide spectrum. Shark protein data base and specifically proteoglycan data base is not available in NCBI protein database. Therefore complete search of NCBI database with available peptide sequences was done to identify the presence of proteoglycans in our samples. Some hits which had noteworthy similarities with proteoglycans namely, aggrecan, decorin and epiphycan of available species were observed in the search. Trypsin digestion of shark cartilage-extracted PGs sample generated a peptide fragment AGWLSDGSVR which has been recognized as having sequence similarity to aggrecan core protein of *Xenopus (Silurana) tropicalis* with an accession no. 301605297. Tryptic digested peptides were searched using Sequest HT against NCBI full protein database with the 5 ppm tolerance used for MS and for 0.02Da for MS-MS. The peptides searched were then filtered against 1% FDR NCBI protein data base which then revealed the presence of aggrecan core protein, decorin and epiphycan peptide hits in the proteoglycans sample. Two peptides VGVVNYASTVK and VGIVFTDGR were observed to be derived from decorin protein of accession no. 296207278 as per the data base search. Also a leading peptide ie, LDGNPINLSK presumed to have similarity to protein epiphycan of *Cricetulus griseus* with an accession no. 354483477 was identified in the sample. Epiphycan is an important low molecular weight proteoglycan present in cartilage tissue.

Proteomics technology is increasingly gaining popularity as a technology to understand the pathological and biochemical changes during disease. It is being used to identify typical proteins expressed during a specific condition and is also being employed to develop novel drugs for treatment. (Hanash, 2003). Peptide-mass fingerprinting is a vital technique to establish peptide sequences, even when the quantity of protein available is very limiting. This method is

superior for a precise identification and sequencing of proteins of interest (Pappin *et al*.,1993). Trypsin generated sequences are found to be most appropriate for identification of protein by combining liquid chromatography with mass spectrometry (LC/MS) (Huang and Henion, 1990). Study of proteomics of extracellular matrix protein of cartilage has reported the presence of unique proteins namely, aggrecan, collagen type 2, decorin etc. Aggregan the prominent proteoglycan was characterised by trypsin digestion followed by peptide based protein identification by LC-MS/MS method. Core protein domain of aggrecan was identified and reported in human articular cartilage protein analysis.. The aggrecan core protein was analyzed individually for its three globular domains and documented by Müller *et al*. (2014) in human arthritic studies. Identification and characterization of aggrecan and other proteoglycans have been reported based on their peptide sequence employing LC MS/MS based proteomics approach (Gesteira *et al*., 2011).

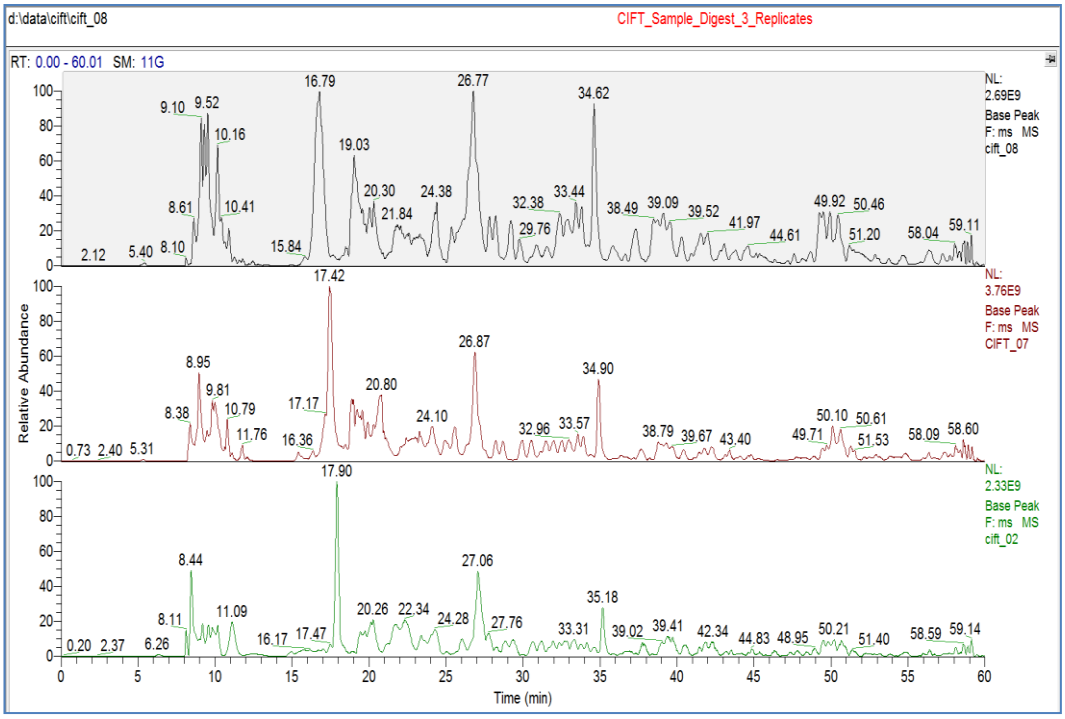

Fig. 3.7 LC-MS/MS Spectra of trypsin digested peptide of proteoglycans

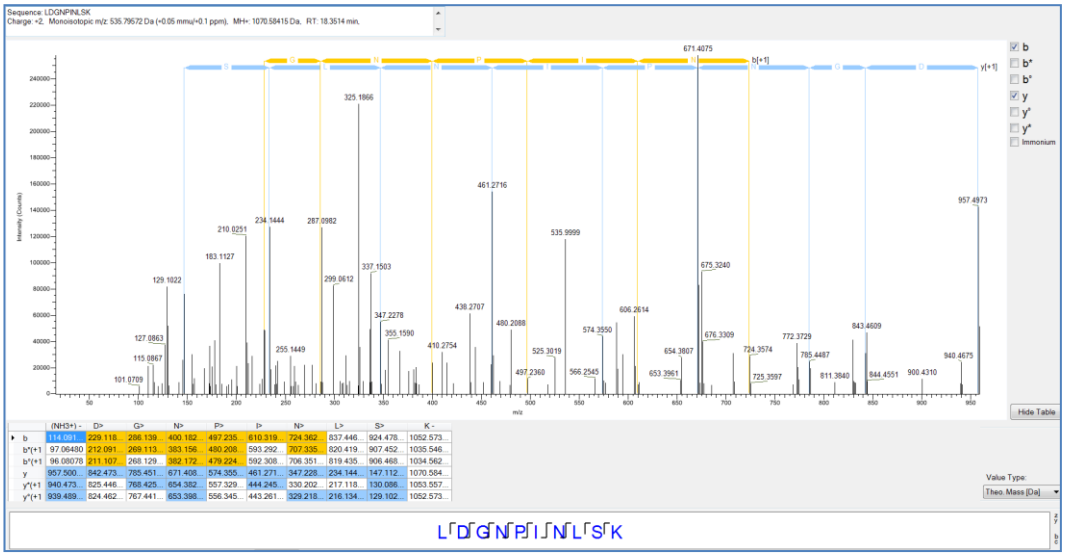

Fig. 3.8 Spectrum of epiphycan-like protein peptide (Accession No. 354483477): LDGNPINLSK. consensus view: b and y ions are denoted with color

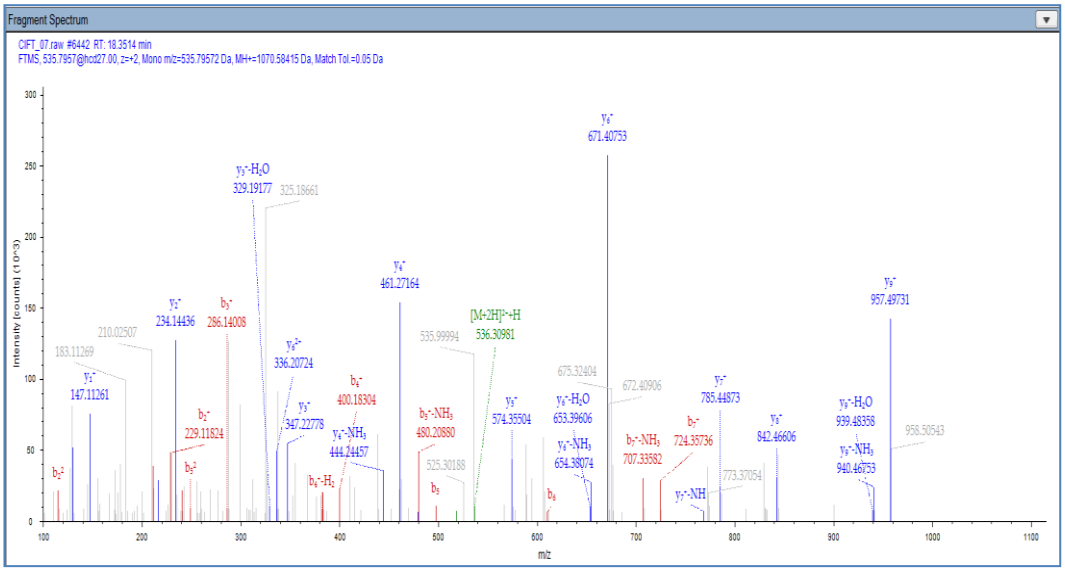

Fig. 3.9 Peptide annotation (LDGNPINLSK) of b and y ions in the MSMS spectra

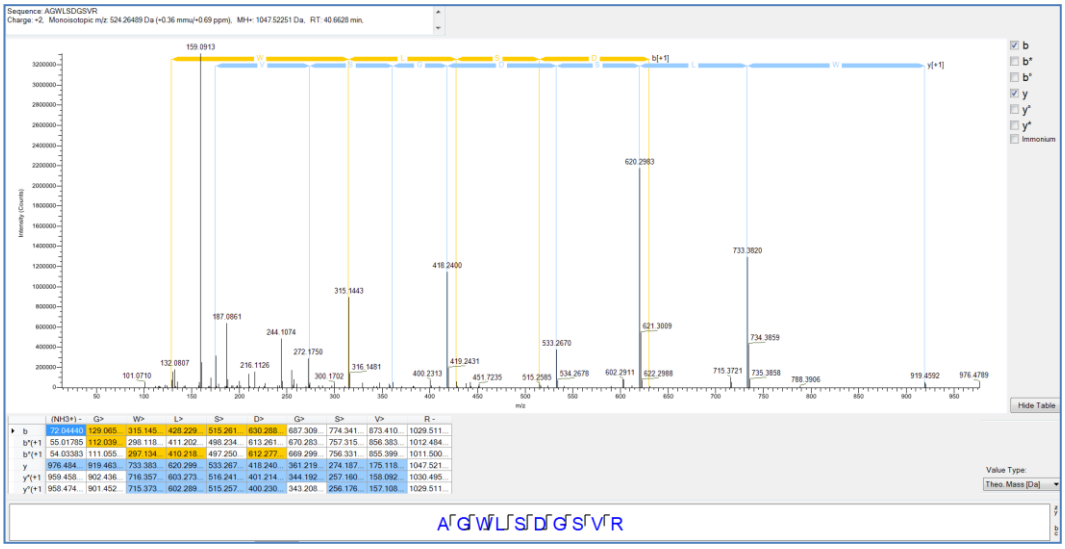

Fig. 3.10 Spectrum of aggrecan core protein-like protein peptide AGWLSDGSVR (Accession No. 301605297). Peptide consensus view: b and y ions are denoted with color

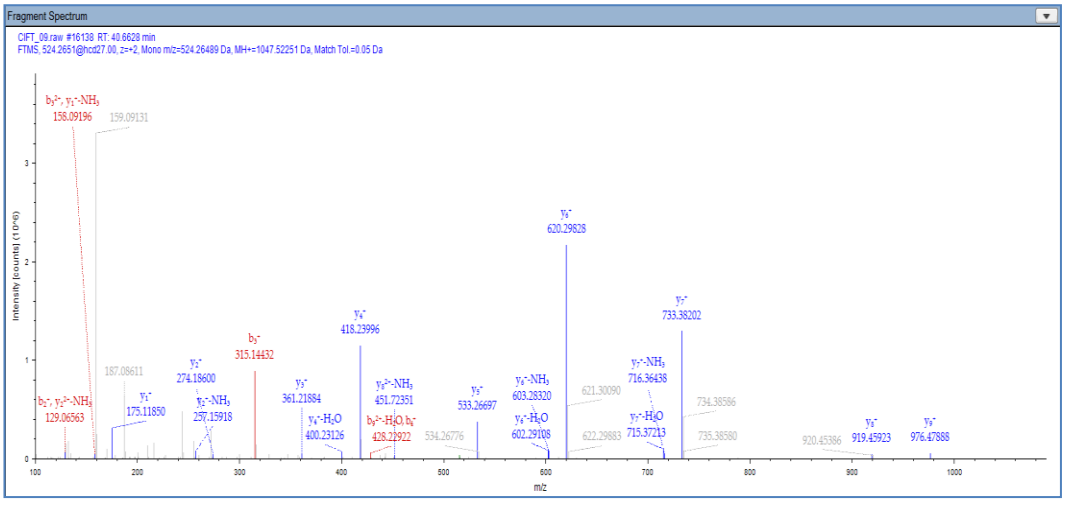

Fig. 3.11 Peptide Annotation (AGWLSDGSVR) of b and y ions in theMSMS spectra

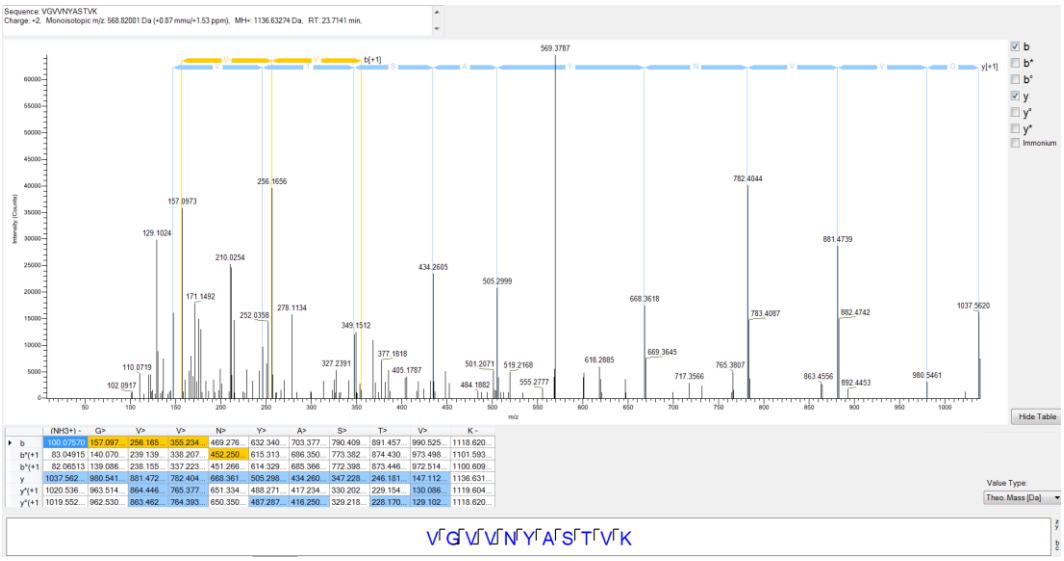

Fig. 3.12 Spectrum of Decorin protein peptides (Accession No. 296207378) 1) VGVVNYASTVK & 2) VGIVFTDGR. Peptide consensus view: b and y ions are denoted with color for peptide 1) VGVVNYASTVK

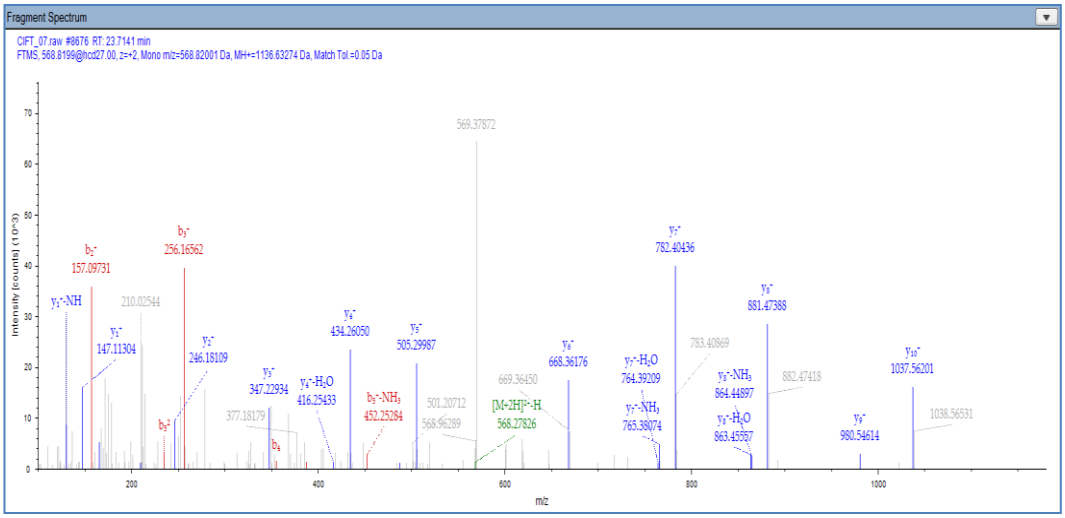

Fig. 3.13 Peptide Annotation (1) VGVVNYASTVK) of b and y ions in the MSMS spectra

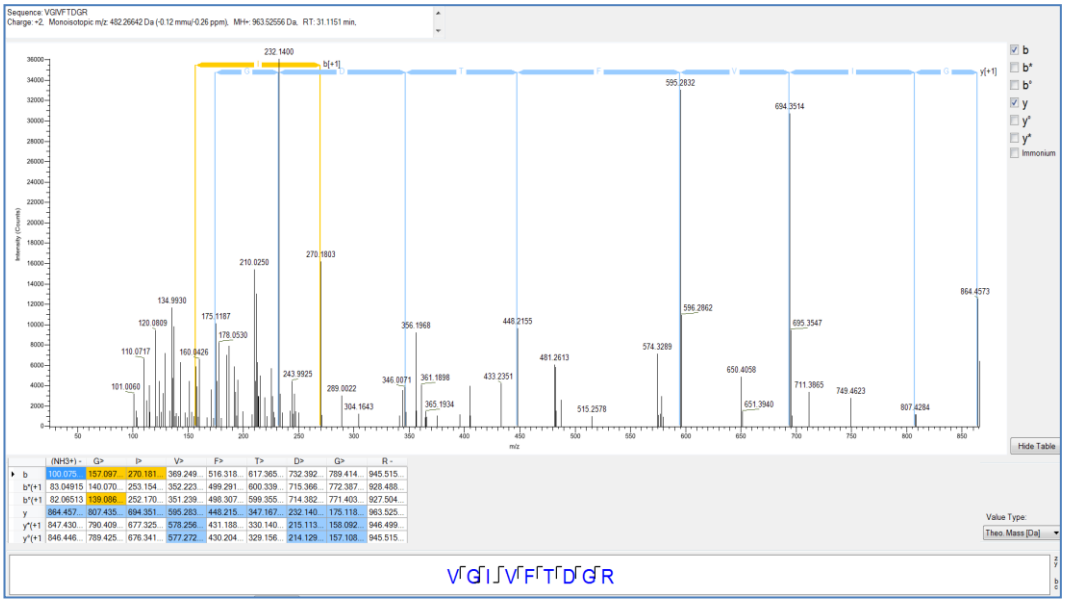

Fig. 3.14 Peptide consensus view: b and y ions are denoted with color for peptide Peptide2: VGIVFTDGR

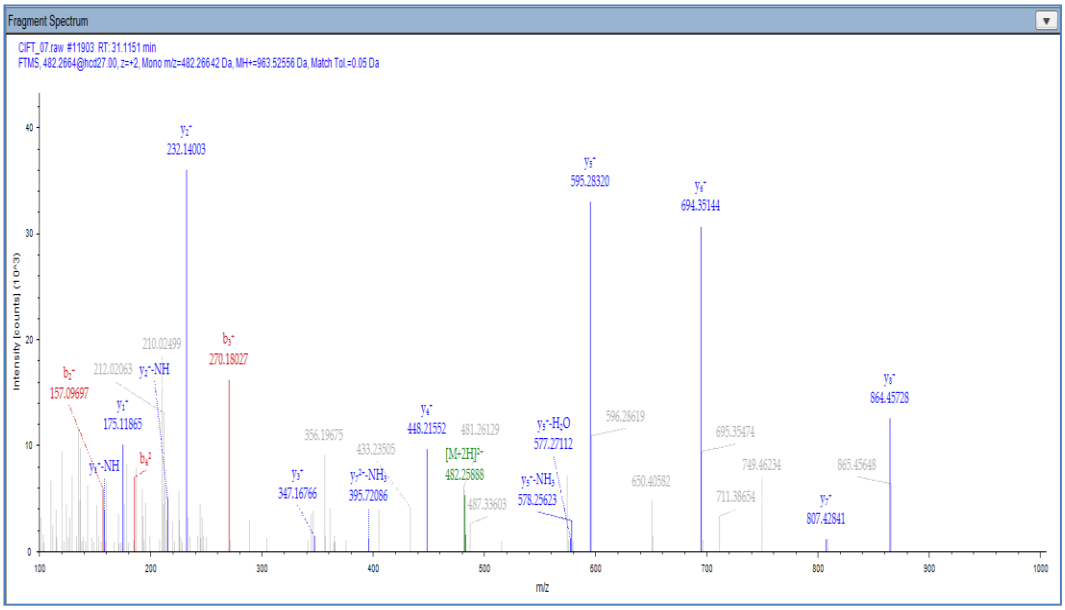

Fig. 3.15 Peptide Annotation (Peptide2: VGIVFTDGR) of b and y ions in the MSMS spectra

3.4 Conclusion

Characterization of proteoglycans got attention due to their nutracuetical and pharmaceutical properties. Deeper characterization, especially structural characterization is used to confirm the purity of the proteoglycans. Structural characterization by FTIR, 1H-NMR revealed the presence of proteoglycans by obtaining the fuctional groups response corresponds to glycosaminoglycans and protein. Characterization of trypsin-digested shark cartilage-extracted protoeglycans by LC MS-MS and subsequent NCBI whole protein database search has led us to conclude for the first time that *E. brucus* cartilage has 3 unique peptide fragments which share sequence similarity with arggrecan core protein, decorin and epiphycan respectively. Also characterization of GAG part of PGs of shark cartilage by tandem mass spectroscopy with ESI revealed that the m/z ion pattern in the 200-1500 range were similar to that of standard GAGs like chrondrotin sulphates A & B and hylauronic acid. Characterization of PGs and the associated GAGs was carried out as a prelude to further understand their biological roles and their possible utility as effective agents against arthritis and cancer are being explored.

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Chapter 4

In *vitro* anti-cervical cancer effect of proteoglycans isolated from deep sea shark *Echinorhinus brucus* cartilage by inducing apoptosis and cell cycle arrest

4.1 Introduction

Cervical cancer is the fifth most common cancer in humans and the second most common cancer in women living in less developed regions with an estimate of 84% of the new cases worldwide (Manos *et al*.,1999). As per the statistics of WHO in 2015, approximately 270, 000 women died from cervical cancer, more than 85% of these deaths occurring in low- and middle-income countries like India. This high level of incidence makes it a serious concern especially in Indian women population and there is urgent need for studies that can offer solutions. Reports says that India is likely to have over 17.3 lakh new cases of cancer and over 8.8 lakh deaths due to the disease with cancers of cervix, breast and lung topping the list (ICMR, 2016).

In vitro cell line studies has become an excellent option to study the anti-cervical cancer effects of PGs to evaluate the effect of different drugs and their possible mechanisms of action. HeLa cell line is the most widely used cervical cancer cell line to study the role of drugs against cervical cancer (Liu *et al*., 2011).

More than 100 chemotherapeutic drugs are in use today either alone or in combination with other drugs or treatments. Chemotherapy drugs have different side effects such as hair loss and thinning, nausea and vomiting, mouth and dental problems, loss of fertility, fatigue etc. High dose of doxorubicin, a chemotherapy drug causes cardiotoxicity in humans (Vishnu *et al*., 2017). It has an IC_{50} value of 1000 nM on HeLa cells (Larasati *et al*., 2011) which shows that it is not too sensitive with the treatment of doxorubicin (Maruti *et al*., 2015). Hence, it is needed to develop a new approach of treatment with a little or no side effects.

For over 40 years, natural products are being used for fighting against cancer. Researchers have isolated approximately 7000 marine natural products especially from marine invertebrates to fight against different diseases including cancer. However, vertebrates like shark especially shark cartilage proteins are seldom being exploited.

Proteoglycans (PGs) are important macromolecules of extracellular matrix found in vertebrate and invertebrate tissues. It is involved in various cellular functions including cell growth, adhesion, and differentiation (Kjellen & Lindahl., 1991; Iozzo *et al.,* 1997). They occur in virtually almost all mammalian tissues and are especially prominent in cartilage. The predominant proteoglycans present in cartilage is the large chondroitin sulfate proteoglycan 'aggrecan'. The other important types of proteoglycan present in cartilage are decorin, biglycan, epiphycan, versican etc. Studies reported that a special type of proteoglycan, Proteoglycan (P1) isolated from *Phellinus linteus* mushroom was found to exhibit anti-cancer activities (Li *et al*., 2011). Apart from this small leucine rich proteoglycans (SLRP) like lumican and decorin are also found to prevent different types of cancer. (Vuillermoz *et al*., 2004; Hamid *et al*., 2013)

Till date, there is no report regarding the anti-cervical cancer activity exerted by proteoglycans of shark origin. Hence current work is designed to investigate the anti-cervical cancer effect of proteoglycans isolated from deep sea shark *Echinorhinus brucus* cartilage.

4.2 Materials and Methods

BD FACS, multimode reader, confocal microscope, phase-contrast microscope, refrigerated centrifuge, incubator, Annexin V – FITC apoptosis kit, propdium iodide, RNAse, Hoechst 33342 stain, crystal violet, DMEM, trypsin, foetal bovine serum, phosphate buffer saline, DMSO, MTT[3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide], hydrogen peroxide, antibiotic-antimycotic were purchased from Gibco Invitrogen (Carlsbad, CA, USA). All the chemicals used were of high quality analytical grade chemicals.

4.2.1 Cell culture and treatment

HeLa cells (human epithelial cervix carcinoma) were obtained from NCCS (National Centre for Cell Science), Pune and were cultured in Dulbbecos modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (GibcoBRL) and 2% antibiotic-antimycotic mixture (GibcoBRL) were added at 37°C in a humidified atmosphere containing 5% CO_2. Cells were seeded at appropriate density on plate for each analysis. After attachment, cells were incubated in 37°C with different concentrations of PGs sample. Initial study started with MTT to confirm anti-proliferative activity. Untreated cells served as negative control. The positive control of apoptosis was induced with 500 µM H_2O_2 (Sigma) in the medium for 10 min.

4.2.2 Cell viability assay and cell morphology

Cell proliferation was determined by MTT assay (Mosmann *et al*., 1989). HeLa Cells were trypsinized and seeded on 96 well plate. After 80% confluency, different concentrations of sample (10 µg/ml, 50 µg/ml, 100 µg/ml, 250 µg/ml, 500 µg/ml PGs) were incubated at 37°C for 24 h. Sample-free media was used as negative control. Cell morphology was observed after 24 h incubation with sample using phase-contrast microscope. 100 µl MTT reagent (50 µg/well) was added and incubated for 4 h in dark. After incubation, the reagent was removed and 200 µl of DMSO was added to all wells, covered with aluminium foil and cells were agitated on shaker for 45 min. After shaking absorbance was read at

570 nm using multimode reader. Each assay was carried out three times, and the results were expressed as mean.

Percentage viability = (OD of test /OD of control)*100

4.2.3 Colony formation assay

Colony formation assay serves as a useful tool to test whether a compound can reduce the clonogenic survival of tumor cells. A colony is defined as a cluster of at least 50 cells that can often only be determined microscopically. It is the method of choice to determine the effectiveness of cytotoxic agents. HeLa cells were seeded onto 6-well plates with a density of 2×10^5 cells per well. After 24 h incubation, three different concentrations (10, 100 and 250 µg/ml) of PGs sample were added to the cells and incubated for 24 h after incubation, the cells were stained with 0.5% crystal violet solution and further incubated at RT for 30 minutes. The stained cells were observed using phase contrast microscope.

4.2.4 Annexin-V apoptosis assay

HeLa cells were seeded onto 6-well plates with a density of 2×10^5 cells per well. After 24 h incubation, three different concentrations (10, 100 and 250 µg/ml) of PGs sample were added to the cells and incubated for 24 h. Cells were then trypsinized, aspirated and re-suspended in 1000 µL assay binding buffer. This was mixed well and centrifuged at 400x g for 5 min. The supernatant was discarded and cells were further stained with FITC-Annexin-V (2 µL) and propidium iodide solution (2 µL) in binding buffer, from the Annexin-V apoptosis detection kit (cayman) and then kept undisturbed for 10 min at room temperature in darkness. After 5 min, this was centrifuged at 400x g for 5 min and then re-suspended in 1 ml assay binding buffer. This was followed by flow cytometry analysis. Ratio of apoptotic cells was measured by as described by manufacturer's instructions.

4.2.5 Nuclear staining with Hoechst 33342 (Chromatin condensation)

The nuclear morphology of the cells was evaluated using the cell-permeable DNA dye, Hoechst 33342. Presence of chromatin condensation or fragmentation is an indication of apoptosis. The HeLa cells were seeded in 24-well plates at a concentration of 1.0×10^5 cells/ml. After cells attachment, the cells were treated with various concentrations of the PGs sample and further incubated for 24 h at 37 ^{0}C in a humidified atmosphere. Then Hoechst 33342, a DNA-specific fluorescent dye, was added at a final concentration of 10 µg/ml, followed by 10 min of incubation at 37^{0}C. The stained cells were then observed under a confocal fluorescence microscope to examine the degree of nuclear condensation.

4.2.6 Cell cycle analysis

Cell cycle distribution was detected using propdium iodide staining. HeLa cells were seeded onto 6-well plates for 24 h and rinsed twice with PBS. Three different concentrations (10, 100 and 250 µg/ml) of PGs were added to the cells and incubated for 24 h. After 24 h, the cells were washed twice with PBS and then trypsinized and fixed using 70% absolute ethanol. This was vortexed gently and kept under ice for 30 minutes. The samples were then centrifuged at 2000 rpm for 5 minutes. Further, the cells were resuspended in 0.25 ml PBS and 5µl RNase A and incubated at 37 ºC for 30 minutes. After incubation 10 µl propidium iodide was added and kept in dark at 4ºC. The cells were further washed with PBS to remove the unbound PI and then centrifuged for 5 min at 1500 rpm. The supernatant was discarded and the cell pellet was further re-suspended in 1 ml PBS.

4.2.7 Statistical analysis

One way analysis of variance (ANOVA) was carried out to split the total variability in the data with variability due to treatment and error. Then, variability due to treatment was compared with variability due to error once. ANOVA was found significant, tukey's test was performed to compare the treatment means at 5 % level of significance ($p < 0.05$). All the statistical analysis was carried out by using Proc GLM SAS g.3.

4.3 Results and discussion

4.3.1 Cell viability and morphological analysis

The results of anti-proliferation study done with MTT assay are shown in fig. 4. 1. It was observed that the proteoglycans extracted from shark cartilage showed cytotoxic effect in a dose-dependent manner. High rate of cell cytotoxicity observed indicates the effectiveness of extracted proteoglycans against proliferation of HeLa cell lines. Cell cytotoxic effect of PGs against HeLa cell lines was further confirmed by cell morphology analysis (Fig. 4. 2). The morphology of the HeLa cells was examined using a phase contrast microscope. PGs treated cells showed a round morphology of cells with shrinkage. A proportion of the cells revealed swelling, cell membrane lysis and disintegration of organelles, suggesting PGs induced toxicity in cells. Through phase-contrast microscopy, a decrease in the total number of cells and an accumulation of cells floating in the culture medium, indicating cell death was observed. In the control group, the cells exhibited an intact morphology of nucleus and cytoplasm. The cell morphology became more round and floated compared to the untreated healthy cells, showing a dissimilar cytoskeleton. Based on the MTT assay three different concentrations viz., 10, 100 and 250 µg PGs were used for further analysis.

Cell cytotoxicity analysis using MTT is the widely accepted method to determine cell cytotoxicity exerted by bioactive compounds (Mosaman, 1983). Significant reduction of cell viability, i.e., cell cytotoxicity was observed for 250 µg/ml PGs-treated samples, exhibiting 73% cell cytotoxicity. The above results are in good accordance with the work done by Vuillermoz et al., 2004. Proteoglycans extracted from mushrooms exhibited similar cytotoxic effect on colorectal carcinoma cells and it suggests the usefulness of proteoglycans in controlling tumour growth (Jambunathan *et al*., 2014). The results obtained from the cytotoxicity assays indicate that inhibition of HeLa cells was gradually increased by the addition of PGs in MTT assay. It reveals that HeLa cell line is susceptible to PGs. Cell cytotoxicity was visually confirmed by reduction of HeLa cell count of PGs-treated samples by microscopic view.

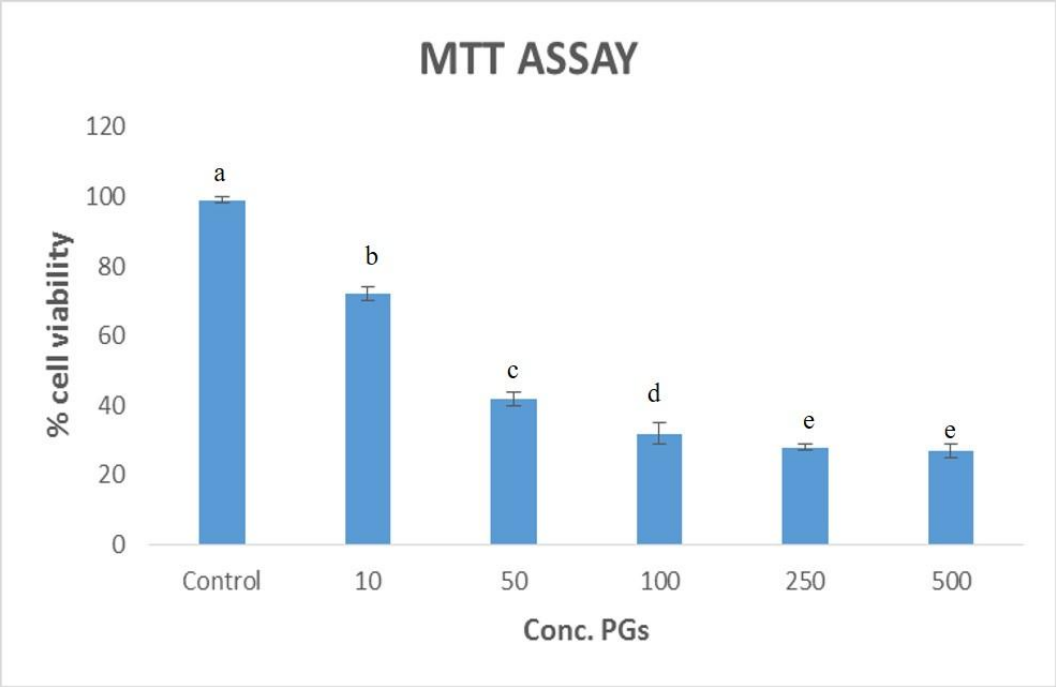

Fig. 4.1 Cell cytotoxicity of PGs (proteoglycans) on HeLa cell lines in dose dependent manner. Treatment means with different letters (a, b, c, d and e) indicate significant difference ($p< 0.05$).

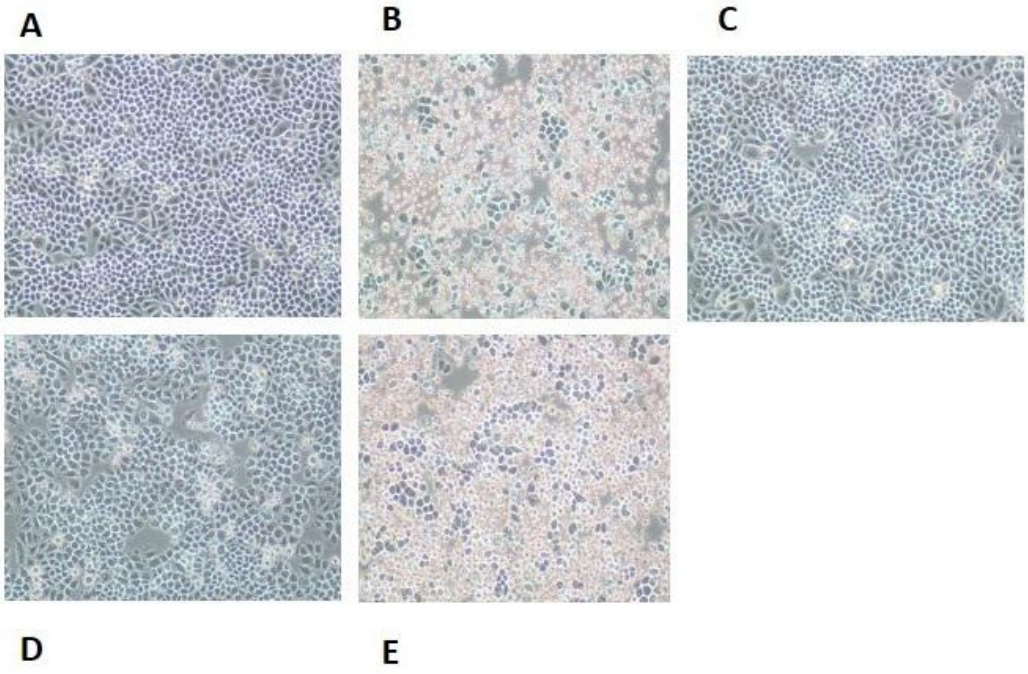

Fig. 4.2 Morphological analysis of HeLa after treatment by light microscopy : morphological changes of HeLa cells treated with A) untreated as negative control B) H_2O_2 treated as positive control C) 10 µg/ml D) 100 µg/ml and E) 250 µg/ml PGs and cells were observed using phase-contrast microscopy. Original magnification, 40X

4.3.2 Colony formation assay

To explore the anti-proliferative properties of PGs, colony formation assay was performed. The results showed that the sample can inhibit the colony formation capacity, mainly at 250 µg/mL concentration. PGs clearly reduce the colony to small scattered colonies as that of 500 µM H_2O_2 treated ie, positive standard groups. PGs showed a promising inhibitory effect on colony formation of HeLa cells in a dose dependent manner. Colonies were photographed using phase-contrast microscope (Fig. 3).

Confirmation of anti-proliferative activity of PGs was clearly observed in colony formation assay. Reduction of HeLa cell colonies indicated the role of PGs in the cytotoxicity of the HeLa cells by certain mechanism. Thus the overall study evaluates that PGs isolated from cartilage of *Echinorhinus brucus* has potential activity on HeLa cells.

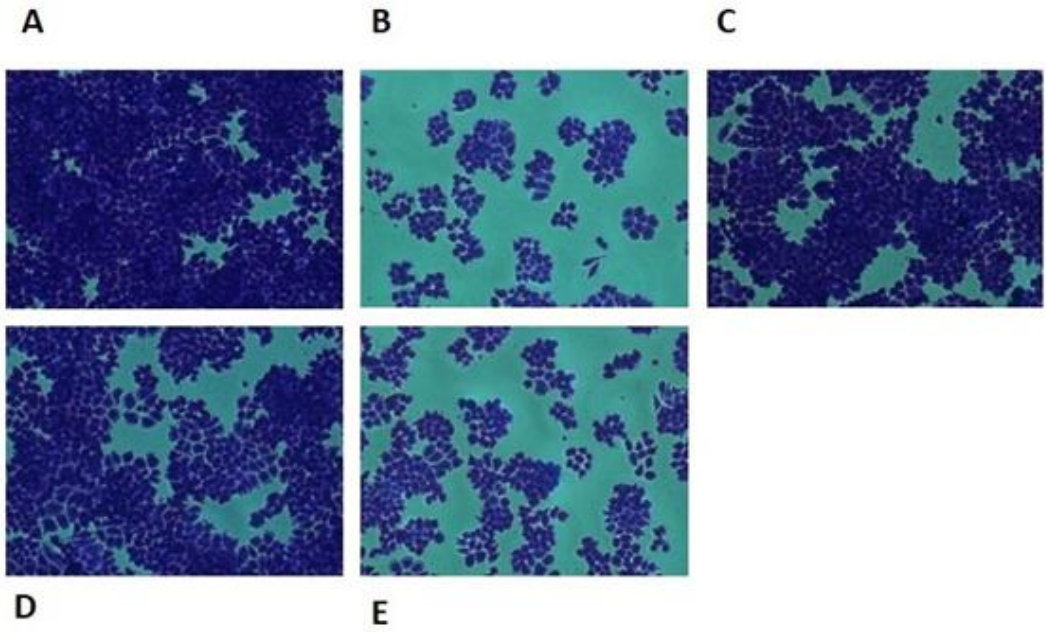

Fig. 4.3 Inhibitory effects of PGs (proteoglycans) on colony formation capacity of HeLa cells. A) untreated as negative control B) H_2O_2 treated as positive control C) 10 µg/ml PGs treated D) 100 µg/ml PGs treated and E) 250 µg/ml PGs treated HeLa cells

4.3.3 Chromatin condensation

The Hoechst 33342 staining was used for the detection of chromatin condensation (DNA damage). Representative stained images are shown in Fig. 4.4 The number of apoptotic HeLa cells (white arrows) which displayed a round and shrunken cell body and chromatin condensation inside the nucleus suggest the PGs-induced apoptosis. Control cells show an intact uniform nucleus and H_2O_2 used as positive control with highly condensed chromatin.

Apoptosis which is also called programmed cell death, is the protective pathway of the body to kill mutated or damaged cells to prevent the cells from becoming cancerous. So compounds that are able to trigger the apoptotic pathway always have an advantage to resist cancer development. Many drugs developed by researchers could possibly activate apoptosis and hence could have anti-cancer activities (Fulda and Debatin, 2006; Lin *et al*., 2007). There are assays like chromatin condensation and flow cytometry assisted identification of apoptosis activation of PGs by Annexin V-FITC staining.

Chromatin condensation is a vital process that is specific to apoptosis mechanism. After the activation of apoptosis, cells reach the final stages of cell death by the action of both extrinsic and intrinsic pathways. Chromosomes of the cells become condensed and finally cell death occurs and this process is highly specific to apoptosis. These chromatin condensation changes of cells can be better visualized by staining with nuclear stain called Hoescht stain which imparts blue colour after staining and binds to chromatin of cells. This enables the visualization of chromatin condensation after treating with apoptotis-activating anti-cancer compounds. Prominent chromatin condensation observed in PGs-treated sample indicated cell cytotoxicity activity of PGs against HeLa cell lines through the activation of apoptosis. Similar reports were observed by Zhao *et al*., 2011 who have reported the anti-proliferative activity of compound called ergone, a plant steroid isolated from *Russula cyanoxantha*, towards HepG2 cells by the activation of chromatin condensation. *In vitro* and *in vivo* anti-colon cancer effects of *Garcinia mangostana xanthones* extract has been reported by the activation of

apoptosis and involvement of chromatin condensation (Aisha *et al*., 2012). Above studies are in accordance with our observation of involvement of chromatin condensation by PGs against HeLa cell lines. Hence, we assumed that cell cytotoxicity exerted by PGs could be due to the activation of apoptosis.

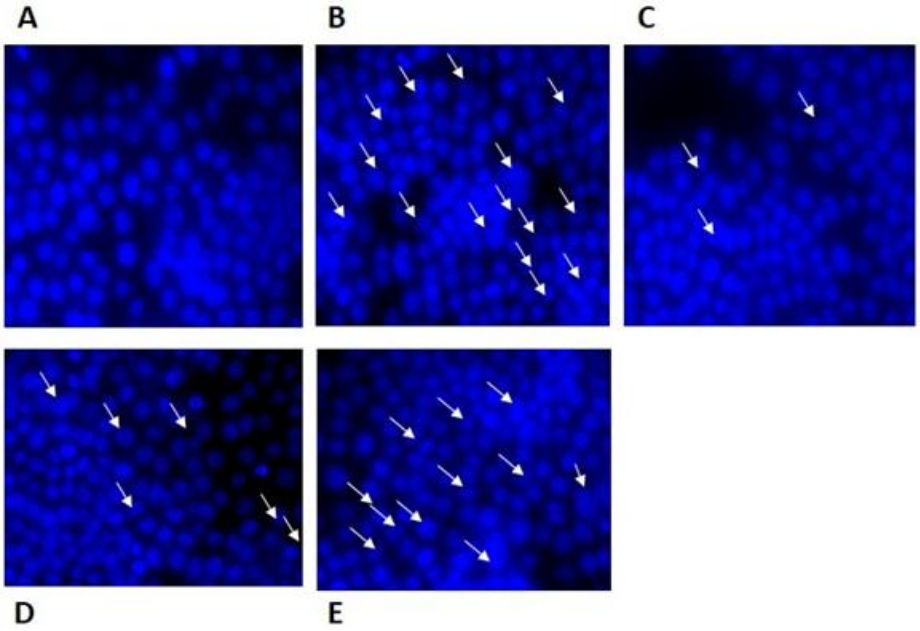

Fig. 4.4 Cell apoptosis observed in treated HeLa cells using Hoechst 33342 staining: HeLa cells were treated with A) untreated as negative control B) H_2O_2-treated as positive control C) HeLa cells treated with 10 µg/ml D) 100 µg/ml and E) 250 µg/ml PGs. Photographs were taken under a fluorescence microscope (200X original magnification). Arrows represent apoptotic cells with chromatin condensation inside the nucleus

4.3.4 Effect of PGs on the degree of apoptotic cells by Annexin V-FITC staining

To further verify PGs-induced apoptosis in HeLa cells, cells were stained with annexin V-FITC and PI using Annexin V- FITC apoptosis kit (Cayman). Results of flow cytometry are presented in Fig. 4.5 The lower right quadrant (Q4) depicts the percentage of early apoptotic cells (annexin V-FITC-stained cells) and the upper right quadrant (Q2) represents the percentage of late apoptotic cells (annexin V-FITC and PI-stained cells). The fully apoptotic cells are those in the lower right and upper right quadrants. H_2O_2-treated cells were taken as positive control and untreated cells as negative control. As shown in Fig. 4. 6, no apoptotic cells were detected in the control group. However, after treatment with

PGs sample at different concentrations 10 µg/ml, 100 µg/ml, 250 µg/ml and H_2O_2, ratio of apoptotic cells were 4.8%, 14.9%, 27.5% and 77.7% respectively.

Apoptotic activation of PGs in *in-vitro* anti-cervical cancer study was confirmed by Annexin V-FITC and PI staining with the aid of flow cytometry. Response for late and early apoptotic cells were obtained in annexin V-FITC and PI analysis. Cells that underwent late and early apoptotic stages were clearly observed in PGs-treated samples which confirm the triggering of apoptosis by PGs to kill HeLa cells. Anticancer activity and apoptosis activation by quercetin in human lung cancer cell line A-549 has been already reported (Zheng *et al*., 2012). Our results also agree with the above studies and substantiated the evidence of activation of apoptosis by PGs against HeLa cell lines. In the present study, PGs isolated from deep sea shark *E. brucus* has shown apoptotic-triggered anti-cancer effect on in vitro HeLa cell lines. Importance of natural products to kill cancer cells by activating apoptosis is well established (Youn *et al*., 2009). Hence PGs can be added to the list of natural compounds which could evoke the apoptotic pathway to fight against cancer.

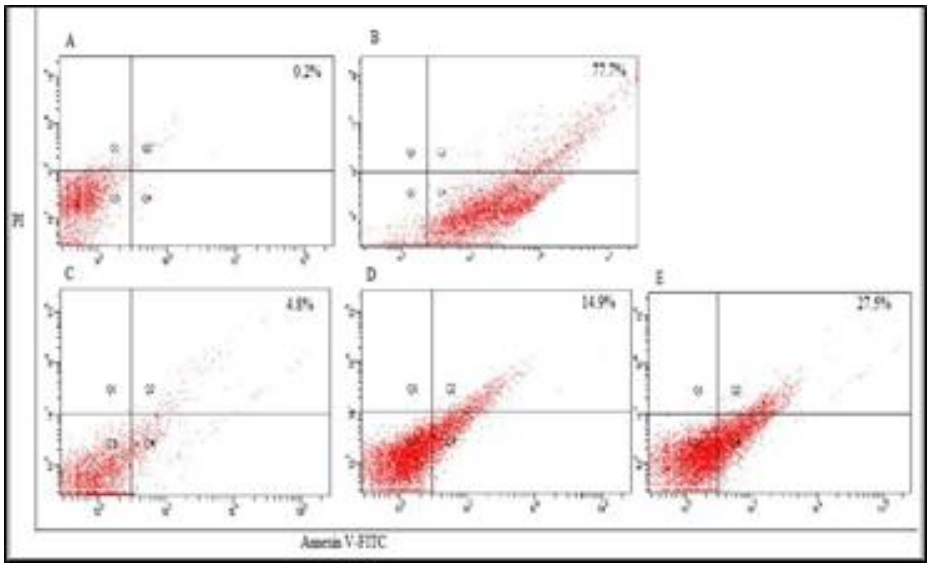

Fig. 4.5 PGs-induced apoptosis in HeLa cells was determined by flow cytometry using annexin FITC-PI staining method. A) untreated as negative control B) H_2O_2 treated as positive control C) HeLa cells treated with 10 µg/ml D) 100 µg/ml and E) 250 µg/ml PGs. The lower right quadrant (Q4) indicates the percentage of early apoptotic cells (FITC-stained cells) and the upper right quadrant (Q2) indicates the percentage of late apoptotic cells (FITC+PI-stained cells)

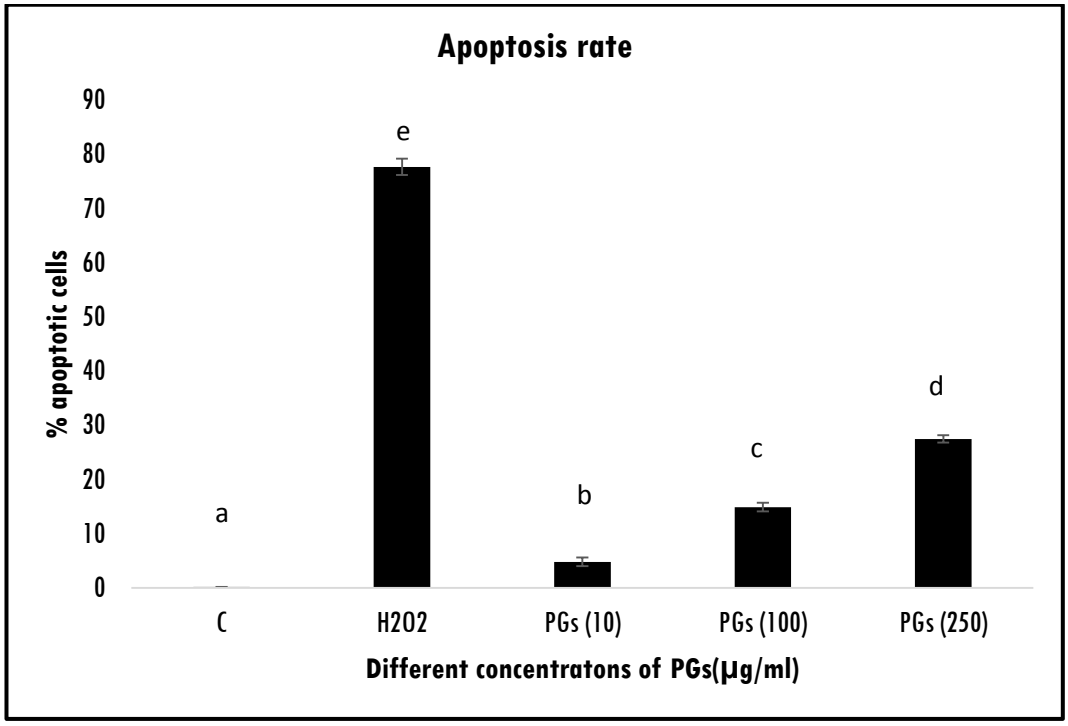

Fig. 4.6 PGs -induced apoptosis rate in dose dependent manner. Treatment means with different letters (a, b, c, d and e) indicate significant difference ($p< 0.05$).

4.3.5 Cell cycle distribution

Fig. 4.7 depicts PGs-induced cell cycle distribution in dose dependent manner. Cell cycle analysis was done by using propidium iodide staining. Cell cycle analysis revealed three distinct cell populations in HeLa cells, which were indicative of cells in the G0/G1, S and G2/M phases of the cell cycle. There were no significant differences in the cell population analysis between the negative control and the cells treated with PGs at different concentrations. Our result shows that the most effective concentration of 250 µg/ml that induced the highest apoptosis in cells, also caused the accumulation of cells at G2/M phase of the cell cycle. G2 phase in the cell cycle is where DNA repair might occur in cells, along with preparation for mitosis in M phase. Increasing arrest of the cells in G2/M phase has been shown to be associated with enhanced apoptosis.

Cell cycle arrest enhances the activation of apoptosis and facilitates cell cytotoxicity against cancer cells. Reports suggested the involvement of apoptosis

and cell cycle arrest in chitosan induced anti-cancer activity on oral cancer cell lines (Wimardhani *et al*, 2014). Activation of apoptosis in human cervical carcinoma HeLa cells was previously reported which highlight the role of apoptosis to kill cervical cancer cells (Liu *et al*., 2011). Similar trend was observed in this study where PGs induced apoptotis-activated cell cytotoxicity on HeLa cervical cancer cell lines and clear cell cycle arrest at G1/S and G2/M phases were observed. Researchers suggest the role of cell cycle regulation at G1, S, G2 and M phases of the cell cycle to resist cancer progression (Tuteja and Tuteja, 2000). Compounds which arrest cell cycle phases especially at G2/M phases facilitate better protection from cancer occurrence and recovery. Role of cell cycle arrest in anti-proliferative action of mushroom extract against leukemia cells has been reported by Gu and Belury, 2005. Similarly anti-tumor activity exerted by the arrest in the G2/M phase and its apoptotic association has been reported in human anti-gastric adenocarcinoma (SGC-7901 cells) studies. They confirmed cell cycle arrest at G2/M phase and enhanced apoptotic events to prevent cancer progression in their study (Shi *et al*., 2007; Cui *et al*., 2007). Youn *et al*. (2009) evaluated the anti-proliferative activity of water extract of *Inonotus obliquus* extract on murine melanoma (B16- F10) cells. They observed better apoptotic response followed by arrest of cell cycle at G0/G1 phase indicating the importance of cell cycle arrest to block cancer cell proliferation and activation of apoptosis. All the findings confirm the better protection rendered by bioactive molecules against cancer cells by arresting cell cycle and by enhanced activation of apoptosis. Similar response was exerted by PGs isolated from deep sea shark cartilage which emphasize the anti-cancer potential of PGs against HeLa cell lines. So we confirm that PGs used in this study exhibited anti-cancer activity on cervical cancer through combined action of apoptosis and cell cycle arrest.

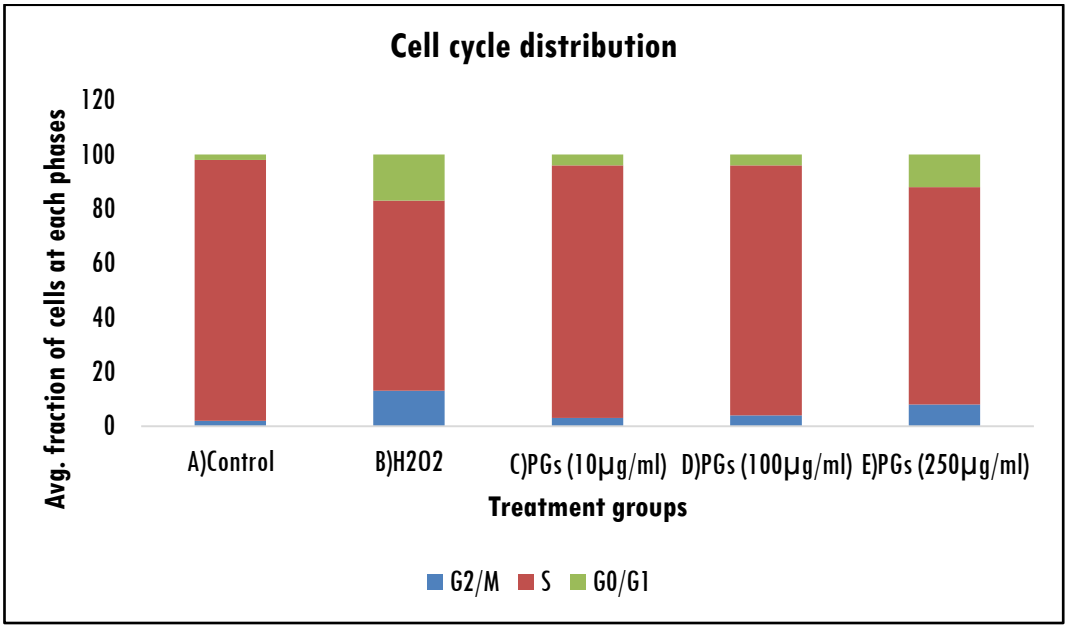

Fig. 4.7 Cell cycle distribution in HeLa cell linenof different treatment A) untreated as negative control B) H_2O_2-treated as positive control C) HeLa cells treated with 10 µg/ml D) 100 µg/ml and E) 250 µg/ml PGs

4.4 Conclusion

Cervical cancer is a big concern among women since numerous cases are increasingly being reported each day. At the same time current treatment with chemotherapeutic drugs are associated with several side effects. There is a continuous search for natural compounds with negligible side effects in the current scenario. Proteoglycans from marine sources especially from shark cartilage can be better utilized to fight against cervical cancer and other cancers too. Compounds having the capacity to activate apoptosis play vital role to fight against cancer. We observed higher dose of proteoglycans (250µg/ml) isolated from deep sea shark cartilage has a greater potential to fight against cancer through the activation of apoptosis and cell cycle arrest. However, detailed investigations are further needed to elucidate the exact mechanisms of action of proteoglycans in apoptosis involved anti-cervical cancer effect.

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Chapter 5

Proteoglycans isolated from deep sea shark, *Echinorhinus brucus* cartilage inhibits proliferation of MCF-7 human breast cancer cells by inducing apoptosis

5.1 Introduction

Breast cancer is the most common cancer form in women; with an estimated 1.4 million new cases worldwide each year, breast cancer constitutes about 25% of all cancer cases in women and is the second most common cancer form overall **(**Youlden *et al*., 2012). As life styles change in India, the country which is home to 17-18% of world population is seeing a steep rise in the occurrence of breast cancer especially among the younger women. From a current figure of 1, 55, 000 new emerging cases, it is predicted to rise to 200, 000 by the year 2030. During the last decade, focus has been on prevention, early detection and introduction of new drugs and treatment modalities. The largest survival improvements were seen in cases where early detection was combined with introduction of effective drug therapies**.** Chemotherapy is one of the commonly used strategies in breast cancer treatment. This therapy is usually associated with adverse side effects, ranging from nausea to bone marrow failure, digestive problems, leucopenia, hair loss and development of multidrug resistance (MDR) **(**Visvanathan *et al*., 2013)**.**

Because of the heterogeneous nature of breast cancer both histopathologically and genetically, the etiology remains uncertain. Use of *in vitro* cell lines has proven to be an excellent option for evaluating the effects of various drugs and also for determining possible mechanisms of action. MCF-7 cell line is the most widely used breast cancer cell line in the world, which was first introduced in 1973 at the Michigan Cancer Foundation and has become the ideal model for studying breast cancer *in vitro* **(**Holliday and Speirs, 2011**).**

Apoptosis, also called programmed cell death, plays a key role in protection against uncontrolled proliferation of cells, enabling the body to eradicate unwanted cells, thereby maintaining proper homeostasis **(**Elmore, 2007**).** Cancer cells respond to treatment with chemotherapeutic drugs because the latter trigger apoptosis pathway. Activation of apoptosis is characterized by the activation of the caspase cascade of enzymes belonging to aspartate proteases family (eg. caspase 3, capase 8, caspase 9 etc) **(**Fulda and Debatin, 2006**)**. The enzymes are responsible for various mechanisms involved in apoptosis such as DNA laddering, cytochrome *c* activation, breakdown of different proteins and finally cell death **(**Brown and Attardi, 2005**).**

p53 gene is considered as one of the major regulators of cell division and is a gene increasingly associated with mutations that eventually lead up to cancer development**.** It is also a potential inducer of positive and negative mediators of apoptosis such as BAX, Bcl_2 etc **(**Kuo *et al*., 2002**).** *p53* gene is a subject of extensive investigations in relation to its role in apoptosis-induced cell death. The possible ways in which these mediators control apoptosis is by either regulating mitochondrial permeability or by controlling cytochrome *c* release (Singh *et al*., 2005**;** Ferlini *et al*., 2003) . Therefore a study of expression of these proteins could indicate the involvement of apoptosis during investigations into anti-cancer effect of biomolecules.

Proteoglycans (PGs) are specialized biomolecules that are conjugated forms of protein and glycosaminoglycans or GAGs . The GAG portions have negative charge, due to the presence of acidic sugar moieties and/or derivatization

by sulphate groups (Iozzo, 1998). Cartilage is a rich source of different mixture of proteoglycans namely, aggrecan, decorin, biglycan, fibromodulin and lumican. They play a vital role in many metabolic functions because of the presence of sulfated glycosaminoglycan chains and protein core **(**Kjellen and Lindahl, 1991).

Some proteoglycans have been found to be effective against the growth of melanoma cancer **(**Vuillermoz *et al*., 2004) and proteoglycans extracted from some species of mushrooms have been observed to control colorectal carcinoma proliferation **(**Li *et al*., 2011)**.** But there are few or no reports of anti-cancer studies with proteoglycans of marine origin. Shark is a rich source of proteoglycans (PGs) and is being considered as a better source of PGs over the mammalian sources (Higashi *et al*., 2015). Moreover, no evidence is available in literature to establish the involvement of apoptosis in anti-proliferative activity against MCF-7 cell lines by proteoglycans. Therefore the objective of this study has been to study the anti-proliferative activity of proteoglycans extracted and purified from the cartilage of deep sea shark *Echinorhinus brucus* and to delineate the mechanisms involved.

5.2 Materials and Methods

5.2.1 Sample preparation

A deep sea shark *E. brucus* sample was brought to the laboratory in chilled condition. PGs were extracted from the separated cartilage with help of chaeotropic agent 4 M guanidine hydrochloride, 0.05 M EDTA, 0.1 M sodium acetate buffer and protein inhibitor iodoacetamide and 0.05 M benzamidine hydrochloride at 4^{o}C for 12 h. After extraction isolation was carried out by the help of DEAE- cellulose anion exchange chromatography with 7 M urea and 0.1, 0.2 and 0.4 M NaCl. Column eluent was dialyzed and freeze dried for analysis.

5.2.2 MTT assay (Cell viability and cell toxicity)

The viability of cells was assessed by MTT assay (Mosmann, 1983) using cancer cell lines. The assay is based on the reduction of soluble yellow tetrazolium salt to insoluble purple formazan crystals by metabolically active

cells. Only live cells have the activity to take up the tetrazolium salt. The enzyme (succinate dehydrogenase) present in the mitochondria of the live cells is able to convert internalized tetrazolium salt to formazan crystals, which are purple in colour. Then the cells are lysed and dissolved in DMSO solution. The colour developed is then determined in an ELISA reader at 570 nm.

5.2.3 Caspase- activation assay

Caspase-3 is an intracellular cysteine-requiring aspartate protease that exists as a proenzyme that is activated during the cascade of events associated with apoptosis. Activities of caspases were determined by chromogenic assays using caspase-3 activation kits according to the manufacturer's protocol (Calbiochem, Merck) **(**Stankovic *et al*., 2011). After treating with test compound, the cells were lysed using lysis buffer (50 mM HEPES, 100 mM NaCl, 0.1% CHAPS, 1 mM DTT, 100 mM EDTA). Lysates were centrifuged at 10,000 rpm for 1 min. The supernatants (cytosolic extract) were collected and protein concentration was determined by Lowry's method **(**Lowry *et al*., 1951**)** using BSA as a standard. 100 µg protein (cellular extracts) was diluted in 50 µl cell lysis buffer for each assay. Cellular extracts were then incubated in 96 well microtiter plates with 5 µl of the 4 mM p-nitroanilide (pNA) substrates, DEVD-pNA (acetyl-Asp-Glu-ValAsp p-nitroanilide) (caspase-3 activity) and LEHD-pNA (tetrapeptide, Leu-Glu-His-Asp) for capase-9 activity for 2 h at 37°C. Caspase activity was measured by cleavage of the above substrates to free pNA. Free pNA (cleaved substrates) was measured at 405 nm in a micro-titer plate reader. Relative caspase-3 activity was calculated as a ratio of the absorbance of treated cells to untreated cells.

5.2.4 DNA laddering assay

The experiment is characterized by the activation of endogenous endonucleases with subsequent cleavage of chromatin DNA into inter nucleosomal fragments of roughly 50 base pairs (bp) and multiples thereof (100, 150 etc.). This effect can be used to detect apoptosis via the DNA laddering assay.

$2x10^5$ MCF-7 cells were incubated in 24 flat-wells plate (triplicate wells of 10^5 per well) with different concentrations of sample (10 µg/ml, 25 µg/ml, 50 µg/ml and 100 µg/ml) (10^5 target cells per well). Fresh DMEM medium was added and allowed for 24 h incubation. Cell sample was collected the in 1.5 ml eppendorf tube, spun down, resuspended with 0.5 ml PBS in 1.5 ml eppendorf tubes, and 55 µl of lysis buffer was added (40 ml of 0.5 M EDTA, 5 ml of 1 M TrisCl buffer, pH 8.0, 5 ml of 100% Triton X-100 and 50 ml of H_2O) for 20 min on ice (4^oC). Eppendorf tubes were centrifuged in cold at 12,000 g for 30 minutes. Samples were transferred to new 1.5 ml eppendorf tubes and then extracted the supernatant with 1:1 mixture of phenol: chloroform (gentle agitation for 5 min followed by centrifugation) and precipitated in two equivalence of cold ethanol and one-tenth equivalence of sodium acetate. Precipitates were spun down, decanted, and resuspended in 30 µl of deionized water-RNase solution (0.4 ml water + 5 µl of RNase) and 5 µl of loading buffer for 30 minutes at 37°C. 2 µl of DNA ladder (marker) was also inserted on the outer lanes. 1.2% gel was run at 5V for 5 min before increasing to 100V. After the dye front reached $3/4^{th}$ of the gel, the DNA shearing image was observed in 312 nm UV illuminator.

5.2.5 DUAL staining for apoptosis

5.2.5.1 Fluorescence microscopic analysis of dual staining for cell death analysis

In this study, we used acridine orange/ethidium bromide (AO/EB) dual staining assay (Baskić *et al*., 2006; Liu *et al*., 2015). Acridine orange is taken up by both viable and nonviable cells and emits green fluorescence if intercalated into double stranded nucleic acid (DNA) or red fluorescence if bound to single stranded nucleic acid (RNA). Ethidium bromide is taken up only by nonviable cells and emits red fluorescence by intercalation into DNA. We distinguished four types of cells according to the fluorescence emission and the morphological aspect of chromatin condensation in the stained nuclei. Viable cells have uniform bright green nuclei with organized structure. Apoptotic cells have orange to red nuclei with condensed or fragmented chromatin. Necrotic cells have a uniformly orange to red nuclei with condensed structure.

5.2.5.2 Dye and drug preparation

200 µL of dye mixture (100 µl/mg AO and 100 µl/mg EB in distilled water) was mixed with 2 ml cell suspension (30,000 cells/ml) in 6-well plate. The suspension was immediately examined and viewed under Olympus inverted fluorescence microscope (Ti-Eclipse) at 200x and 400 x magnification. Untreated cells were taken as controls and test group were treated with drug at different concentration 10-100 µg/ml concentrations for 24 h of exposure. A minimum of 100 cells were counted in each sample.

5.2.5.3 Sample treatments

MCF-7 cells were seeded in a 24-well plate (50,000 cells per well). After 24 h of incubation, the medium was replaced with 100 µl medium containing various doses of samples at different concentrations for different time intervals 1st h, 12th h and 24th h. Untreated cells served as control. After 12 h, the media was aspirated and treated with prepared dye and observed under the fluorescent microscope at emission and excitation wavelength of 460-490 nm.

5.2.6 Apoptotic gene expression assay by mRNA isolation and RT-PCR

This assay was done to study the apoptotic gene expression (Grifantini *et al*., 2003; Maniatis *et al*., 1982; Livak and Schmittgen, 2001). Total RNA was isolated from TRIZOL-(Sigma, India) according to the manufacturer's instructions. Briefly, the sample in TRIZOL was repeatedly pipetted to disrupt cells. The samples were incubated for 5 min at room temperature to permit complete dissociation of nucleoprotein complexes, 0.25-ml portions of chloroform were added, and the samples were centrifuged at 12,000 xg for 15 min at 4°C. Upper aqueous phase was mixed with 5 mg of RNase-free glycogen and 0.5 ml of isopropyl alcohol were introduced to precipitate nucleic acids for 15 min at room temperature, and the pellets were washed with 75% ethanol (in DEPC-treated water) (Invitrogen, U.S.A.). Pellets were resuspended in RNase free water, and DNase I (Invitrogen) treatment was performed according to the manufacturer's instructions. RT-PCR was performed in triplicate using SuperScriptTM two Step RT-PCR with platinum® Taq kit according to manufacturer's recommendations (Invitrogen, Carlsbad, CA, U.S.A.).

For cDNA synthesis, complementary DNA was synthesized from 1 µg total RNA from each sample in 20 µL of reaction buffer (contained 50 mM Tris-HCl, pH 8.3, 75 mM KCl and 3 mM $MgCl_2$) using SuperScript II reverse transcriptase enzyme (Genetech, RT-PCR mix- Germany) in a 20 µl volume reaction containing 10 mM dithiothreitol, 10 U RNase inhibitor (Promega, Madison, WI, USA), 1 mM dNTPs and 2.5 µM random hexamers. Each sample was incubated for 45 min at 45° C, followed by 10 min at 72° C in a Agilent amplicon system (AGILENT Biosystems), the prepared cDNA was stored in -20° C for further use. The cDNA (1µl) was then amplified in 20 µl of reaction buffer for 40 cycles of denaturation (96°C for 30 s), annealing (56°C for 30 s), and extension (72°C for 30s) using primers.

Bcl-2, Fwd: 5′-CATGCTGGGGCCGTACAG-3 Rev: 5′-GAACCGGCACCTGCACAC-3′ β-actin, Fwd: 5'-TTCTACAATGAGCTGCGTGTG-3' Rev: 5'-GGGGTGTTGAAGGTC TCAAA-3' p53, FW, 5'-AGGGTTAGTTTACAATCAGC-3', RW, 5'-GGTAGGTGCAAA TGCC-3'; BAX, Fwd:5′-CATGCTGGGGCTACAG-3 Rev:5′ GAACCGGCACCTGCAC-3′

The PCR products were stained with 1 or 2% ethidium bromide (depending on the size of the amplification products) agarose gels (1 % for Bcl-2) in TBE (9). A 100 bp DNA ladder molecular weight marker (Life Technologies, Rockville, MD) was run on every gel to confirm expected molecular weight of the amplification product.

Acquisition of gel images and quantitative analysis of images of the RT-PCR ethidium bromide-stained agarose gels were acquired with a Cohu High Performance CCD camera (Cohu Inc. San Diego, CA) and quantification of the bands was performed by image J software (Boston university). Band intensity was expressed as relative absorbance units. The ratio between the sample RNA to be determined and β-actin was calculated to normalize for initial variations in sample concentration and as a control for reaction efficiency. Mean and standard deviation of all experiments performed were calculated after normalization to β-actin.

5.2.7 Statistical analysis

One way analysis of variance (ANOVA) was carried out to split the total variability in the data with variability due to treatment and error. Then, variability due to treatment was compared with variability due to error once. ANOVA was

found significant, tukey's test was performed to compare the treatment means at 5 % level of significance ($p < 0.05$). All the statistical analysis was carried out by using Proc GLM SAS g.3.

5.3 Results and Discussion

5.3.1 MTT assay (Cell viability and cell toxicity)

Cell cytotoxicity analysis with the help of MTT assay is widely adopted method to find out cell cytotoxicity activity of any bioactive compounds. (Mosaman, 1983). It was observed that the proteoglycans extracted from shark cartilage showed cytotoxic effect in a dose-dependent manner. It is shown in Fig. 5.1. IC_{50} was found to be 25 µg/ml and it indicates the effectiveness of extracted proteoglycans against proliferation of MCF-07 cell lines. Significant reduction of cell viability i.e., cell cytotoxicity was observed for 50 and 100 µg/ml samples, the later exhibiting 73% cell cytotoxicity. Cell cytotoxicity revealed the importance of proteoglycans to kill MCF-7 cancer cells could be by activating cell death pathways. The above results are in good accordance with the work done by Jambunathan *et al*., 2014 and work reported by Vuillermoz *et al*., 2004. Proteoglycans extracted from mushrooms exhibited similar cytotoxic effect on colorectal carcinoma cells and it suggests the usefulness of proteoglycans in controlling tumour growth (Li *et al*., 2011).

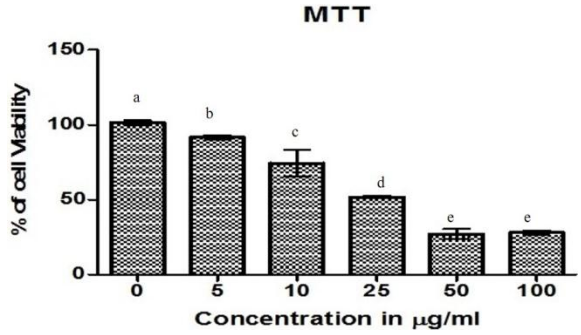

Fig. 5.1 MTT assay showing the cell death at different concentration of PGs treatment ie., 5, 10, 25, 50 & 100 µg/ml PGs after 24h incubation. Treatment means with different letters (a, b, c, d and e) indicate significant difference ($p < 0.05$).

5.3.2 Dual staining for apoptosis

In this study, the double fluorescence assay showed the presence of significant number of orange-red fluorescent cells in the MCF-7 cell line treated

with 100 µg of PG indicating active cell death. It is illustrated in Fig. 5. 2. Double fluorescent staining with AO and EB is very helpful to assess the morphological changes during cell death. AO stains the viable cells and produces green fluorescence whereas EB is taken up by the dead cells that have lost their cytoplasmic membrane integrity and show uniform orange fluorescence. Renvoize *et al*., 1998 described methods to identify apoptosis mechanism during cell death. He has mentioned dual staining technology to track cells undergoing apoptosis. Previous reports emphasize the dual staining method to differentiate apoptosis and necrotic cells during cell death. AO/EB dual staining technology was employed to identify apoptosis induction by plant bioactive compounds as one of the reliable methods (Stankovic *et al*., 2011). Relevance of dual staining technology to confirm apoptosis involvement by using acridine orange/ethidium bromide is described in cycloheximide-induced apoptosis in human leukocytes (Baskić *et al*., 2006). Our results agree with research findings on apoptotic involvement by using dual staining technology. Hence confirmed cell death induced by PGs on MCF-7 cell lines by apoptosis activation.

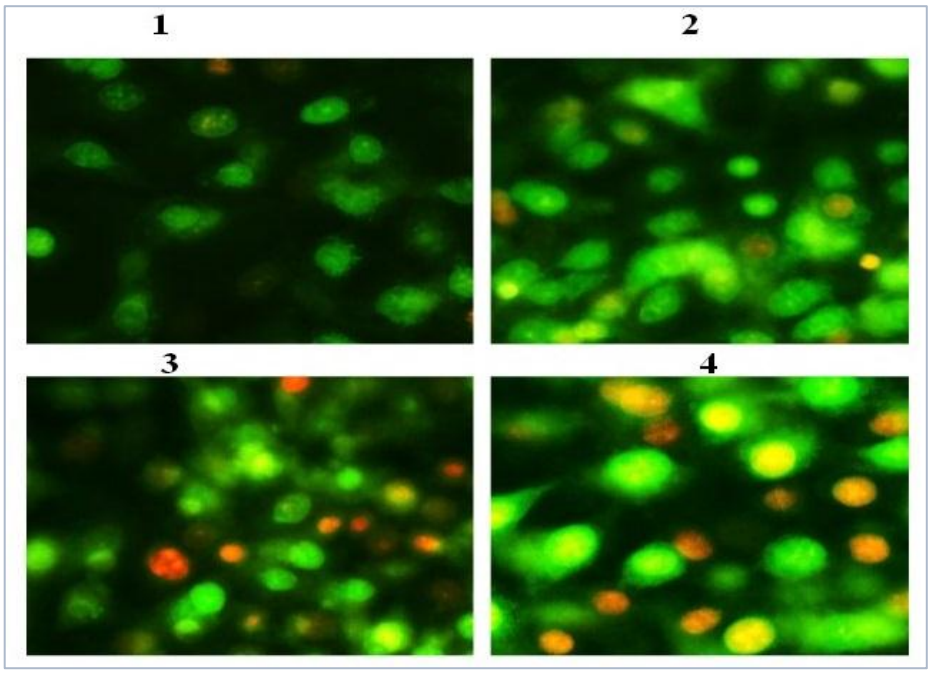

Fig. 5.2 Double fluorescent images at different concentration of PGs treatment ie., (1) 5 µg/ml, (2) 10 µg/ml, (3) 50 µg/ml & (5) 100 µg/ml on MCF-7 cell line. Uniform orange fluorescent cells with condensed nucleus indicates the presence of apoptosis in treated sample compare to control

5.3.3 Caspase- activation assay

Caspase 3 & 9 levels are summarized in Fig. 5.3. Significant amount of caspases 3 and 9 ie, 2.81 and 3.1 µg/ ml respectively were noticed in 100 µg of PG-treated sample. Gradual increase of caspase levels were observed in PG treated samples. Apoptosis is commonly called as programmed cell death with proper and ordered biochemical and molecular pathways that play a vital role in the regulation of homeostasis in normal cells. It helps to kill and remove damaged or unwanted cells to maintain the healthy balance between normal cells and mutated or damaged cells (Cotter, 2009; Kerr *et al*., 1972). Studies have reported the importance of apoptosis to remove potentially dangerous mutated cells to block cancer and about lack of activation of apoptosis that leads to cancer (Halazonetis *et al*., 2008). Hence, active apoptotic mechanism helps to safeguard the body from cancer (Fulda, 2010). Apoptosis could be the reason for cell cytotoxicity effects of proteoglycans from shark cartilage on MCF-7 cell line. A family of special proteins called as caspases is specifically activated in the course of apoptosis. Caspases are normally present as inactive zymogenes (pro-caspases) that are activated by specific apoptotic stimulation (Wang *et al*., 2012). This cascade starts with activation of autocatalytic initiator proteins called caspases, which cleave and activate the effector caspases that, in turn are involved in cell lysis. These proteins cause breakage of major cellular components that are essential for normal cellular function. Caspase 3 and 9 are the major apoptotic markers that are up regulated and whose expression is enhanced during activation of apoptosis. Caspase 9 is the main upstream caspase for the intrinsic pathway whereas in associated with extrinsic pathway. Both the intrinsic and extrinsic pathways together caspase 8 trigger the activation of caspase 3. Caspase 3 breaks down the inhibitor of the caspase-activated deoxyribonuclease, which triggers the initiation of nuclear apoptosis. Caspase-3 is an intracellular cysteine protease that exists as a proenzyme, becoming activated during the cascade of events associated with apoptosis. Noori *et al*., (2012) have reported similar findings with respect to caspase activation during anti-proliferation studies on MCF-7 cell lines. Production of caspase-3 is vital for the control of cell division during tumour growth and decreased production could lead to active tumour growth and

resistance to chemotherapy (Devarajan *et al*., 2002). This indicates the importance of caspase production during anti-proliferative action and the therapeutic agents that increase caspase production could be effective anti-proliferative agents. Activation and execution of apoptosis can be identified by the increased activation of caspase 9 and 3. Researchers suggested the active apoptotic pathway by tracking the caspase 3 & 9 protein and similar enhanced expression of caspase 3 & 9 was reported in anti-gastric carcinoma effect of ethanolic extract of *Ganoderma lucidum* (Jang *et al*., 2010). In this study, we observed increased caspase 3 and caspase 3 expression which corroborates the anti-cancer effect of PG extracted from shark cartilage.

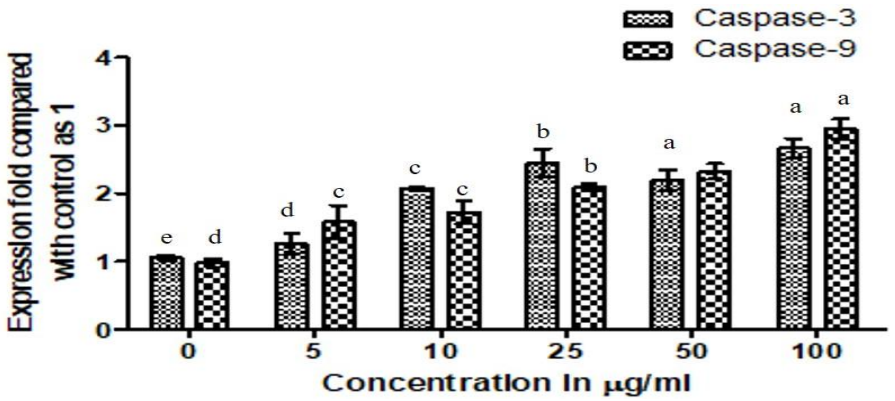

Fig. 5.3 Level of caspases 3 & 9 at different concentration of PGs treatment ie., 5, 10, 25, 50 & 100 µg/ml on MCF-7 cell line. Treatment means with different letters (a, b, c, d and e) indicate significant difference ($p< 0.05$).

5.3.4 DNA laddering assay

DNA laddering assay was successfully used to establish the involvement of apoptosis and helps in ruling out the involvement of necrosis as the cause of cell death .The assay showed the formation of inter nucleosomal fragments of roughly 50 base pairs (bp) and multiples thereof (100, 150 etc.) in DNA isolated from cells treated with 100 µg of PG and are shown in Fig. 5.4. The caspases can trigger the production of several degradative enzymes such as DNases, which execute breakdown of the DNA in the nucleus. These nucleosomal fragments were generated as a result of activation of endogenous endonucleases with subsequent cleavage of chromatin DNA; a feature characteristically observed in apoptotic cell death. Fragmentation of DNA is a major event of apoptosis in which dfinite

breakdown of DNA into 50 to 300 kilobase pieces occur (Vaux and Silke, 2003) DNA ladder pattern is often visible during early apoptotic stages and it has been accounted as an evidence of cell death due to apoptosis (van Breda *et al*., 2008). Similar DNA fragmentation pattern was reported in MCF-7 cell lines after sulforaphane treatment, an isothiocyanate present in cruciferous vegetables (Pledgie-Tracy *et al*., 2007). Hence assumed the involvement of apoptosis mediated cell death by PGs treatment on MCF-7 cell lines.

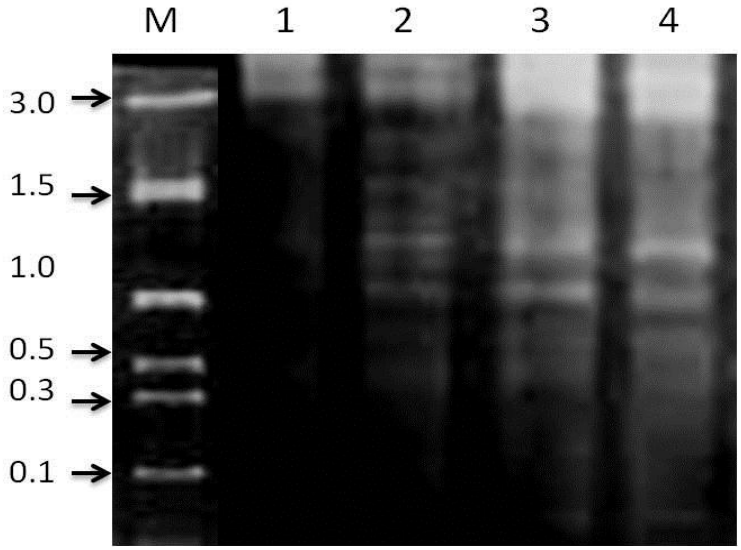

Fig. 5.4 DNA laddering images at different concentration of PGs treatment ie., lane (1) 10 µg/ml (2) 25 µg/ml (3) 50 µg/ml and (4) 100 µg/ml on MCF-7 cell line. Fragments of roughly 50 base pairs (bp) and multiples thereof (100, 150 etc.) have seen maximum in lane 3 & 4 indicates apoptotic involvement

5.3.5 Apoptotic gene expression assay by mRNA isolation and RT-PCR

Gene expression studies have been widely used to confirm apoptosis activity. Pro-apototic and anti-apoptotic regulator genes such as BAX and Bcl-2 are actively expressed in order to regulate apoptosis (Wei *et al*., 2001). The gene *p53* is well known for its ability to control cell division. Expressions of these genes are quite common during cell division and apoptosis. An increase or decrease in expression of these genes provides valuable information with respect to apoptosis and proliferation. Gene expression details are is illustrated in Fig. 5.5. In the present study, significant increase in expression of BAX and decreased expression of Bcl-2 was observed in cells treated with 100 µg of PGs. These

events indicate effective activation of apoptosis which contribute to prevention of MCF-7 cell proliferation. Also a, significant increase in *p53* gene expression was observed in cells treated with 100 µg of PG which shows the active involvement of *p53* gene product in bringing about effective cell division control. PGs may activate apoptosis by the activation of the expression of Bcl-2 & BAX by *p53* mediated pathway. Apoptosis is characterized mainly by two pathways, extrinsic (cytoplasmic) pathway and/or the intrinsic (mitochondrial) pathway (Luo *et al*., 2008). High Bax: Bcl-2 ratio could favour release of enzymes namely, cytochrome *c*, caspase and eventually lead to active apoptosis **(**Shafi *et al*., 2009**)**. A healthy ratio of pro-apoptotic to anti-apoptotic factors is significant for active apoptosis pathway. Effective chemotherapeutic agents should be capable of inducing these factors in ideal proportion to illicit a good anti-proliferative effect. Our results showed up-regulation of Bax and subsequent down-regulation of Bcl-2 gene expression which could be one of the mechanisms by which PG induced apoptosis activation against MCF-7 cell line proliferation **(**Grifantini *et al*., 2003**)**. Intrinsic pathways consists of Bcl-2 family member protein a kind of pro-apoptotic proteins and Bax protein, a anti-apoptotic protein. A healthy ratio of ratio of Bax/Bcl-2 favours better activation and execution of apoptotic events (Reyes-Zurita *et al*., 2011). During proper apoptosis a decrease in the mitochondrial membrane potential occurs which favours up-regulation of Bax, down regulation of Bcl-2. A special type of gene called *p*53 which codes for *p*53 protein induces up-regulation of Bax and down-regulation of Bcl-2 in *p*53-related apoptosis (Martin and Elkon, 2004). So increased expression of *p*53 accelerate the expression of BAX and down regulation of Bcl-2. Thus an increase in Bax/BCL-2 facilitate the proper execution of apoptosis. Down regulation in the expression of in the p53 tumour suppressor gene expression leads to the major reason behind the occurrence of more than 50% of human cancers (Bai and Zhu, 2006). *p*53 gene expression facilitates the activation and deactivation of Bax/Bcl_2 gene expression and in turn controls apoptotic pathway during cell death. So up regulation and expression of *p*53 activated apoptosis through the up regulation of BAX and down regulation of Bcl_2 (Lee *et al*., 2013). Reports substantiate the increased expression

of *p*53 and subsequent up regulation of BAX and down regulation of Bcl_2 expression in anti-cancer effect and apoptosis induction of cordycepin through DR3 pathway in the human colonic cancer cell HT-29 (Lee *et al*., 2013). Similar observation was noticed In this study in which PG treatment caused the increase in expression of p53 gene and BAX, at the same decreased expression of Bcl_2 was also been noticed. So we assumed that PGs were able to induce apoptosis by controlling the gene expression of p53, BAX and Bcl_2.

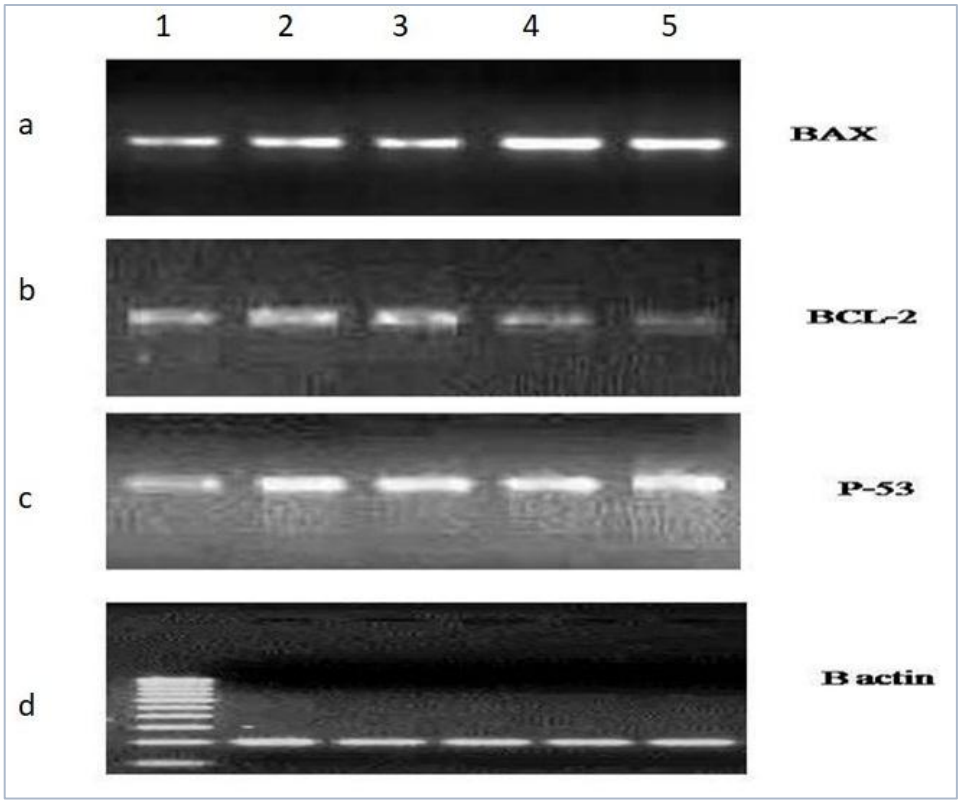

Fig. 5.5 Apototic gene expression at different concentration of PGs treatment ie., (1) 5, (2) 10, (3) 25, (4) 50 & (1) 100 µg/ml on MCF-7 cell line. a) BAX expression b) Bcl-2 expression c) *p53* gene expression d) β-actin

Several studies have reported the triggering of apoptosis by natural compounds (Igney and Krammer, 2002). Our results also support the above observation as it was shown that PGs activated apoptosis-mediated cell death against MCF-7 cell lines. In mammals each and every mutated/damaged or old cell has the capacity to be committed to apoptosis leading to its death. Whereas due to some uncertain reasons some cells escape from apoptosis mechanism and

they have many fold chances to become cancerous. And it was reported that eventual development of cells devoid of mechanisms for apoptosis activation could be the reason behind the induction of resistance to chemotherapy in tumour tissues. So uses of agents that can effectively activate the process of apoptosis have evolved to be the best way to fight against cancer cells (Koff *et al*., 2015).

5.4 Conclusion

Compounds which have the ability to evoke body's own anti-cancer mechanisms are gaining increasing attention due to their lower side effects and better anti-cancer potential. PGs can also be considered as the vital macromolecules that can be effective as anti-cancer agents. Shark cartilage is a rich source of PGs that have shown to activate apoptotic mediated anti-proliferative activity against MCF-7 cell lines and highest activity was observed for 100 µg/ml PGs treatment. Apoptosis involvement in anti-proliferative activity of PGs isolated from deep sea shark cartilage was confirmed by caspase 3 & 9 activation, DNA laddering assay, double fluorescent staining with acridine orange/ethedium bromide and gene expression analysis of apoptotic markers such as Bcl-2, BAX and p53. Individual PGs from shark cartilage are to be isolated and further studies need to be done in order to elucidate the effect of each of the PG type. All these results point out the importance of marine sector as a tremendous source of anti-cancer and other bioactive agents. In order to strengthen the fight against cancer and other deadly diseases and for well being of human being marine source can be better exploited.

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Chapter 6

Anti-osteoarthritic effect of proteoglycans isolated from bramble shark *Echinorhinus brucus* cartilage in monosodium iodoacetate (MIA) induced rat model

6.1 Introduction

Osteoarthritis (OA) is the most reported joint disorder mainly seen in elderly people (Busija *et al*., 2010). OA is characterized by degeneration of cartilage structure and biological function, and loss of regulation of proinflammatory and anti-inflammatory pathways (Goldring and Otero, 2011). Symptoms of OA include joint failure due to damage of synovial joints and articular cartilage causing severe pain during weight-bearing including activities viz, standing and walking (Krasnokutsky *et al*, 2008). Studies have shown pathogenesis of OA is due to inflammation at the site of synovium joints (Smith *et al*., 1997; Fiorito *et al*., 2004). Articular cartilage is the major affected area in OA due to degeneration of cartilage protein mainly, proteoglycans and type II collagen. Proteolytic enzymes produced during OA condition specifically cleave aggrecan, a major proteoglycan of the joint cartilage and collagenases breakdown type II collagen (Mankin and Lippiello, 1970).

Proper cure for OA is yet to be reported since it is almost not possible to restore the cartilage once it is damaged or destroyed (Goldring and Otero, 2011).

The objective of treatment in OA currently is to alleviate symptoms viz., pain, maintain or improve joint mobility, make the joints stronger. Drugs which are used in the treatments of OA try to decrease pain so that patients can improve joint function and quality of life. Major treatment of OA targets inflammatory mediators and protection of chondrocytes in order to relieve pain and maintain matrix integrity in the joints of OA patients. Therefore, alleviated inflammation will play key role in OA treatment. Precisely, reducing the articular cartilage inflammation and progress of cartilage destruction, through their ability to associate with joint tissues and its surroundings result in the reduction of joint pain (Burrage *et al*., 2006).

Proteoglycans (PGs) are specialized biomolecules that are conjugated forms of protein and glycosaminoglycans (GAGs). The GAG portions have negative charge, due to the presence of acidic sugar moieties and/or derivatization by sulphate groups (Nakano *et al*., 2002). Major GAGs are present in association with PGs are dermatan sulfate (DS), keratan sulfate (KS) heparin sulfate (HS), heparin chondroitin sulfate (CS), and hyaluronic acid (HA). GAGs either present on the cell membrane surface in connection with core proteins covalently, form proteoglycans, or as part of the extracellular matrix (ECM). GAGs are known to control several physiological functions such as tissue regeneration, cell differentiation, cell cell signaling etc. (Esko & Lindahl, 2001; Iozzo, 1998; Turnbull *et al*., 2001). Cartilage is a rich source of different mixture of proteoglycans namely, aggrecan, decorin, biglycan, fibromodulin and lumican. They play a vital role in many metabolic functions because of the presence of sulfated glycosaminoglycan chains and protein core (Roughley and Lee, 1994). Studies have reported the importance of glycosaminoglycans against cartilage regeneration in arthritic condition. Anti-inflammatory effects of glycosaminoglycan (GAG) isolated from *Gryllus bimaculatus, Gb* were reported in a chronic arthritic rat model induced (Ahn *et al*., 2014). So natural bioactive molecules are increasingly popular to treat arthritis.

Monosodium iodoacetate (MIA)-induced animal model is an ideal model to study osteoarthritis. MIA is a protein inhibitor which blocks GAG and PG

biosynthesis by inhibiting corresponding enzymes and leading to osteoarthritic development (Kalbhen, 1985). There are no reports of studies conducted on anti-osteoarthritic effect of proteoglycans in MIA-induced osteoarthritis in rat model. Therefore an attempt was made to study anti-osteoarthritic effect of proteoglycans isolated from deep sea shark *Echinorhinus brucus* cartilage on MIA induced albino rat model.

6.2 Materials and method

6.2.1 Evaluation of *in vivo* anti-osteoarthritis activity

Wistar strain female albino rats (200-250 g) were used for this experiment. They were housed individually in polyurethane cages under hygienic conditions, maintained at room temperature (28 ± 2^{o} C) and provided food and water ad libitum. Animal experiments were performed as per the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India and approved by the Institutional Animal Ethics Committee (IAEC), Central Institute of Fisheries Technology (ICAR-CIFT), Cochin, Kerala, India.

6.2.2 Induction of OA with MIA in rats

The animals were randomized and assigned to treatment groups before the initiation of the study (n = 6 per group). MIA solution (3 mg/50 μL of 0.9% saline) was directly injected into the intra-articular space of the right knee under anaesthesia induced with a mixture of ketamine and xylazine. After 10^{th} day of arthritis induction rats were divided randomly into five groups:

Group I - Normal group with no MIA injection

Group II - MIA injection without treatment

Group III - Indomethacin- (IM) treated group (2 mg/kg) with MIA injection (Standard drug)

Group IV - 200 mg/kg PGs treated group with MIA injection

Group V - 100 mg/kg PGs treated group with MIA injection

Group III, IV and V were administered orally with indomethacin and PGs for 41 days after inducing osteoarthritis with MIA respectively.

6.2.3 C-reactive protein (CRP) and uric acid analysis of rat serum

Rat serum was separated and stored at -20^{o}C until analysis. CRP level were estimated by CRP/C-Reactive Protein ELISA Kit (Sigma Aldrich) as per by manufacture's protocol. Estimation of uric acid was done by method of Lorentz and Berndt, 1967. Reagents for uric acid analysis are A) urizyme buffer: polyhalogenated benzoic acid in Tris buffer at pH 7.5 + 0.05 B) urizyme reagent: 4-Aminoantipyrine, peroxidase, uricase. C) uric acid standard (5 mg/dl): An aqueous solution containing 5 mg/dL uric acid. 100 to 500 µg uric acid standard was added to standard test tubes, S1 to S5. 1 ml of urizyme buffer and 0.1 ml urizyme enzyme were added to all the tubes. 100 µl serum from each group sample was taken as test and urizyme buffer and urizyme enzyme was added as that of standard. All the tubes were incubated at 37^{o}C for 5 min. After incubation absorbance was measured at 510 nm.

6.2.4 X-ray and histopathology analysis

For histopathology study, the knee joint specimens were first fixed in formalin, and then decalcified with 5% nitric acid overnight. Blocks were made and paraffin sections were cut 5-7 µm thick, stained with haematoxylin and eosin. Fixed and stained specimens were observed under light microscope Leica ICC50 optical microscope. X-ray of right knee lateral view was taken by using digital Siemens 300MA machine with factors KVP-50 and MAS-8

6.2.5 Anti-oxidant defense system and lipid peroxidation assay

GSH levels in gastric mucosa were determined as described earlier (Sedlak and Lindsay, 1968). Joint tissue homogenate of each rat group was prepared by homogenising 1 g joint tissue in 50 mM phosphate buffer and were mixed with 12.5% trichloroacetic acid and centrifuged for 4000 rpm, 15 min at 4°C. The absorbance of supernatant, Tris buffer (0.4 M, pH 8.9) and 5, 5′-dithiobis 2-nitrobenzoicacid (DTNB, 0.01 M) absorbance of the supernatant was measured at

420 nm and expressed as mg GSH/g of tissue. The activity of SOD was determined as described previously by Marklund and Marklund (1974). Aliquots of tissue homogenate were mixed with pyrogallol (1 mM) and buffer solution (Tris-HCl- 1 mM, EDTA- 5 mM, pH 8.5). The reaction was incubated for 20 min, stopped with the addition of 1N HCl and then centrifuged for 4 min at 14,000 rpm. The absorbance of the supernatant was measured at 405 nm. The amount of SOD that inhibited the oxidation of pyrogallol by 50%, relative to the control, was defined as one unit of SOD activity. The enzymatic activity was expressed as U/mg of protein. Catalase activity was measured as described earlier (Aebi, 1984). Sample aliquots of supernatant were mixed with a solution containing 30% H_2O_2, milli-Q water and buffer 5 mM Tris EDTA, pH 8.0. Absorbance was determined by spectrophotometry at 240 nm for 60 s. The enzymatic activity was expressed as U/mg of protein. Changes in secondary oxidation products were determined by measuring the thiobarbituric acid reactive substance (TBARS) value as described in literature (McDonald & Hultin, 1987). 0.1 ml of joint tissue homogenate was mixed with 4 ml of 0.085 N H_2SO_4 and 0.5 ml of 10% phosphotungstic acid. Centrifuged the sample for 10 min at 5000 rpm and supernatant was discarded. 4 ml of distilled water and 1 ml of TBA reagent was added to the pellet and incubated in boiling water bath for 1h. A standard of malonlaldehyde (MDA) of concentration 100 µg/ml was prepared. Five tubes containing concentration of 10 µg to 50 µg was taken into test tube s and followed the above procedure without sample. After incubation tubes were cooled and absorbance was measured at 532 nm.

6.2.6 PAGE of joint proteins

Electrophoretic patterns of the different rat serum proteins were analysed according to the method of Laemmli (1970). Serum samples of each group was dissolved in 50 mM phosphate buffer and buffer containing 4 mg/ml protein from above buffer were then heated at 85^{o}C for 5 min. Samples were then mixed (1:1 v/v) with the sample buffer (0.5 mol/l Tris-HCl, pH 6.8 containing 4% SDS, 20% (v/v) glycerol in the presence or absence of 10% b mercaptoethanol). The digested samples were loaded onto a polyacrylamide gel wells having a 10 % resolving gel

(10% SDS: 100 ml, acrylamide: 2.5 ml, ammonium persulphate (APS) 10%: 50 ml, distilled water: 4.85 ml, TEMED: 5 ml, Tris-HCl, 1.5 M: 2.5 ml) and a 4 % stacking gel (10% SDS: 100 ml, acrylamide: 1.33 ml, APS 10%: 50 ml, distilled water: 6.1 ml, tetramethyl ethylene diamine (TEMED): 10 ml, Tris-HCl, 1.5 M: 2.5 ml). The electrophoresis was done at a constant current of 20 mA per gel using a Bio-Rad Tetra Mini Protean II unit (Bio-Rad Laboratories, Inc., Richmond, CA, USA). After electrophoresis, gels were stained with 5% Coomassie blue R- 250 in methanol: acetic acid (3:1) mixture for 1 h. followed by destaining with 7% acetic acid for 1 h for 45 min. High molecular weight protein marker (Biorad-precision plus, dual colour) with a wide molecular weight range (10-250 Da) was used to track the molecular weight of proteins.

6.2.7 Western blotting

COX 2 (D5H5) XP® Rabbit mAb, M/s Cell Signalling technology (CST), Rabbit Anti-Rat IL-1β and Rabbit Anti-Rat TNF-α antibodies from M/s PeproTech and Rabbit Anti-Rat MMP-13 antibodies from Sigma Aldrich were used for immunoblotting analysis as per manufactures protocol. Concentration of anti-bodies were made to 0.1µg/ml (1:1000 dilution from stock) to obtain optimum anti-bodies for reaction. After PAGE gels were embedded in the readymade western blotting stacking system (Invitrogen) and transferring of protein was done by iBlot (Invitrogen) transfer system. After PAGE gel was incubated in blocking solution ie, 5% bovine serum albumin (BSA) in a tray for 1 h at room temperature followed by the addition of primary antibodies corresponding to anti-rat rabbit TNF- α, IL-1β, COX-2 and MMP-13 10 antibodies at a concentration of 1:1000 (0.1µg/ml) and incubated at 4^{o}C for overnight with gentle shaking. After incubation gels were washed with washing buffer ie, 1X tris buffer saline triton (TBST) for three times for 5 min each. After that washing solution was removed and secondary antibody ie, anti-rabbit IgG embedded with alkaline phosphatase at a concentration 1:2000 was added and incubated for 1 h at room temperature with gentle shaking. After incubation washing step by using TBST was again done to remove unbound anti-bodies for three times for 5 min each. Washing solution was removed and substrate solution ie,

NBT/BCIP (nitro-blue tetrazolium chloride/5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt) for alkaline solution was added to the tray and incubated for 1 h at room temperature with gentle shaking. After incubation bands were formed which correspond to protein of interest as visualized in blotting imager and documented.

6.2.8 Expression study of inflammatory markers

Total RNA was isolated from joint tissues using RIBOZOL™ RNA Extraction Reagent (Amaresco) at a concentration of not more than 100 mg tissue/ml RIBOZOL™ reagent as described in the manufacturer's protocol. RNA concentrations were determined by OD260 measurements and 2 µg of total RNA was treated with RNase-free DNase 1 (Life Technologies) prior to reverse transcription with the first-strand cDNA Synthesis Kit (Origin) with Oligo dT as the primer following manufacturers' instructions. After reverse transcription, the first-strand cDNA samples in a final volume of 20 µl were stored at -20 ^{0}C until used for PCR. The conventional PCR was performed in a thermocycler (Applied Biosystem) with the 2X PCR master mix (Thermo Scientific) in a volume of 50 µl with final concentration of 1 X PCR master mix, 0.4 mM of each primer and 2 µl of first strand cDNA sample. The quantitative real-time PCR was performed for each sample in triplicate for each primer in Applied Biosystems® StepOne™ Real-Time PCR with the Real Time PCR master mix with SYBR Green (Origin) in a volume of 20 µl with final concentration of 1 X master mix, 0.5 mM of each primer and 2 µl of first strand cDNA mixture. Quantitative real-time PCR was carried out for 40 cycles and fluorescence readings were acquired at the end of each amplification cycle at 72 ^{0}C. Melting curve analysis was performed with continuous fluorescence acquisition from 65 to 95 ^{0}C at a temperature transition rate of 0.1 ^{0}C/s to determine the amplification specificity.

6.2.9 Statistical analysis

One way analysis of variance (ANOVA) was carried out to split the total variability in the data with variability due to treatment and error. Then, variability due to treatment was compared with variability due to error once. ANOVA was found significant, tukey's test was performed to compare the treatment means at 5

% level of significance ($p < 0.05$). All the statistical analysis was carried out by using Proc GLM SAS g.3.

6.3 Results and discussion

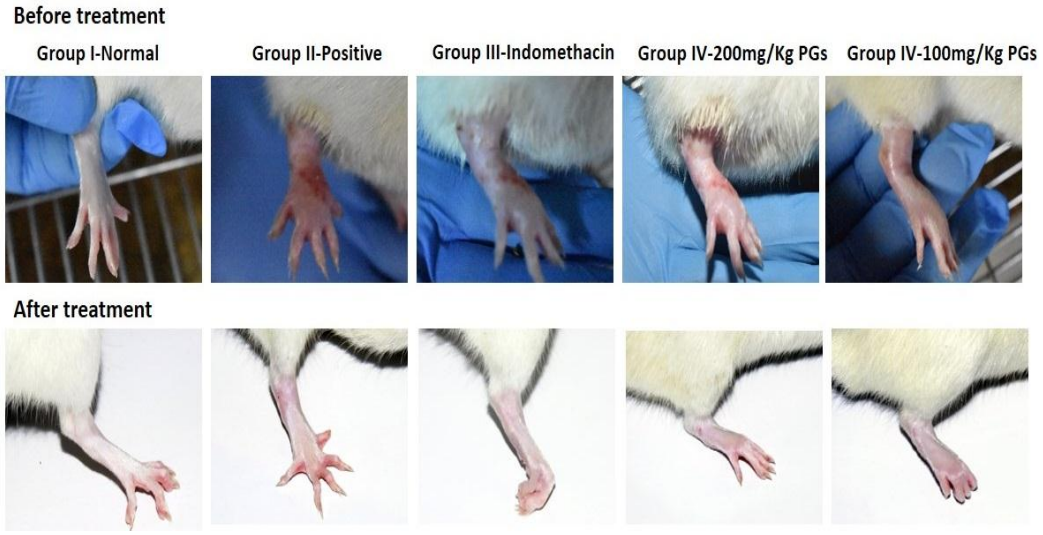

Fig. 6.1 Experimental rats before and after treatment

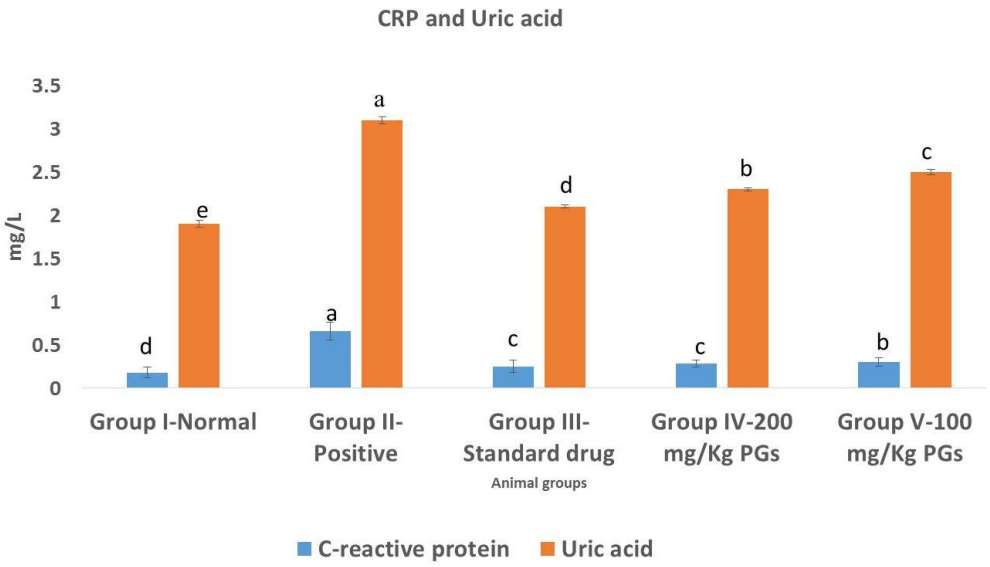

Fig. 6.2 Serum C-reactive protein and uric acid levels of experimented rat groups. Treatment means with different letters (a, b, c, d and e) indicate significant difference ($p < 0.05$)

6.3.1 C-reactive protein and uric acid analysis of rat serum

Serum CRP and uric acid levels depicted in figure 6.2. C-reactive protein (CRP) is a major component of innate immunity based inflammation through the activation of complement system (Du Clos and Mold, 2011; Jin *et al*., 2015). Uric acid (UA) is a product of purine nucleotides metabolism and mutation in the human uricase gene results in serum UA levels which ultimately lead to inflammation in the body (Ruggiero *et al*., 2006). Significant variation was evident in serum uric acid and C-reactive protein (CRP) analysis among studied groups. CRP and uric acid level decreased rats treated with 200 mg/Kg PGs (group IV) ie, 0.30 mg/L and 2.5 mg/L respectively, has similarity with standard drug indomethacin treated group (group III) ie, 0.25 mg/L and 2.1 mg/L. Both CRP and uric acid levels were observed high in group II, positive group. Above results indicate the ability of PGs in decreasing the severity of osteoarthritis development and could be a promising molecule in osteoarthritic treatment. Hence results indicate administration of PGs might have decreased the tissue damage at the joint area by decreasing the inflammation. Above statement is well supported by the decreased levels of C-reactive protein and uric acid levels in PGs treated and indomethacin drug treated group during analysis.

Researchers reported robust correlation between increased CRP and aggravated physiological and biochemical symptoms of OA (Chun *et al*., 2016). Previous report pointed increased uric acid levels accelerate the severity of OA by the activation of inflammatory cytokines like IL-18 and IL-1β (Denoble *et al*., 2011; Ruggiero *et al*., 2006). It was reported that C-reactive protein and UA levels have strong synchronised involvement in inflammation (Frohlich *et al*., 2000). All these data suggest the importance of decreased uric acid and CRP for better recovery from osteoarthritis. Interestingly both the markers were significantly reduced in proteoglycans treated groups indicating its effectiveness in alleviating OA progression.

6.3.2 Anti-oxidant defence system and lipid peroxidation (TBARS)

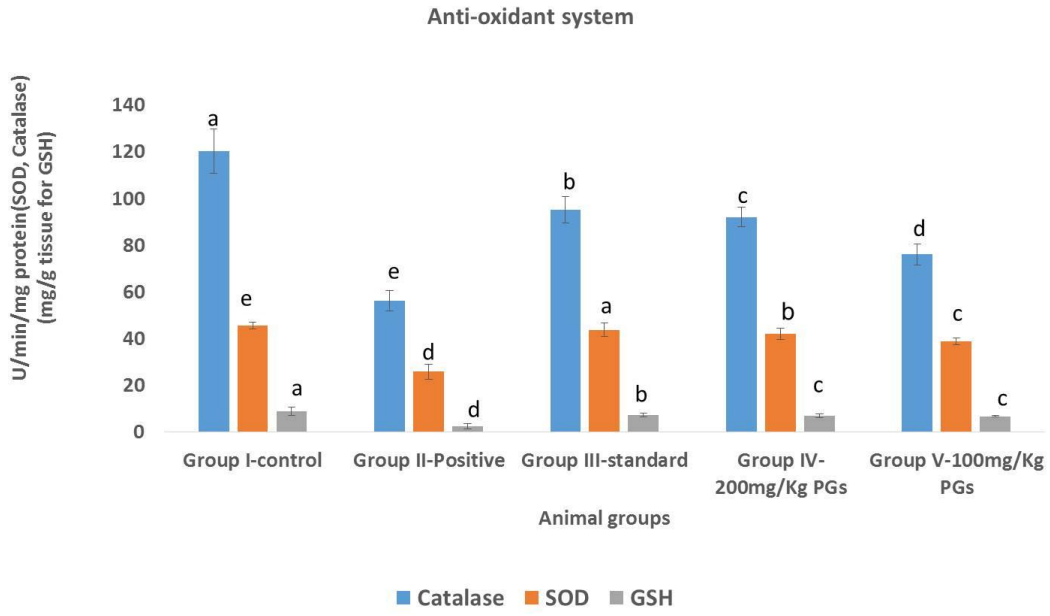

Fig. 6.3 Anti-oxidant defence parameters (SOD, Catalase and GSH) of experimented rat groups. Treatment means with different letters (a, b, c, d) indicate significant difference ($p< 0.05$)

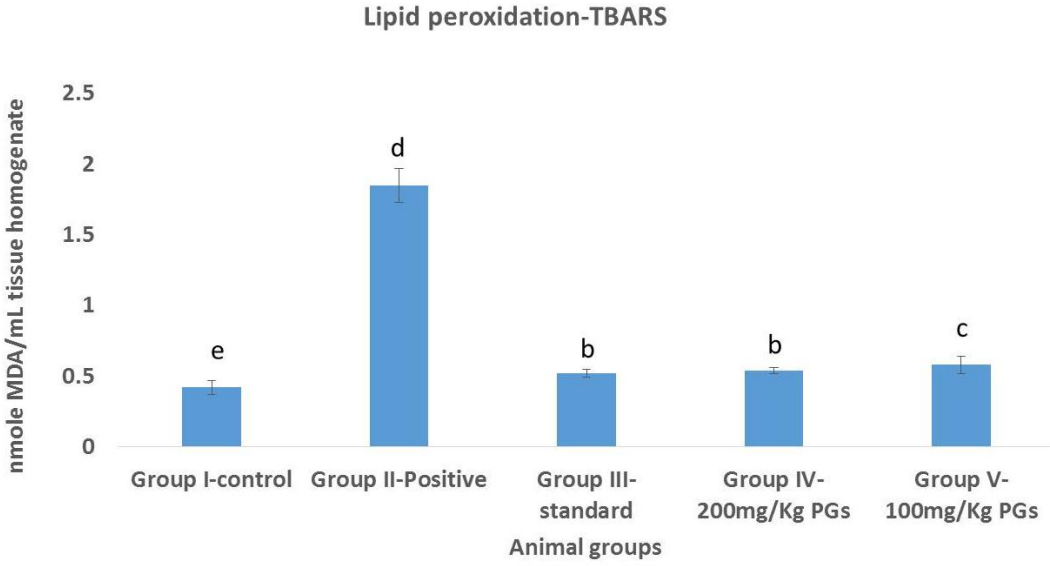

Fig. 6.4 Levels of thiobarbituric acid reactive substances (TBARS) in knee joint of experimental rat groups. Treatment means with different letters (a, b, c, d) indicate significant difference ($p< 0.05$)

Anti-oxidant defence system and lipid peroxidation (TBARS) were depicted in figure 6.3 and 6.4 respectively. Oxidative stress is a major cause of acceleration of osteoarthritic progression. Anti-oxidant enzymes namely catalase (CAT) and SOD levels were increased in proteoglycans PGs treated (200 mg/Kg) sample i.e. 120.3 and 45.56 U/min/mg protein respectively. Group IV results has similarities with indomethacin treated groups (group III) with values 95 and 43.58 U/min/mg protein for catalase and SOD respectively. High concentration of reduced glutathione (GSH) attributes to the active glutathione anti-oxidant defence involvement. Increased GSH content was observed for PGs treated rats (group IV) i.e. 6.9 mg/g tissue which was similar to indomethacin drug treated group (group III) during analysis i.e. 7.1 mg/g tissue. Lipid peroxidation value (TBARS) was high in positive group whereas decreased lipid peroxidation was found in standard and proteoglycans treated especially, 200 mg/Kg PGs treated group. Increased levels of anti-oxidant parameters such as SOD, catalase and GSH and decreased lipid peroxidation results were observed for both indomethacin and high concentration i.e. 200 mg/Kg PGs treated group. Whereas contrast results were noticed in group II, positive group. Oxidative stress develops due to loss of balance between reactive oxygen species (ROS) and anti-oxidant defence system. This results in the breakdown of functional macromolecules in the body and cellular damage (Betteridge, 2000; Jones, 2008). The anti-oxidant system always detoxifies unwanted ROS and maintains proper environment for cellular functioning. Anti-oxidant defence system of non-enzymatic anti-oxidants include enzymatic α-tocopherol, ascorbic acid, paraoxonases, reduced glutathione (GSH) and carotenoids and enzymatic anti-oxidants such as catalase (CAT), glutathione peroxidise (GPX), NADPH ubiquinone oxidoreductase (NQO1), superoxide dismutase (SOD) (Gibson *et al*., 2009; Fattman *et al*., 2003). This system is actively involved in the scavenging of free radicals and maintenance of the proper cellular potential (Trachootham et al., 2008).

The development of OA is well correlated with the increased free radical production and thus leads to articular cartilage degradation. Loss of oxidative balance at joints cause the inhibition of cartilage matrix formation and leads to

acceleration of cartilage degradation. (Henrotin *et al*., 2005; Aigner *et al*., 2006; Altindag *et al*., 2007). Role of anti-oxidant defence system is well studied in monosodium iodoacetate-induced osteoarthritic condition and in screening of osteoarthritic treatment. It was reported that drug like atorvastatin, a HMG-CoA reductase inhibitor can be effectively used to prevent cartilage degradation by alleviating the oxidative stress. This study reveals the importance of active anti-oxidant defence system to reduce osteoarthritic pain development. (Pathak *et al*., 2015). The pathogenesis of osteoarthritis is complicated and several factors are involved in it. Many researchers demonstrated the role of ROS in cartilage erosion at the jonts (Valko *et al*., 2007; Griffiths, 2005). ROS damage the equilibrium between anabolic and catabolic processes of the cartilage and activate many metalloproteinases eventually leading to cartilage degradation (Liu and Malik, 2006). Chondrocytes produce a wide variety of antioxidant defences against the free radicals with the help of CAT, GPX and SOD. Decreased anti-oxidant enzymes facilitate the rise in lipid peroxide formation which creates loss of homeostasis in the joints and leads to articular cartilage degradation (Poole, 2002). Normal chondrocytes are known for strong anti-oxidant system that includes CAT, SOD and GP_X (Matsumoto *et al*., 1991). There is a decrease in the expression and activity of these anti-oxidant enzymes in osteoarthritic cartilage which indicates the importance of strong anti-oxidant system to resist OA development (Scott *et al*., 2010). Above reports emphasize the active anti-oxidant system to prevent osteoarthritic progression. In this study PGs treated sample has showed increased anti-oxidant parameters like SOD, Catalase and GSH. It indicated the role of PGs isolated from deep sea shark cartilage to boost the anti-oxidant defence system to alleviate osteoarthritic progression.

6.3.3 Histopathology and X-ray

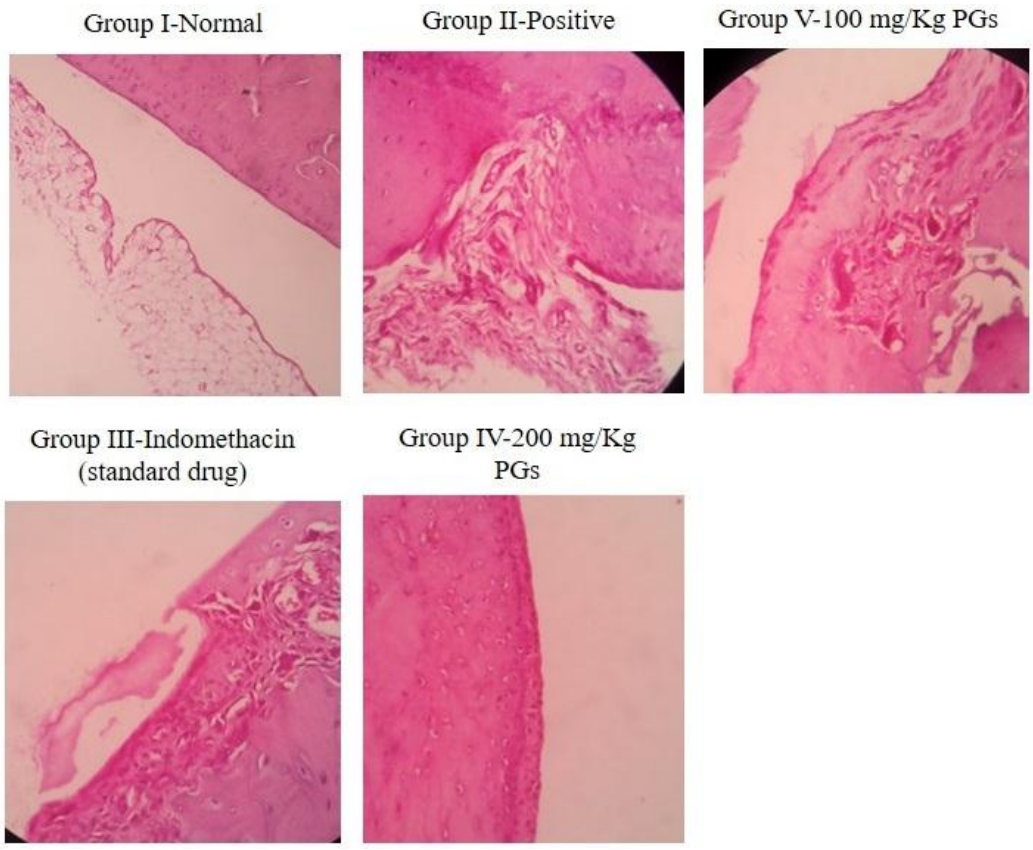

Fig. 6.5 Histopathology analysis of joint tissue of experimental rat groups

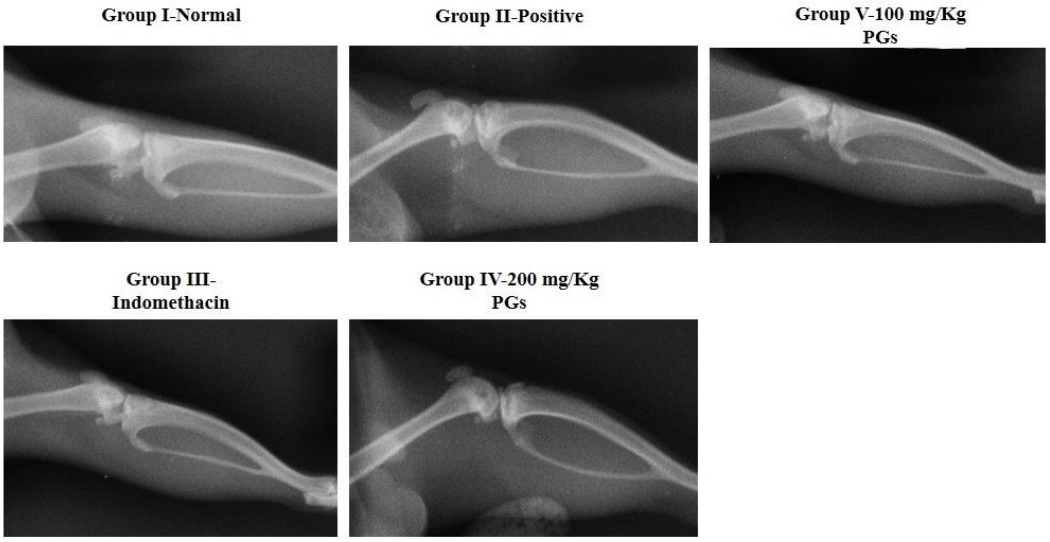

Fig. 6.6 X-ray images of joint tissue of experimental rat groups

Figure 6.5 illustrates the histopathology analysis of joint section of articular cartilage of experimental rats. In normal control group, rats show joint with preserved surface articular cartilage having chondrocytes organised into tangential, transitional and radial layers. Underlying trabecular bone is seen with interspersed fibroadipocytic marrow. Cartilage erosion was absolutely absent in histopathology analysis. Positive group (group II) sections show joint with few preserved foci of surface articular cartilage having increased chondrocytes in disarray. Extensive areas of erosion of cartilage were noted with increased vascularization of cartilage. Underlying trabecular bone was seen with interspersed fibroadipocytic marrow. In standard drug indomethacin treated group (group III), joint with few preserved foci of surface articular cartilage having chondrocytes organised into tangential, transitional and radial layers was seen. Limited areas of erosion of cartilage were noted with slightly increased vascularization and disarray in organisation of chondrocytes in cartilage. Underlying trabecular bone was seen with interspersed fibroadipocytic marrow. In 200 mg/Kg PGs treated group show joint with thinning of surface articular cartilage and disarray of chondrocytic organization. Less loss of articular cartilage or neovascularisation of cartilage was seen. Underlying trabecular bone seen with interspersed fibroadipocytic marrow. In 100 mg/Kg PGs treated animal (group V), sections show joint with surface articular cartilage having chondrocytes in disarray. Slight increase in areas of erosion of cartilage was noted with increased vascularization of cartilage. Underlying trabecular bone was seen with interspersed fibroadipocytic marrow.

Figure 6.6 depicted the X-ray analysis of joint section of articular cartilage of experimental rats. In case of X-ray analysis joint space appears normal and articular surface appears for normal group (group I) which indicate the functional joint anatomy. Whereas narrowed joint space and asymmetry were evident in positive group (group II). Clear sclerosis at articular joint and cyst like lucensies noted involving proximal bone for this group. These observations are specific for severe osteoarthritic condition. In all the remaining groups including group III, IV

and V narrowed joint space was noticed but reduction in the sclerosis and cyst like lucensies were are not seen in these groups.

Severity of arthritis can easily be recognised by microscopic visualization of histological changes of joint especially the articular cartilage. The major changes accounting for joint damage are synovial hyperplasis, cartilage erosion, well arranged chondrocytes arrays, preserved foci of surface articular cartilage and infiltration of inflammatory cells into synovium. In this study, such changes were prominent in positive group (group II) whereas better protected joint morphology ie., decreased histological evidence was observed for group IV and is in good similarity with indomethacin treated group (group III). Observed findings in this study agreed with previous reports (Chun *et al*., 2016; Cifuentes *et al*., 2010). X-ray images of joints provide vital information regarding the internal structure in osteoarthritis with respect to synovial space narrowing, increased bone intensity, cyst appearance etc. Preliminary evidence of decreased osteoarthritic progression was noticed for high dose PGs treated group (group IV) which agreed with histopathological and X-ray results.

6.3.4 PAGE, Western blotting and Expression studies

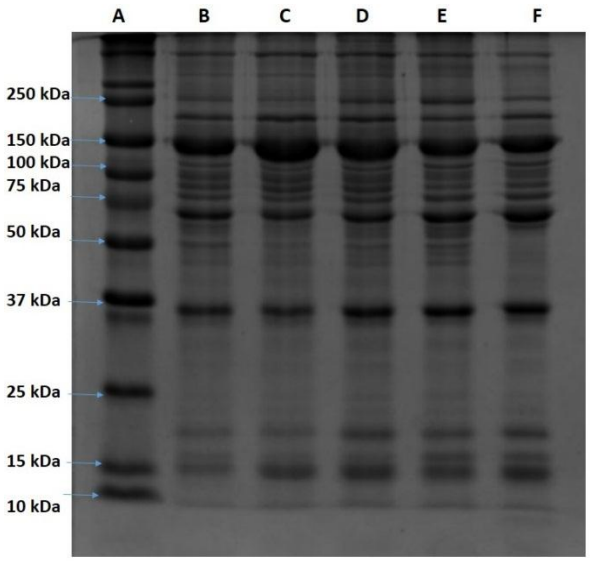

Fig. 6.7 PAGE of joint protein of experimental rat groups A) Wide range molecular marker B) Normal group C) Positive group-II D) Indomethacin treated-group III E) 200 mg/Kg PGs treated – group IV F) 100 mg/Kg PGs treated – group V

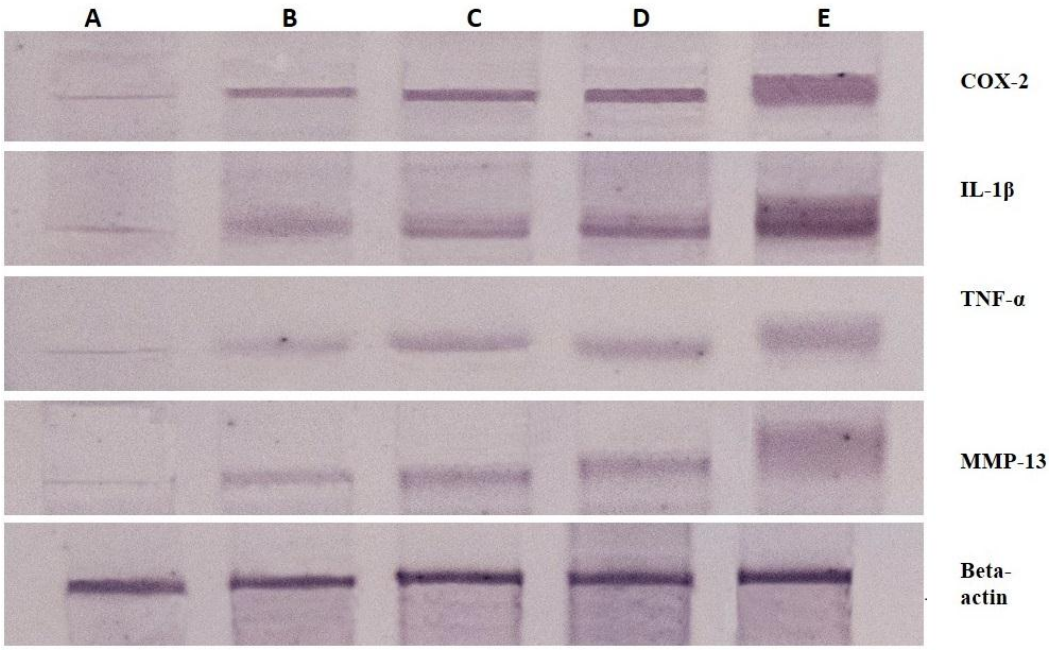

Fig. 6.8 Western blot analysis of inflammatory markers of joint protein in experimental rats, COX-2, IL-1β, TNF-α, MMP-13, β-actin of experimental rat groups A) Normal group B) Indomethacin treated-group III C) 200 mg/Kg PGs treated – group IV D) 100 mg/Kg PGs treated – group V E) Positive group-II

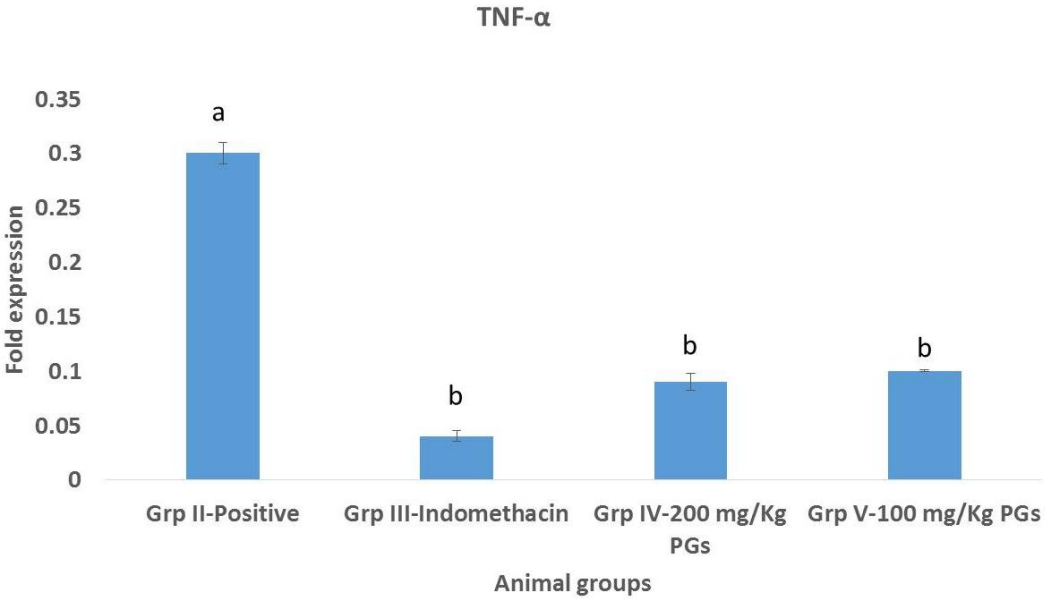

Fig. 6.9 mRNA fold expression of TNF-α (Tissue necrotic factor- α) gene of experimental rat groups. Treatment means with different letters (a, b) indicate significant difference ($p< 0.05$)

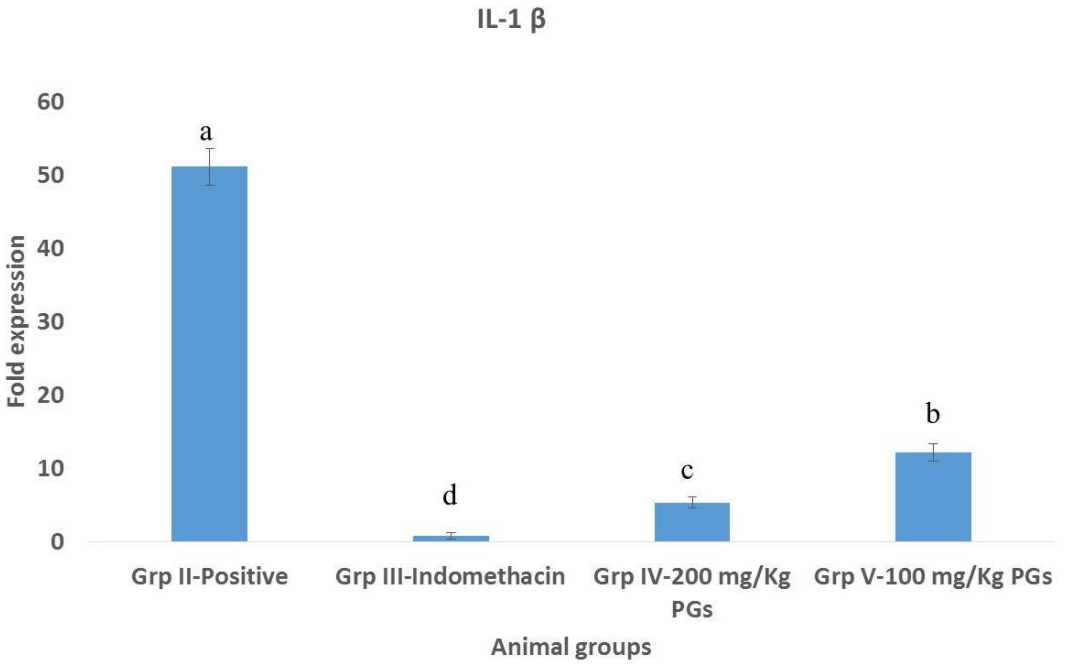

Fig. 6.10 mRNA fold expression of IL-1 β (Interleukin 1 beta) gene of experimental rat groups. Treatment means with different letters (a, b, c, d) indicate significant difference ($p< 0.05$)

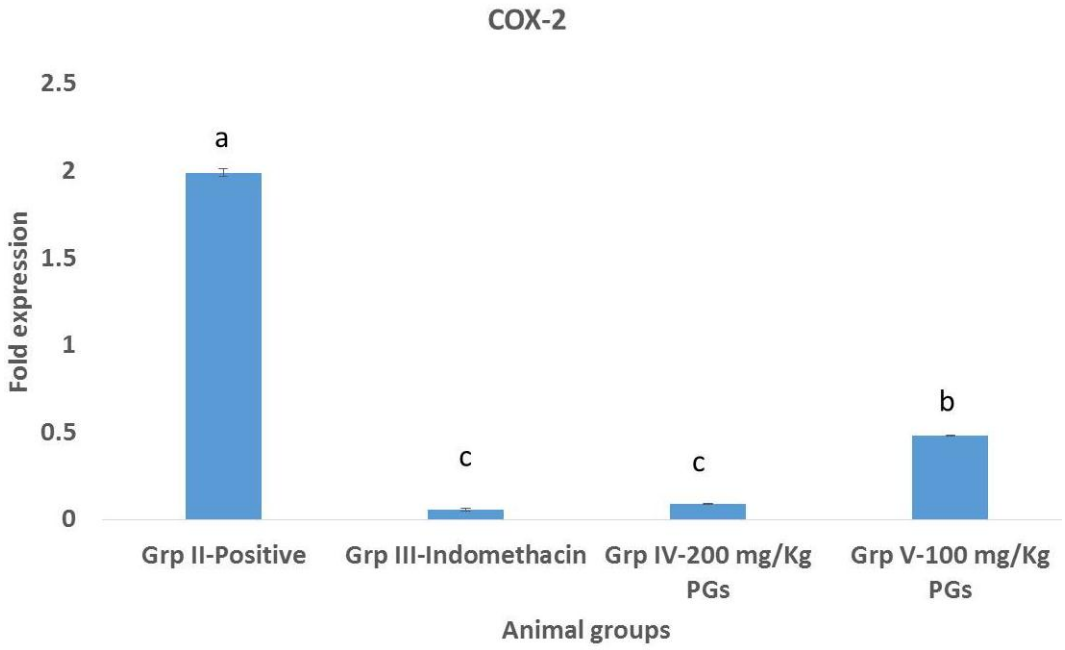

Fig. 6.11 mRNA fold expression of COX-II (cyclooxygenase-2) gene of experimental rat groups. Treatment means with different letters (a, b, c) indicate significant difference ($p< 0.05$)

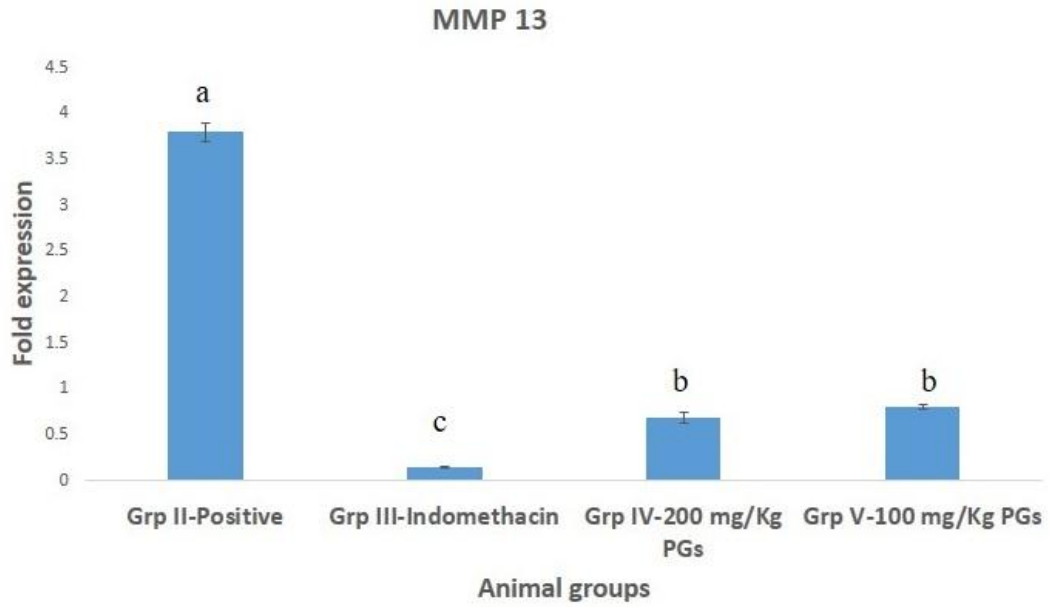

Fig. 6.12 mRNA fold expression of MMP-13 (Metalloproteinase-13) gene of experimental rat groups. Treatment means with different letters (a, b, c) indicate significant difference ($p< 0.05$)

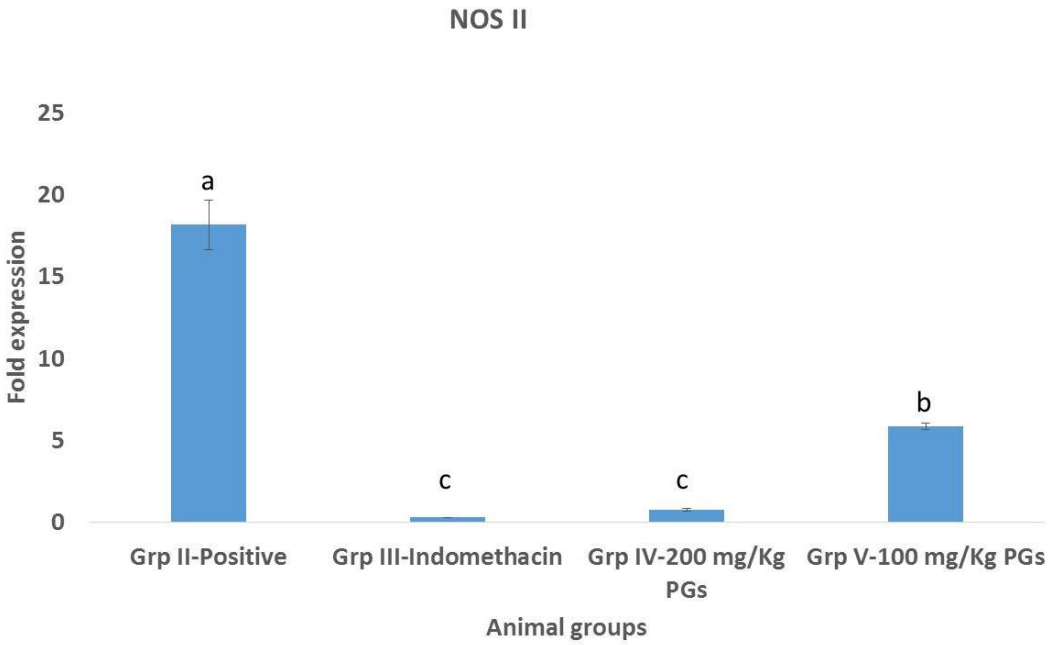

Fig. 6.13 mRNA fold expression of NOS II (nitric oxide synthase II) gene of experimental rat groups. Treatment means with different letters (a, b, c) indicate significant difference ($p< 0.05$)

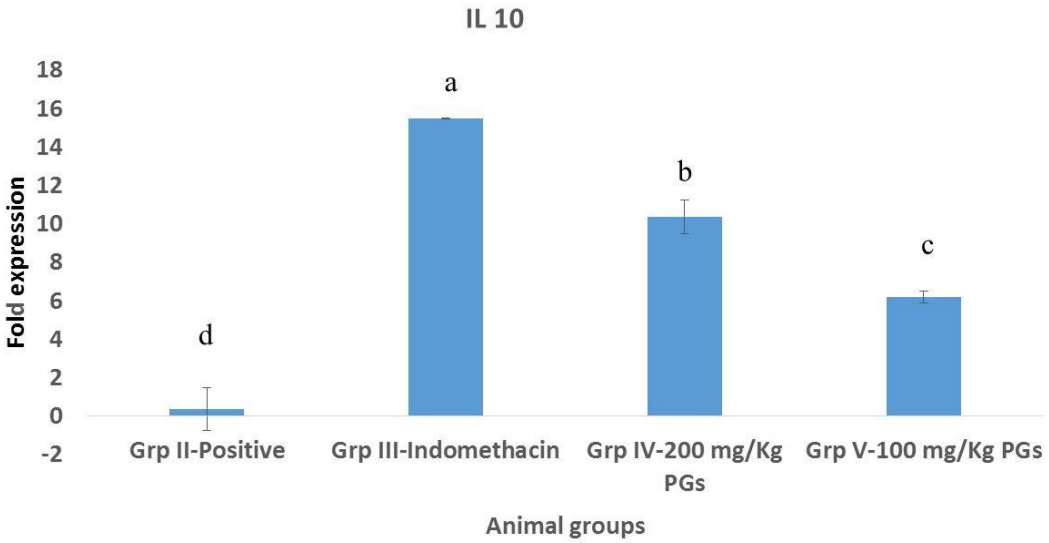

Fig 6.14 mRNA fold expression of IL-10 (Interleukin-10) gene of experimental rat groups. Treatment means with different letters (a, b, c, d) indicate significant difference ($p< 0.05$)

Fig. 6.7 illustrated protein pattern of joint in different groups. Clear change in protein pattern was evident in PAGE and it indicates the up and down production of various protein rather inflammatory mediators at the joint during the course of the study. Fig. 6.8 summarized western blot analysis of inflammatory markers (TNF- α, IL-1β, COX-2 and MMP-13) in the rat knee joint tissues of each groups. Western blotting analysis of inflammatory markers of osteoarthritic disease helps to study the status of the OA disease. There are clear visible changes in the inflammatory markers such as TNF- α, IL-1β, COX-2 and MMP-13. An increase in inflammatory markers was observed in MIA-induced groups i.e., positive group (group II) and decreased levels were observed in indomethacin treated (group III) and 200 mg/Kg PGs treated group (group IV) showed promising response when compared to standard drug. In 100 mg/Kg PGs treated group (group V) also decrease in inflammatory markers was observed but less than that of 200 mg/Kg PGs treated group (group IV).

Figure 6.9, 6.10, 6.11, 6.12, 6.13 and 6.14 depicted the mRNA expression analysis of inflammatory markers such as TNF- α, IL-1β, COX-2, MMP-13, NOS II and IL-10 respectively. Expression analysis of inflammatory mediators in rat

knee joint tissues such as MMP-13, IL-1β, TNF-α, IL-10, COX-2 and NOS-II revealed promising effect of proteoglycans against osteoarthritis. Fold expression of major enzyme inflammatory mediators such as MMP-13, COX-2 and NOS-II were decreased in PGs treated sample and was similar to standard drug treated group. This helped to decrease the arthritic symptoms in the joints. Other important inflammatory proteins like IL-1β, TNF-α expression were clearly decreased in 200 mg/Kg PGs treated group (group IV) and similar trend was observed in indomethacin treated group (group III). The above results suggest the protective effect of shark proteoglycans against osteoarthritic development.

Inflammatory markers play a key role in the development of osteoarthritic condition and associate pain generation. Increased number of macrophages are commonly seen in the arthritic joints and they are responsible for the production of the most severe pro-inflammatory cytokine which causes articular cartilage degradation called TNF-α. TNF-α is further involved in the recruitment of other inflammatory cells into the synovium and trigger the production of other prominent pro-inflammatory cytokines like IL-1β. IL-1β accelerates the production of certain metalloproteinsases mainly MMP-13 in osteoarthris which causes the degradation of articular cartilage and finally joint destruction (Arend and Dayer, 1995). Certain proinflammatory agents called prostaglandins mainly PEG2 derived from arachidonic acid triggers inflammatory responses and pain development in osteoarthritic disease. They are produced by a special type of enzyme known as COX-2 which is activated mainly during inflammatory conditions. Alleviated expression of COX-2 produces many fold PEG2 than in normal condition and is the main reason behind arthritic symptoms (Braza-Boils *et al*., 2011).

Tracking the presence of these inflammatory proteins could help to understand the status of osteoarthritis with or without treatment. Indomethacin is a non-selective inhibitor of COX-2 thus reducing the production of PGE2 levels in joints. Studying the levels of inflammatory proteins such as TNF- α, IL-1β, COX-2 and MMP-13 in normal group (group I), positive (group II), standard drug

indomethacin treated (group III)and PGs treated groups (group IV & V) will help to assess comparison of the effectiveness of the treatment. Improvement in the articular cartilage in arthritic condition by the down-regulation of COX-2 expression and PGE2 production has been reported. This finding emphasises the importance of inhibition of COX-2 expression and successive production of PEG2 in osteoarthritic treatment (Park *et al*., 2009). In this study both western blot analysis and mRNA expression studies revealed the reduction in the COX-2 in PGs treated groups (group IV & V) especially in 200 mg/Kg PGs treated group. This work has good accordance with the above reports. So alleviation of osteoarthritic inflammation observed in this study may be due to the inhibition of COX-2 by PGs administration.

IL-1β and TNF-α are actively involved in the cartilage degradation by recruiting other inflammatory mediators. The available data suggests the importance of targeting these proinflammatory mediators in osteoarthritic treatment (Chevalier *et al*., 2013). IL-1β is more potent inflammatory cytokine since it triggers the production of many other inflammatory mediators and proven most important target for anti-osteoarthritic drugs (Pelletier *et al*., 1997; Fernandes *et al*., 1999). Previous studies reported that both IL-1β and TNF-α cause chondrocyte damage by inducing necrosis and apoptosis (Caramés *et al*., 2008). Previous reports confirmed the induction of necrosis of chondrocytes thus worsening the arthritic condition by synergic action with TNF-α (Caramés *et al*., 2008; López-Armada *et al*., 2006). Exact mechanism of induction of death of chondrocytes by apoptosis and triggering of necrosis by IL-1β and TNF-α is not fully understood, but involvement of NO is well documented in such cases (Vuolteenaho *et al*., 2002; Pelletier *et al*., 1996).Western blot analysis and mRNA expression studies showed decrease of TNF-α in PGs treated groups (group IV & V) especially in 200 mg/Kg PGs treated group. So reduction of inflammatory symptoms in proteoglycans treated rats may be due to the inhibition of TNF-α production at the knee joint area and hence anti-osteoarthritic response observed in this study

Interleukin-1β (IL-1β), is found to accelerate degradation of extracellular matrix components by promoting the synthesis of metalloproteinases. Thus formed metalloproteinases degrade cartilage proteins resulting in arthritic condition (Arend and Dayer, 1995). Studies reported the active role of nitric oxide in the degeneration of joints affected by osteoarthritis. Nitric oxide was found to accumulate in the arthritic joints and the production is triggered by proinflammatory cytokine IL-1β (Farrell *et al*., 1992). Correlation between decreased inflammatory mediators expression and reduction in osteoarthritic symptoms has been reported in previous studies. Decreased expression of TNF-α, IL1-β and metalloproteinases was found to decrease the inflammation in the joints of MIA induced osteoarthritic animal models (Jeong *et al*., 2015). Western blotting and mRNA expression results showed low expression of IL1-β in indomethacin treated group (group III) and 200mg PGs/Kg treated groups (group IV). This results are supported by the previous works reported above. So anti-osteoarthritic effect observed in the proteoglycans treated animals may also be due to the decreased expression of IL1-β.

Increased expression of iNOS by IL-1β TNF-α and other factors was documented which ultimately produce high amount of NO affecting the physiology of joint cartilage (Abramson, 2004). Increased NO production subsequently leads to the synovial death and negatively affect the smooth functioning of joints (Maneiro *et al*., 2005). Drugs which inhibit the production of NO by downregulating iNOS gene expression has found attention and has been reported in MIA-induced osteoarthritic rat model study. Thus decreased production of NO resulted in less cartilage degradation and retained better cartilage structure (More *et al*., 2013). In this study high concentration proteoglycans treatment ie, 200mg PGs/Kg reduced the expression COX-2, TNF-α and IL1-β. Similarly NO expression was also decreased. As mentioned previously TNF-α and IL1-β favour the production of NO by activating iNOS gene. So decreased TNF-α and IL1-β could be the reason behind decreased expression of iNOS gene. Thus proteoglycans might have reduced the progression of osteoarthritis in rats.

Matrix metalloproteinases (MMPs) belong to the class of zinc-dependent proteases with several roles and mainly participate in breakdown of multiple extracellular matrix (ECM) proteins. MMP-13, also called collagenase-3 produced by macrophages at the site of inflammation, degrades various proteins especially cartilage proteins such as collagen I, II, II, IV, aggrecan, fibronectin, gelatin, perlectan etc. (Mittal *et al*., 2016; MacColl and Khalil, 2015). Studies reported over expression of MMP-13 in the cartilage tissue of osteoarthritis affected patients. During onset of osteoarthritis, many fold expression of MMP-13 occurs at the chondrocytes leading to the development of arthritis at joints. Low MMP-13 expression is observed in normal cartilage tissue and this finding has helped to develop drugs for osteoarthritis targeting MMP-13. Reduction in the MMP-13 expression in osteoarthritic cartilage indicates the improvement in joint regeneration and decreased complications of articular cartilage (Wang *et al*., 2015; Yu *et al*., 2015; Mashimo *et al*., 2016). These results have good accordance with the above report. Similar to indomethacin treated group (group III) decreased MMP-13 expression was observed in 200mg PGs/Kg treated groups (group IV). Proteoglycans used in this study is found to prevent the expression of MMP 13 thus protecting articular cartilage structural integrity.

Interleukin-10 (IL-10) is an important cytokine well known for its role in chondrocyte protective activity (Hulejova *et al*., 2007). IL-10 acts as a simulator of chondrocyte proliferation and helps to resist articular cartilage degradation in osteoarthritic condition (Jung *et al*., 2013). Meegeren *et al*., 2013 reported the decreased articular cartilage degeneration by the administration of IL-10 in mice models. Possible mechanism of action of IL-10 would be the stimulation of production of inhibitors of metalloproteinases and IL1-β (Lacraz *et al*., 1995). Anti-osteoarthritic effect of IL-10 has been demonstrated in human trials and this knowledge suggests the importance of measuring IL-10 levels to confirm the anti-osteoarthritic effect (Helmark *et al*., 2010). It was reported that IL-10 could possibly help to resist cartilage degradation and extracellular matrix destruction by inhibiting the GAG release from cartilage and down regulating the metalloproteinases production (Behrendt *et al*., 2016; Rojas-Ortegaa *et al*., 2015).

In this experiment we observed increased production of chondroprotective IL-10 production in proteoglycans treated group. So anti-osteoarthritic effect of proteoglycans was confirmed by this result.

O´Driscoll, 1998 demonstrated proteoglycan erosion and cartilage degradation in osteoarthritis. So any compound enhancing the production of proteoglycans or resisting proteoglycan degradation could be a promising biomolecule for the treatment of osteoarthritis. Other works have also signified the better regeneration of osteoarthritic cartilage by the administration of GAG rich supplements such as chondroitin sulfate or glycosamine sulphate in animal arthritic model studies (Handl *et al*., 2007; Reginster *et al*., 2001; Piperno *et al*., 2000). In this anti-osteoarthritic study we observed reduction in the inflammation and better joint recovery by oral supplementation of proteoglycans. We also witnessed decrease in CRP, uric acid, pro-inflammatory mediators, inflammatory enzymes, anti-inflammatory cytokine IL-10, better anti-oxidant status and better histopathological evidence of articular cartilage attributed to decrease in osteoarthritic progression in proteoglycans treated groups.

6.4 Conclusion

Compounds which are able to alleviate the production and counteract the action of inflammatory mediators in osteoarthritis get increasing attention in the treatment of OA. Progression of osteoarthritis is characterized by degradation of cartilage. Reasons behind cartilage degradation include loss of ant-oxidant activities of GSH, SOD, catalase, increase of CRP, uric acid in serum, increased lipid peroxidation, production of inflammatory mediators like IL-1β, TNF-α, MMP-13, COX-2 and decreased production of anti-inflammatory cytokine IL-10. Synthetic drugs like NSAIDs are commonly using to reduce arthritic symptoms but are known for side effects. In this experiment proteoglycans treatment to osteoarthritis induced rat revealed promising anti-osteoarthritic effect. Anti-oxidant defence system parameters like SOD, catalase and GSH was activated, at the same time lipid peroxidation, CRP and uric acid level significantly reduced.

More importantly inflammatory mediators expression clearly decreased and anti-inflammatory cytokine IL-10 level raised in proteoglycans treated groups.

Recently natural bioactive molecule got attention to treat arthritis due to seldom or no side effects and natural biomolecules like proteoglycans could possibly restore the structural and physiological integrity of joint especially in the articular cartilage by decreasing arthritic progression. Both structural and biochemical results obtained in this study substantiated the relevance of application of proteoglycans in osteoarthritic treatment. Prolonged usage of this biomolecule could be an ideal option to reduce the osteoarthritic progression and could offer relief to suffering patients.

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Chapter 7

Study on effect of proteoglycans from deep sea shark *Echinorhinus brucus* cartilage on proteome variation in monosodium iodoacetate induced osteoarthritis knee joint in Wistar albino rats

7.1 Introduction

Osteoarthritis (OA) is a leading joint degrading disease that mainly affects elderly people. It is characterized by the erosion of cartilage tissues at the site of joints due to variation in the extracellular matrix components and loss of chondrocytes (Goldring and Goldring, 2004). Osteoarthritis is characterized by articular cartilage degradation, which is assumed to be caused by loss of balance between anabolic and catabolic processes in joint tissues (Wieland *et al*., 2005). Activation of catabolic processes in articular cartilage is associated with triggering of a complex network of inflammatory factors, including proteolytic enzymes such as metalloproteinases (MMPs), whose up-regulation is boosted by proinflammatory cytokines such as IL-1β or TNF-α (Wojdasiewicz *et al*., 2014). Even though the occurrence of OA is very common, treatment is mainly focussed on alleviation of pain and inflammation (Karsdal *et al*., 2013).

Proteome was originally referred as the total protein present in a cell or a tissue. The term proteome was coined by Wilkins in 1994 (Wilkins *et al*., 1996). Several proteins expressed during onset of osteoarthritis and protein biomarkers

signals present in osteoarthritis can be traced by proteomics techniques. Proteomic techniques have the advantage of providing extra levels of understanding to the progression of osteoarthritic disease. Liquid chromatography coupled to tandem mass spectrometry (LC–MS) has been proven to be the most effective technique for proteome identification in biological samples and recently, has been widely employed for mass identification of complex protein mixtures (Schiess *et al*., 2009).

Variety of mass spectrometry (MS)-based proteomic techniques have been employed to study proteome of diseased and normal cases (Kim *et al*., 2011, Waarsing *et al*., 2011; Dreier, 2010; Heinegård and Saxne., 2011). Mass spectroscopy technique was used to characterize proteome of synovial fluid of osteoarthritic patients and revealed numerous bioamarker occurrence (Dreier *et al*., 2010; Heinegård and Saxne, 2011; Ruiz-Romero and Blanco, 2010). Removal of high abundant proteins helps to detect the lower abundance proteins which may be vital in understanding OA progression and also for biomarker identification. Some studies indicated the importance of removing high abundance proteins but removal of high abundant proteins like albumin, being a carrier protein, causes the loss of many important proteins (Heinegård and Saxne, 2011; Schiess *et al*., 2009; Brewis and Brennan, 2010; Neilson *et al*., 2011).

Monosodium iodoacetate (MIA)-induced animal model is the most appropriate model to study osteoarthritis. MIA is a protein inhibitor which inhibits glycosaminoglycan and proteoglycan biosynthesis by inhibiting enzymes involved in their production and leads to osteoarthritic development (Buchmann and Kalbhen, 1985). There are no studies is reported on proteome variation in anti-osteoarthritic effect of proteoglycans in MIA induced osteoarthritis in rat model. Proteomics study will help to find out mechanisms of action of compounds in anti-osteoarthitic effect at molecular level. So this work was aimed at studying the proteome in the rat model of MIA induced osteoarthritis and the effect of PGs isolated from deep shark *Echinorhinus brucus*, cartilage.

7.2 Materials and Method

7.2.1 Animal experiment

7.2.1.1 Evaluation of *in vivo* anti-osteoarthritis activity

Wistar strain female albino rats (200-250 g) were used for this experiment. They were housed individually in polyurethane cages under hygienic conditions, maintained at room temperature (28 ± 2^{o} C) and provided food and water ad libitum. Animal experiments were performed as per the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India and approved by the Institutional Animal Ethics Committee (IAEC), Central Institute of Fisheries Technology (ICAR-CIFT), Cochin, Kerala, India.

7.2.1.2 Induction of OA with MIA in rats

The animals were randomized and assigned to treatment groups before the initiation of the study (n = 6 per group). MIA solution (3mg/50 μL of 0.9% saline) was directly injected into the intra-articular space of the right knee under anaesthesia induced with a mixture of ketamine and xylazine. Rats were divided randomly into four groups:

(1) Normal group with no MIA injection : OA 1

(2) Positive group with MIA injection with no treatment : OA 2

(3) Indomethacin- (IM) treated group (2 mg/kg) with MIA injection : OA 3

(4) 200 mg/kg PGs treated group with MIA injection : OA 4

Rats were administered orally with PG_S and indomethacin for 41 days after inducing osteoarthritis with MIA.

7.2.2 Sample preparation for proteomics

Protein extraction from joint tissue was done by the method of Onnerfjord *et al*., 2012. 100 mg freeze dried tissue from each group digested with 15 times volume of chaeotropic extraction buffer (4 M guanidium-HCl, 50 mM sodium

acetate, 5 nM benzamidine-HCl, 5 mM iodoacetamide, pH-5.8) for 48 h on an orbital shaker at. After digestion samples were centrifuged at 15000 rpm at for 15 mins. Supernatent was collected and protein was quantified by nanodrop machine. 100 µg protein from each group was taken for LC-MS/MS analysis.

7.2.3 Proteomics Analysis

The proteomic protein profiling and relative quantification analysis is performed by liquid chromatography tandem mass spectrometry (LC-MS/MS) as detailed below.

7.2.3.1 In-solution trypsin digestion of rat joint proteins

Approximately 100 µg of proteins from each sample, normalised to a concentration of 1µg/µl, was subjected to in-solution trypsin digestion to generate peptides. Disulfide bonds were reduced by incubation of proteins with 100 mM dithiothreitol in 50 mM ammonium bicarbonate for 30 min at 60^0C. After cooling at room temperature for 5 min, 200 mM iodoacetamide in 50 mM ammonium bicarbonate was added to perform alkylation step in dark at room temperature for 30 min. Proteins were then digested by using sequencing grade modified trypsin (Sigma) at a trypsin: total protein ratio of ~1:25 and incubated for 17 h at 37^0C. The trypsin stock was made in 50 mM ammonium bicarbonate. The enzymatic reaction was stopped by adding formic acid to each sample so that final formic acid concentration was 1.0% and incubated at 37 °C for 20 min. The digested peptide solutions were centrifuged at 20,817 g for 12 min and the supernatant was stored at -20^0C until LC-MS/MS analysis

7.2.3.2 LC-MS analysis of peptides of rat joint proteins

The tryptic peptides were separated using a nanoACQUITY UPLC® chromatographic system (Waters, Manchester, UK). Instrument control and data processing was done with MassLynx4.1 SCN781 software. The peptides were separated by reversed-phase chromatography. The peptide sample was injected in partial loop mode in 5 µL loop (injection volume 3.0 µL).Water was used as solvent A and acetonitrile was used as solvent B. All solvents for the UPLC

system contained 0.1 % formic acid. The tryptic peptides were trapped and desalted on a trap column (Symmetry® 180 µm x 20 mm C18 5 µm, Waters) for 1 min at a flow rate of 15 µl/min. The trap column was placed in line with the reversed-phase analytical column, 75 µm (internal diameter) X 200 mm HSS T3 C18 (Waters) with particle size of 1.8 µm. Peptides were eluted from the analytical column with a linear gradient of 1 to 40% solvent B over 55.5 min at a flow rate of 300 nl/min followed by 7.5 min rinse of 80 % solvent B. The column was immediately re-equilibrated at initial conditions (1% solvent B) for 20 min. The column temperature was maintained at 40^0C. The lock mass, [Glu1]-Fibrinopeptide B human (Sigma) (positive ion mode [M+2H] 2+ = 785.8426) for mass correction was delivered from the auxiliary pump of the UPLC system through the reference sprayer of the NanoLockSpray^{TM} source at a flow rate of 500 nl/min. Each sample was injected in triplicate with blank injections between each sample.

MS analysis of eluting peptides was carried out on a SYNAPT® G2 High Definition MS™ System (HDMS^E System (Waters). The instrument settings were: nano-ESI capillary voltage – 3.4 KV, sample cone - 40 V, extraction cone - 4 V, IMS gas (N_2) flow - 90 (ml/min). To perform the mobility separation, the IMS T-Wave™ pulse height is set to 40 V during transmission and the IMS T-Wave™ velocity was set to 800 m/s. The travelling wave height was ramped over 100% of the IMS cycle between 8 V and 20 V.

All analyses were performed in positive mode ESI using a NanoLockSpray^{TM} source. The lock mass channel was sampled every 45 s. The time of flight analyzer (TOF) of the mass spectrometer was calibrated with a solution of 500 fmole/µl of [Glu^1]-Fibrinopeptide B human (Sigma). This calibration set the analyzer to detect ions in the range of 50 - 2000 *m/z*. The mass spectrometer was operated in resolution mode (V mode) with a resolving power of 18,000 FWHM and the data acquisition was done in *continuum* format. The data was acquired by rapidly alternating between two functions – Function-1 (low energy) and Function-2 (high energy). In Function-1, only low energy mass

spectra (MS) was acquired and in Function-2, mass spectra at elevated collision energy with ion mobility ($HDMS^E$) was acquired. In Function-2, collision energy was set to 4 eV in the Trap region of mass spectrometer and is ramped from 20 eV to 45 eV in the transfer region of mass spectrometer to attain fragmentation in the $HDMS^E$ mode. The *continuum* spectral acquisition time in each function was 0.9 seconds with an interscan delay of 0.024 s.

7.2.3.3 MS Data analysis

The acquired ion mobility enhanced MSE spectra were analysed using Progenesis QI for Proteomics V3.0 (Non Linear Dynamics, Waters) for protein identification as well as for the label-free relative protein quantification. Data processing includes lock mass correction post acquisition. Processing parameters for Progenesis were set as follows: noise reduction thresholds for low energy scan ion – 150 counts, high energy scan ion - 30 counts. The protein identifications were obtained by searching against the *Rattus norvegicus* (Rat) protein database downloaded from UniProt. During database search, the protein false positive rate was set to 4%. The parameters for protein identification was made in such a way that a peptide was required to have at least 1 fragment ion match, a protein was required to have at least 3 fragment ion matches and a protein was required to have at least 1 peptide match for identification. Oxidation of methionine was selected as variable modification and cysteine carbamidomethylation was selected as a fixed modification. Trypsin was chosen as the enzyme used with a specificity of 1 missed cleavage. Data sets were analysed and quantified using Relative Quantitation using Hi-N algorithm, which resolve peptide conflicts and uses the average intensity of the three most abundant unique peptides for a protein. Furthermore, only a fold change higher than 50% difference (ratio of either >0.50 was considered to be indicative of significantly altered levels of expression).

7.2.4 Statistical analysis

One way analysis of variance (ANOVA) was carried out and ANOVA was found significant, tukey's test was performed to compare the treatment means at 5

% level of significance ($p < 0.05$). All the statistical analysis was carried out by using Proc GLM SAS g.3.

7.3 Results and discussion

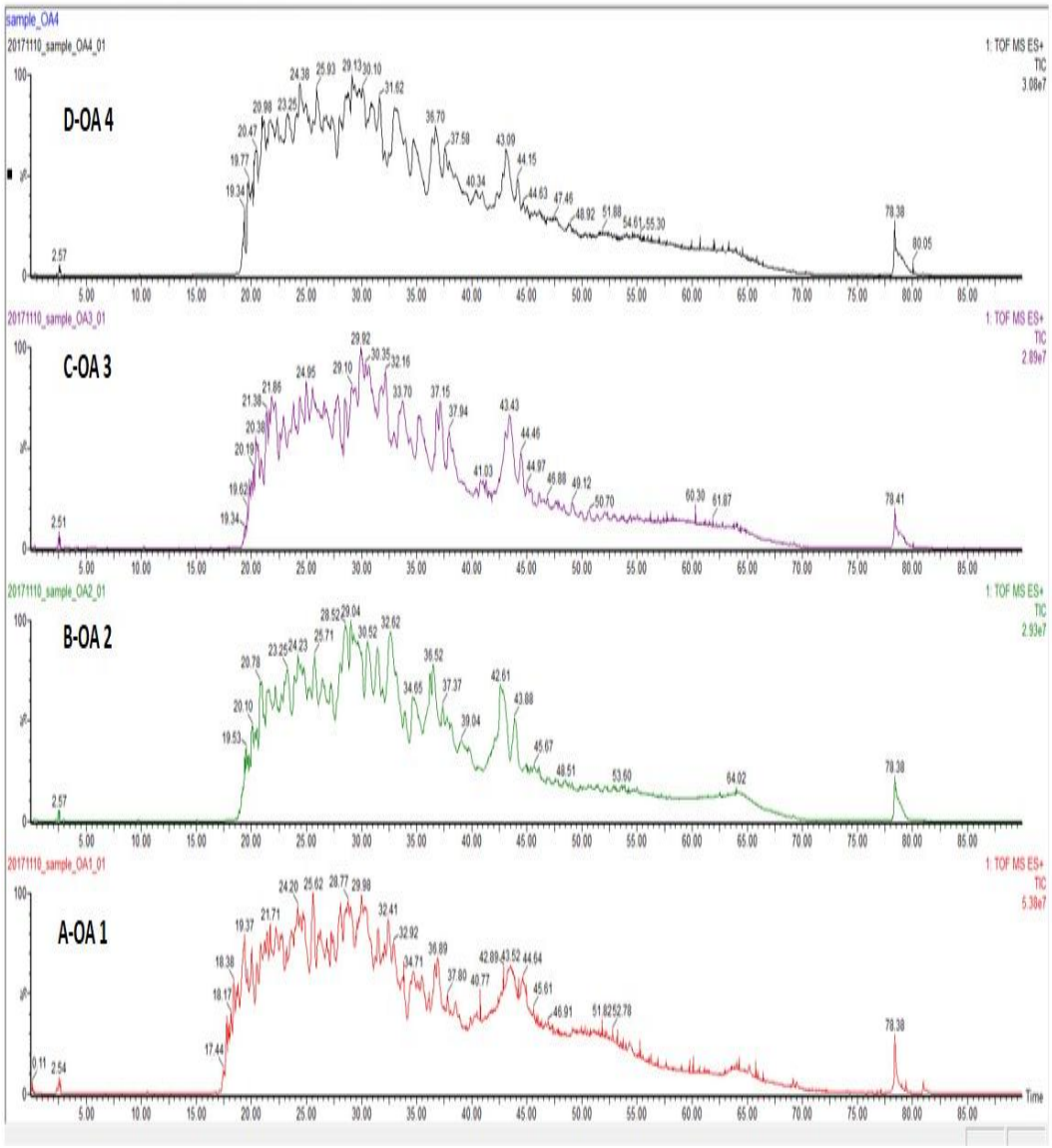

Fig. 7.1: Total peptide spectrum of rat joint proteins four groups A) OA 1 B) OA 2 C) OA 3 D) OA 4

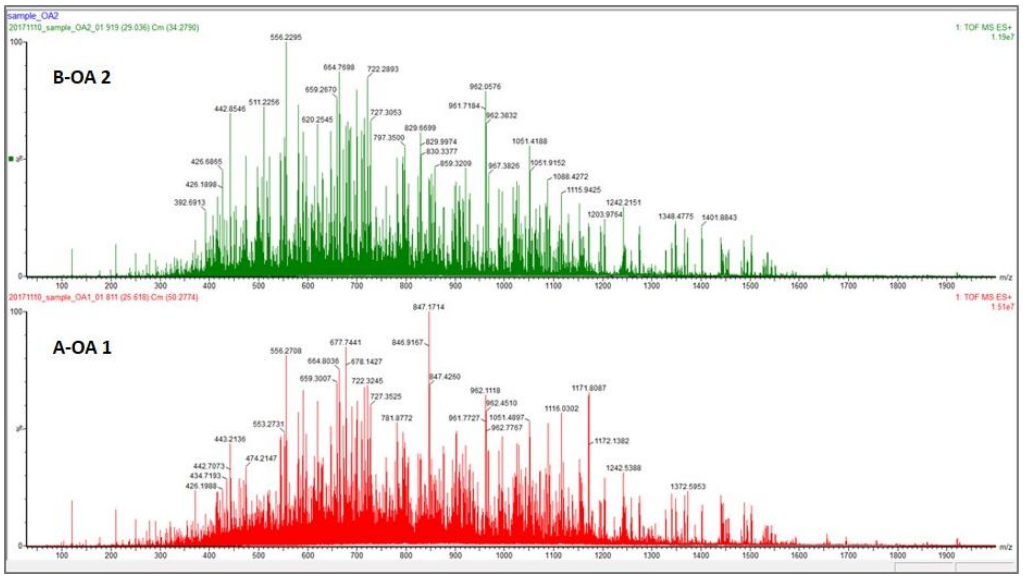

Fig. 7.2: Total peptide spectrum of rat joints group A) OA 1 B) OA 2

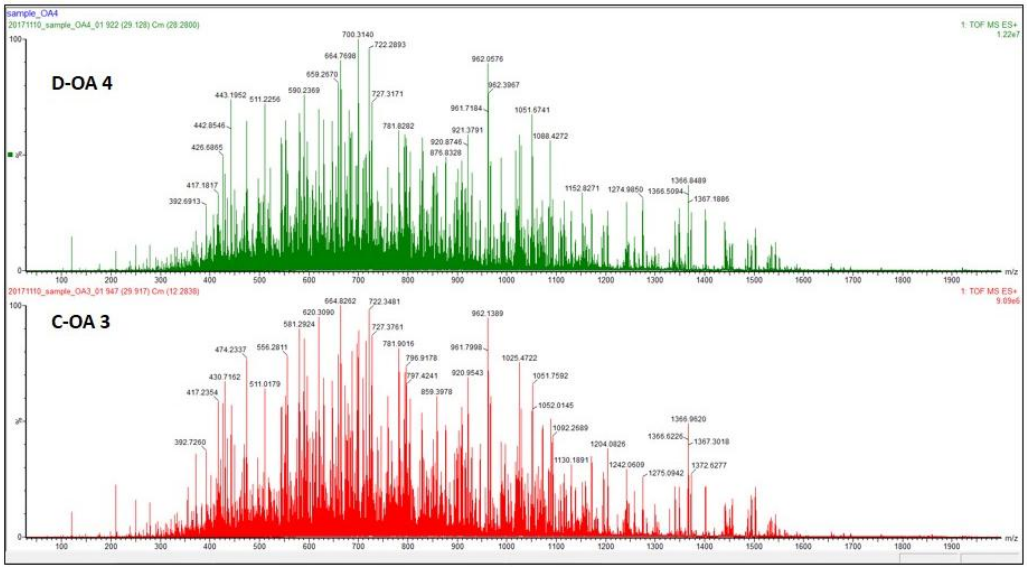

Fig. 7.3: Total peptide spectrum of rat joints group C) OA 3 D) OA 4

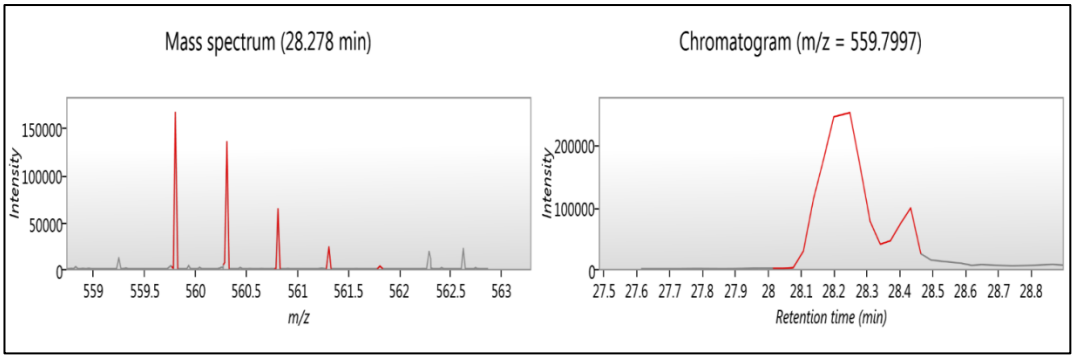

Fig. 7.4 Aggrecan peptide spectrum (m/z 559.799)

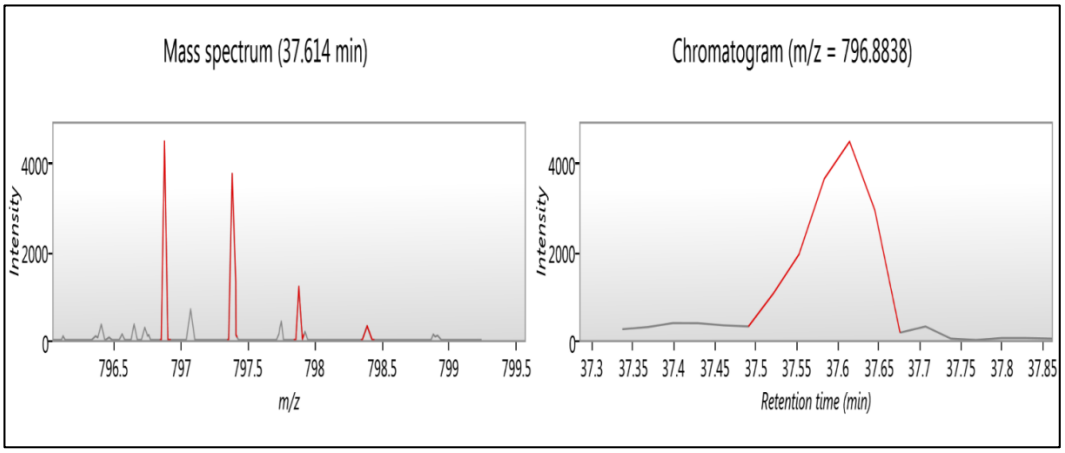

Fig. 7.5: Biglycan peptide spectrum (m/z 559.79)

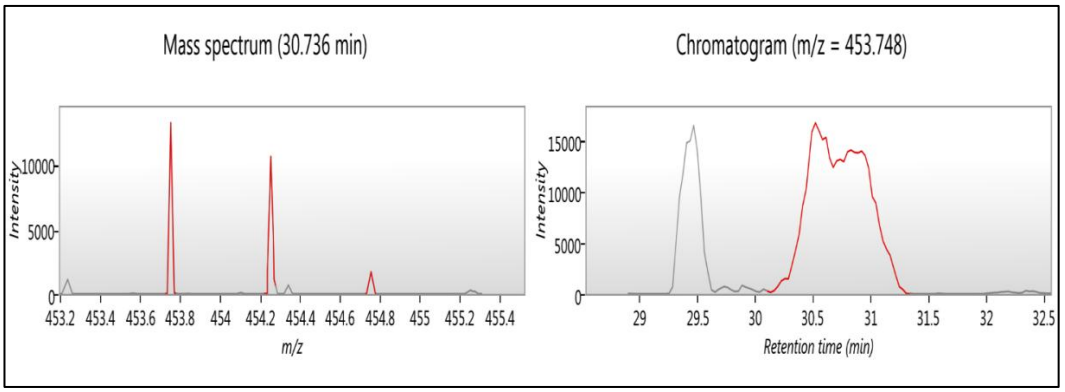

Fig. 7.6: Decorin peptide spectrum (m/z 453.74)

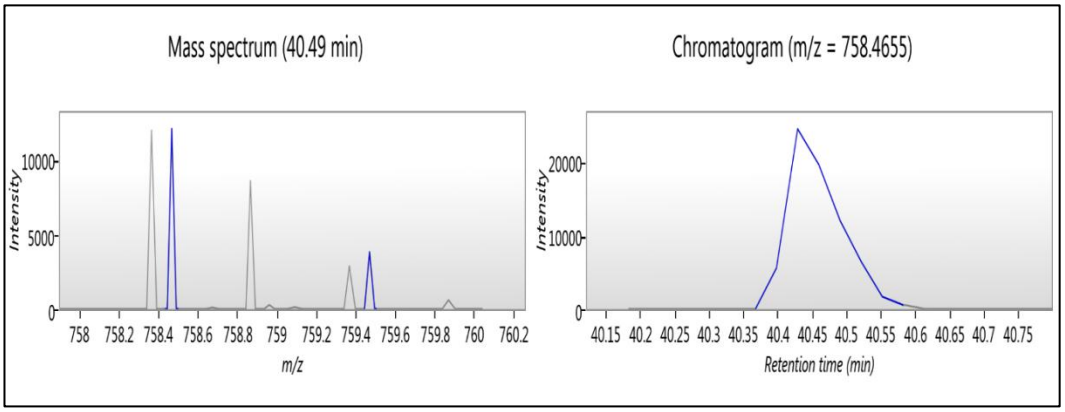

Fig.7.7: Fibromodulin peptide spectrum (m/z 758.46)

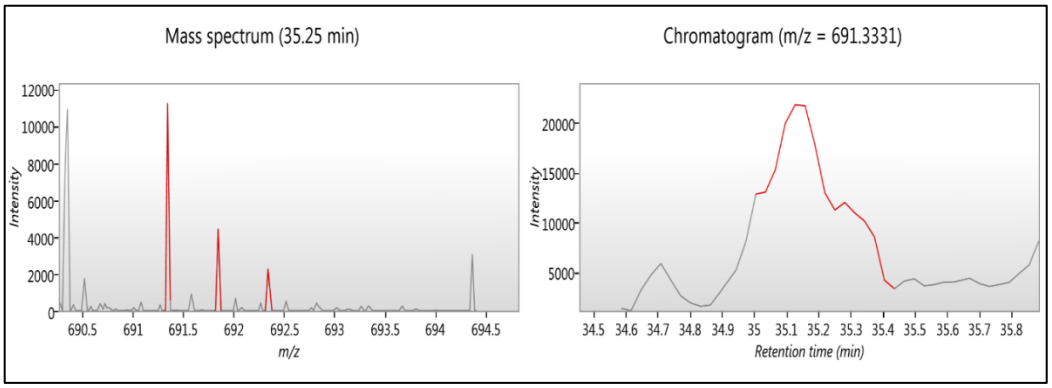

Fig. 7.8: Lumican peptide spectrum (m/z 691.33)

Table 7.1: Proteome comparison, OA 1 VS OA 4

OA 1 VS OA 4

Accession	Peptide count	Unique peptides	Anova (p)	Max fold change	Highest mean condition	Lowest mean condition	Description
P47853	10	10	0.05	5.28	OA4	OA1	Biglycan
P51886	9	9	0.32	1.58	OA4	OA1	Lumican
P07897	15	15	0.06	3.36	OA4	OA1	Aggrecan core protein
Q01129	16	16	0.83	2.94	OA4	OA1	Decorin
P50609	9	7	0.09	3.52	OA4	OA1	Fibromodulin
P20761	5	4	0.91	1.67	OA4	OA1	Ig gamma-2B chain C region
P20760	11	1	0.54	4.43	OA4	OA1	Ig gamma-2A chain C region
P01836	3	2	0.19	1.85	OA4	OA1	Ig kappa chain C region
Q5M842	13	2	0.16	3.51	OA4	OA1	IgG-2a protein
F1LRM7	24	2	0.47	1.02	OA1	OA4	Collagen alpha-1(II) chain
P11232	1	1	0.23	3.90	OA4	OA1	Thioredoxin
P07632	3	3	0.98	2.13	OA4	OA1	Superoxide dismutase [Cu-Zn]
B8K1X9	3	3	0.06	4.34	OA4	OA1	Interleukin 20 receptor beta IL-20
Q91Y94	2	2	0.13	12.05	OA4	OA1	Cytochrome c oxidase subunit 4 isoform 2_ mitochondrial
D3ZD09	2	2	0.09	12.7	OA4	OA1	Cytochrome c oxidase subunit
F1M983	3	3	0.08	7.90	OA4	OA1	Complement factor H

Table 7.2: Proteome comparison, OA 2 VS OA 4
OA 2 VS OA 4

Accession	Peptide count	Unique peptides	Anova (p)	Max fold change	Highest mean condition	Lowest mean condition	Description
P47853	10	10	7.19E-05	2.49	OA4	OA2	Biglycan
P51886	9	9	0.001	1.47	OA4	OA2	Lumican
P07897	15	15	0.004	1.16	OA4	OA2	Aggrecan core protein
Q01129	16	16	0.249	1.09	OA4	OA2	Decorin
P50609	9	7	0.542	1.03	OA4	OA2	Fibromodulin
P20761	5	4	3.53E-05	3.29	OA4	OA2	Ig gamma-2B chain C region
P20760	11	1	0.004	1.59	OA4	OA2	Ig gamma-2A chain C region
P01836	3	2	0.004	1.48	OA2	OA4	Ig kappa chain C region
Q5M842	13	2	0.12	1.10	OA2	OA4	IgG-2a protein
F1LRM7	24	2	0.0001	1.31	OA4	OA2	Collagen alpha-1(II) chain
P11232	1	1	0.0002	3.09	OA4	OA2	Thioredoxin
P07632	3	3	0.29	1.08	OA4	OA2	Superoxide dismutase [Cu-Zn]
B8K1X9	**3**	**3**	0.002	1.94	OA2	OA4	Interleukin 20 receptor beta IL-20
Q91Y94	2	2	0.01	2.46	OA2	OA4	Cytochrome c oxidase subunit 4 isoform 2_ mitochondrial
D3ZD09	2	2	0.15	1.72	OA2	OA4	Cytochrome c oxidase subunit
F1M983	3	3	0.12	1.85	OA2	OA4	Complement factor H

Table 7.3: Proteome comparison, OA 3 VS OA 4
OA 3 VS OA 4

Accession	Peptide count	Unique peptides	Anova (p)	Max fold change	Highest mean condition	Lowest mean condition	Description
P47853	10	10	3.57E-06	7.95	OA4	OA3	Biglycan
P51886	9	9	3.33E-06	1.87	OA3	OA4	Lumican
P07897	15	15	0.09	1.11	OA4	OA2	Aggrecan core protein
Q01129	16	16	6.20E-05	3.56	OA4	OA2	Decorin
P50609	9	7	0.0001	2.04	OA3	OA4	Fibromodulin
P20761	**5**	**4**	0.47	1.07	OA3	OA4	Ig gamma-2B chain C region
P20760	11	1	0.012	1.52	OA4	OA3	Ig gamma-2A chain C region
P01836	3	2	0.348	1.06	OA4	OA3	Ig kappa chain C region
Q5M842	13	2	0.085	1.27	OA3	OA4	IgG-2a protein
F1LRM7	24	2	0.0004	2.08	OA4	OA3	Collagen alpha-1(II) chain
P11232	1	1	0.089	1.40	OA3	OA4	Thioredoxin
P07632	3	3	0.838	1.01	OA4	OA3	Superoxide dismutase [Cu-Zn]
B8K1X9	**3**	**3**	0.264	1.13	OA4	OA3	Interleukin 20 receptor beta IL-20
Q91Y94	2	2	0.0004	8.06	OA3	OA4	Cytochrome c oxidase subunit 4 isoform 2_ mitochondrial
D3ZD09	2	2	0.202	2.55	OA4	OA3	Cytochrome c oxidase subunit
F1M983	3	3	0.031	4.41	OA3	OA4	Complement factor H

Table 7.4: Unique peptide sequences correspond to selected proteoglycans of rat joint proteome

Name (Description)	Natural mass	Unique peptide sequence
Biglycan	1117.58	VVQCSDLGLK
	2413.25	EISPDTTLLDLQNNDISELRK
	2413.24	EISPDTTLLDLQNNDISELRK
	2413.22	EISPDTTLLDLQNNDISELRK
	2285.14	EISPDTTLLDLQNNDISELR
Lumican	1242.65	SLQDLQLANNK
	1380.67	ITNIPDEYFNR
	1380.66	ITNIPDEYFNR
	1380.65	ITNIPDEYFNR
	2508.28	SIPTVNENLENYYLEVNKLEK
Aggrecan core protein	1591.83	NAQDYQWIGLNDR
	1591.74	NAQDYQWIGLNDR
	3162.51	TTRPWGFPEEATRGPDSATAFASEDLVVR
	2310.22	VSLPNYPAIPSDATLEIQNLR
	2310.22	VSLPNYPAIPSDATLEIQNLR
Decorin	905.48	SVFNGLNR
	3340.66	VVQCSDLGLDKVPWEFPPDTTLLDLQNNK
	2126.16	VPWEFPPDTTLLDLQNNK
	2126.07	VPWEFPPDTTLLDLQNNK
	2126.06	VPWEFPPDTTLLDLQNNK
Fibromodulin	1328.66	SAMPVDAPLCLR
	977.535	YLPFVPSR
	977.543	YLPFVPSR
	977.545	YLPFVPSR
	2645.97	DCPQECDCPPNFPTAMYCDNR

Three combinations of comparison was tried to analyse the variation of proteins among each group of rats studied. And the combination were OA1 vs OA 4, OA 2 vs OA 4 and OA 3 vs OA 4. Fig. 7.1. 7.2 and 7.3 represents total peptide spectrum of knee joint proteins of experimental rats of each group. Fig. 7.4, 7.5, 7.6, 7.7 and 7.8 depict selected unique peptise spectrum of proteoglycans aggrecan (m/z = 796.8), biglycan (m/z = 559.79), deorin (453.74), fibromodulin (m/z = 758.46) and lumican (m/z = 691.33) respectively.

Table 7.1 to 7.3 depicts the data highlighting proteome change at the joints in the experimental rats. And table 7.4 depicts selected unique peptides corresponds to proteoglycans of cartilage of experimental rats. Protein data obtained for each groups viz, normal group (OA 1), positive group (OA 2), standard drug treated group (OA 3) and proteoglycans treated rat group (OA 4) has been analysed separately. Table 1 depicts the comparison of proteome of normal group (OA 1) and proteoglycans treated rat group (OA 4). Proteoglycans are one of the major structural biomolecules present in the articular cartilage apart from other molecules like collagen. So symptoms like cartilage erosion of articular cartilage and onset of osteoarthritis is mainly associated with degradation of proteoglycans (Goldring and Goldring, 2004). Therefore, one of the measures by which anti-oteoarthritic effect could be assessed is by determining the content of proteoglycans present by using proteomics technology. Major proteoglycans present in the articular cartilage are biglycan, aggrecan, decorin, lumican and fibromodulin (Iozzo, 1997). Peptides corresponding to cartilage proteoglycans were detected in the joint proteome analysis of rats in each group. Comparison of OA 1 vs OA 4 revealed clear status of proteoglycans distribution between healthy and affected joints. Proteoglycans like biglycan, lumican, aggrecan core protein, decorin and fibromodulins were observed at high levels in OA 4 with 5.28, 1.58, 3.36, 2.94 and 3.52 fold expression respectively. Lumican fold expression in OA 4 was less compare to other proteoglycans mentioned above. Collagen is a major structural protein present in the cartilage especially articular cartilage which accounts for around 60% of total dry weight of cartilage (sophia *et al*., 2009). Collagen is a target for proteolysis by proteolytic enzymes like collagenases that results in acceleration of cartilage erosion during osteoarthritis. Type II collagen is the major collagen present in the cartilage and presence of collagen α-1 (II) was detected during proteomics analysis. Change in content of collagen especially type II collagen was evident between OA 1 and OA 4. Collagen α-1 (II) peptides were high in OA 4 with 1.02 fold expression when compared to OA 1.

Interleukins are the prominent inflammatory mediators which are expressed during the progression of osteoarthritis and these trigger joint

degradation by activating various other inflammatoty agents and metalloproteinases (Wojdasiewicz *et al*., 2014). Interleukin 20 receptor beta IL-20 was detected in the proteome of joint tissue which confirms the active involvement of interleukins at the site of joints indicating the occurrence of osteoarthritis. Interleukin 20 receptor beta IL-20 was high in OA 4 than OA 1with a fold expression of 4.34.

Immunoglobulins are produced during arthritic conditions like osteoarthritis and rheumatoid arthritis because of the involvement of inflammatory mediators. Their presence and increased expression play a vital role in the development of arthritic symptoms (Hyunho *et al*., 2006). We could detect Ig gamma-2B chain C region, Ig gamma-2A chain C region, Ig kappa chain C region and Ig gamma-2a protein which confirm the active involvement of IgG antibodies at the joints during the onset of arthritis. Clear change of expression of IgG antibodies were observed in OA 1 vs OA 4. Ig gamma-2B chain C region, Ig gamma-2A chain C region, Ig kappa chain C region and Ig gamma-2a presence was high in OA 4 group with enhanced fold expression of 1.67, 4.43, 1.85 and 3.51 respectively. Cytochrome oxidases are the mitochondrial enzymes that act as key molecules in mitochondrial respiration and energy production. (Boubriak *et al*., 2009). Proteomic analysis of joint revealed presence of cytochrome c oxidase subunit 4 isoform 2 and cytochrome c oxidase subunit which indicated the involvement of cytochrome c oxidase during osteoarthritis. Both the cytochrome c oxidase subunit 4 isoform 2 and cytochrome c oxidase subunit were high in OA 4 with an increased fold expression of 12.04 and 12.7 respectively. Compliment pathways are vital pathways of body to kill the unwanted cells and are part of inflammatory response (Struglics *et al*., 2016). Compliment pathways are activated in arthritic condition and several compliment factors are activated that induce the onset of arthritis to kill damaged cells. Compliment factor H was detected in this study and its expression was high in OA 1 than in OA 3 with an increased fold expression of 27.90.

Anti-oxidant defense system plays a vital role to neutralize the free radicals generated during osteoarthritis. Thioredoxin is a vital molecule which is involved in the pathway of sulphur containing anti-oxidants such as glutathione (Armstrong *et al*., 2002). Other important anti-oxidant enzymes like super oxide dismutase (SOD) plays a key role in neutralizing super oxides and protects knee joint during osteoarthritis (Scott *et al*., 2010). Peptides specific for thioredoxin and SOD were detected in proteomic analysis which indicated the active involvement of anti-oxidant defense system. Both thioredoxin and SOD levels were high in OA 4 than OA 1 with an enhanced fold expression of 3.90 and 2.13 respectively.

Table 7.2 depicts the comparison of proteome of rats of groups OA 2 vs OA 4. Proteoglycan levels were clearly altered in the joints of OA 2 (positive group) and OA 4 (proteoglycans treated) rat groups. Proteoglycans namely, biglycan, lumican, aggrecan, decorin and fibromodulin expression was observed to be high in rats of OA 4 when compared to those of OA 2 with a fold expression of 2.49, 1.47, 1.16, 1.09 and 1.03 respectively. Significant variation in the content of type II collagen was noticed in OA 1 and OA 4. Collagen α-1 (II) peptides were high in OA 4 with 1.31 fold expression when compared to OA 2. Interleukin 20 receptor beta IL-20 expression was contradictory to proteoglycan expression in OA 2 and OA 4. Interleukin 20 receptor beta IL-20 expression was observed to be high in OA 2 with a fold expression of 1.94. Ig gamma-2B chain C region, Ig gamma-2A chain C region, Ig kappa chain C region and Ig gamma-2a protein showed enhanced fold expression of 3.29, 1.59, 1.48 and 1.10 respectively and were found to be high in OA 2 than OA 4. Cytochrome c oxidase subunit 4, isoform 2 and cytochrome c oxidase subunit expression were found increased in OA 2 with increased fold expression of 2.46 and 1.72 respectively. An expression of 1.85 was noticed for compliment H and was found high in OA 2 compared to OA 4. Peptides specific for thioredoxin and SOD were detected in proteome analysis which showed significant variation in the expression in OA2 and OA 4. Both thioredoxin and SOD levels were high in OA 4 than OA 2 with enhanced fold expression of 3.09 and 1.08 respectively.

Table 7.3 depicts the proteome comparison of OA 3 vs OA 4. Variation in proteoglycan levels were confirmed by proteome analysis and by comparison between OA 3 and OA 4. Mixed response was observed with respect to proteoglycan expression in OA 3 and OA 4 groups. When compared to OA 3 biglycan, aggrecan and decorin expression was high in OA 4 with increased fold expression of 7.95, 1.11 and 3.56 respectively. Whereas, lumican and fibromodulin levels were high in OA 3 with increased fold expression of 1.87 and 2.04 respectively. Type II collagen content was high in OA 4 with an enhanced fold expression of 2.08 when compared to OA 3. Interleukin 20 receptor beta IL-20 was high in OA 4 with a higher fold expression of 1.13 than OA 3. As in proteoglycan expression variation in the expression between groups was noticed for OA 3 and OA 4 with respect to IgG levels. In OA 4, when compared to OA 3, an enhanced fold expression of 1.52 and 1.06 was noticed for Ig gamma-2A chain C region and Ig kappa chain C region respectively. Whereas, Ig gamma-2B chain C region and Ig gamma-2a protein expression was high in OA 3 with increased fold expression of 1.07 and 1.27. Compliment factor H was observed to be high in OA 3 with a fold expression of 4.41 when compared to OA 4. Variation in the expression of thioredoxin and SOD levels were noticed to be high in OA 3 than OA 4. Thioredoxin level was high in OA 3 with increased fold expression of 1.40 whereas enhanced SOD fold expression of 1.01 was observed for SOD in OA 4.

Proteomic technologies is being used to identify biomarkers involved in OA progression and are used to develop a data of protein mediators in osteoarthritic condition. Previous report revealed huge number of proteins namely, cytokines, several proteases, and extracellular matrix fragments in synovial fluid, serum and articular cartilage of patients affected with OA (Chan *et al*., 2015). Proteome profile of normal and osteoarthritis affected joint proteins was done by the aid of liquid chromatography mass spectrometry (LC-MS/MS) followed by peptide-based identification. So proteome identification of proteins involved in osteoarthritis is important for developing therapeutic strategies and LC-MS/MS based proteomics profile which could detect protein markers even at very low concentration (Peffers *et al*., 2015). We have assayed the proteome of joint of

normal, positive, standard drug treated and proteoglycans treated rats by using LC-MS/MS technology. We observed numerous proteins and their level variations between groups which agree with the above reports.

Osteoarthritis progression is highly correlated with the loss of articular cartilage components, especially proteoglycans (PGs), resulting in the destruction of tissue (Venkatesan *et al*., 2004). OA is characterized by the abnormal increase in proinflammatory cytokines, mainly as IL-1β, that prevent biosynthesis of PGs and collagen and accelerate their degradation, damaging the normal homeostasis of cartilage extracellular matrix (Fernandes *et al*., 2002). Alleviation of symptoms of osteoarthritis can occur only if proteoglycans and collagen synthesis take place to restore cartilage structure (Evans and Robbins, 1999). In this study, we observed increase of proteoglycans expression in the joints in proteoglycans treated groups ie, OA 4 when compared to OA 2 which is osteoarthritic group without any treatment. As expected, normal proteoglycans expression was noted for OA 1 ie, normal group. Proteoglycans, mainly aggrecan, biglycan and decorin which are the principal proteoglycans in cartilage were better restored in OA 4 when compared to OA 2 and was slightly higher but similar to OA 3. Previous report suggest, the increased synthesis of aggrecan, an important and dominant proteoglycan in the cartilage could facilitate to restore the cartilage destruction (El-Arman *et al*., 2010). In this study, we noticed that fold expression of aggrecan was high in OA 4 compared to all other groups. We assumed that oral feeding of proteoglycans in rats might have influenced the proteoglycan biosynthesis to counteract osteoarthritic progression. Loss of proteoglycans caused the onset of osteoarthritis (Taylor *et al*., 2006) and similar findings was observed for OA 2 which is the arthritis induced and untreated group. Whereas, reverse observations were noted for OA 3 and OA 4 which indicated the restoring of articular cartilage in proteoglycans fed and standard drug indomethacin treated groups.

Collagen is the most abundant structural protein in extracellular matrix of cartilage which accounts for about 60% of the dry weight of cartilage. Several collagens are present in articular cartilage and among them Type II collagen is the

most abundant and contributes 90% to 95% of the total collagen in extracellular matrix and are associated with proteoglycan aggregates (Sophia *et al*., 2009). Type II collagen is targeted by metalloproteinases (MMPs), mainly MMP 13 during osteoarthritis resulting in the fast break down and degradation of cartilage. Type II collagen synthesis increased in OA and the peptides corresponding to type II collagen were detected and quantified by LC–MS/MS (quadrupole time-of-flight, Q-TOF). It was revealed that collagen catabolism occurs during arthritis where metalloproteinases play a significant role. At the same time, body starts to synthesize collagen at joints to restore cartilage structural integrity (Zhen *et al*., 2008). In this study, we observed the higher fold expression of type II collagen in OA 4 and OA 3 but decreased expression was observed in OA 2. Type II collagen was confirmed by the peptides corresponding to collagen α-1 (II) chain which is a major component of type II collagen triple helix structure. Previous studies reported biosynthesis of the active type II collagen at the joints of osteoarthritic patients (Hermansson *et al*., 2004) and similar findings were noticed in this experiment. From these results, it was clear that proteoglycan supplementation had a positive impact promising action on the regeneration of articular cartilage by facilitating increased biosynthesis of type II collagen.

Several pro-inflammatory cytokines are produced during osteoarthritic progression which accelerate joint degradation by attracting numerous other inflammatory mediators like IL-20, IL-6, TNF-α, nitric oxide etc. Progression of osteoarthritis can be checked by the action of biomolecules which can prevent the production of pro-inflammatory interleukins (Santangelo *et al*., 2012). IL-20, a member of the inflammatory interlukin family is found to be associated with the progression of osteoarthritis and rheumatoid arthritis. Researchers reported the importance of IL-20 in the progression of arthritis (Hsu *et al*., 2006). Enhanced fold expression of interleukin 20 receptor beta IL-20 protein in this study indicated the active role of interleukin 20 in the progression of osteoarthritis. As expected, interleukin 20 receptor beta IL-20 expression was clearly high in OA 2. Whereas decreased interleukin 20 receptor response was noticed for OA 3 and OA 4 which indicated the promising role of proteoglycans to treat osteoarthritis.

Indomethacin-treated group has shown promising decrease of interleukin 20 receptor expression but almost similar response was observed in OA 4 group. So these findings substantiate the decrease of inflammation and recovery of arthritic joint due to the action of proteoglycans isolated from *E. brucus* shark cartilage.

Immunoglobulin mainly IgG plays a vital role in the immunological response during arthritic progression. Studies reporting IgG involvement by identifying IgG receptors are available. Increased expression of IgG is associated with severity of arthritis as it induces immunological reaction at the site of joints. IgG response is high in rheumatoid arthritis but involvement of IgG has also been reported in osteoarthritic progression (Hyunho *et al*., 2013). IgG expression was decreased in OA 4 and in OA 3 indicating the significant role of proteoglycan treatment in reducing immunological response to a degree similar to that of standard drug indomethacin treated group of rats.

Cytochrome oxidases present in mitochondria play a vital role in cellular homeostasis. They are vital enzymes and are part of complex IV of electron transport system of mitochondria. This membrane bound enzyme helps to maintain the membrane potential of mitochondria and hence cellular homeostasis. Decreased expression levels of cytochrome oxidase associated with the oxidative stability and proper cellular metabolism. Increased cytochrome expression and activity has been reported in osteoarthritic progression and reducing of cytochrome activity to normal level is important to prevent osteoarthritic progression (Boubriak *et al*., 2009; Mazat *et al*., 2013). Cytochrome c oxidase subunit 4 isoform 2 and cytochrome c oxidase subunit were identified with increased fold expression in OA 2 when compared to OA 3 and OA 4. These findings agree with the findings put forwarded by Boubriak *et al*., 2009; Mazat *et al*., 2013. Cytochrome oxidase expression was decreased in OA 3 and 4 with emphasizing the protective role of proteoglycans treatment against osteoarthritic progression.

The complement system is responsible for imparting innate immunity. But at some instances complement system becomes so aggressive that it targets and kills body's own cells. Researchers reported the involvement of complement

system in the osteoarthritic progression (Struglics *et al*., 2016). They reported the activation of complement factor leading to increased cell damage during osteoarthritic progression and activation of pro-inflammatory mediators affecting the situation very badly. Fernandez-Puente *et al*. (2011), employed iTRAQ technology to roduce proteome and identify protein biomarkers of OA progression and identified some of the complement protein factors. In this study we observed that expression of complement factor H agrees with the above report. Increased expression of complement factor H was reported in OA 2 and less expression was observed for OA 4. In OA 4 complement expression was observed lower than that of OA 3 which confirm the protective role of proteoglycans against osteoarthric symptoms. Kuroki *et al.*, (2012) also reported the active participation of complement factors during onset of osteoarthritis and this work also is in accordance with the above reports.

Oxidative stress accelerates the joint degradation during osteoarthritis by damaging chondrocytes and joint tissues. In order to resist ROS mediated oxidative stress at joint tissues chondrocytes produce anti-oxidant enzymes like SOD, catalase etc. Involvement of SOD in osteoarthritis is well documented (Henrotin *et al*., 2005). Studies reported the down regulation of superoxide dismutase (SOD) in osteoarthritis progression and enhanced cartilage degradation (Scott *et al*., 2010). Thioredoxin (Trx) and GSH are the major thiol antioxidants which help to protect the tissues from ROS-induced damage. The GSH redox system plays an important role in maintaining oxidative balance in the tissue and thus protects cells from oxidative stress (Armstrong *et al*., 2002). Proteomic analysis of synovial fluid in osteoarthritis using SWATH-mass spectrometry detected the level of thioredoxin (Liao *et al*., 2018). In these results also we could observed presence and variation in the fold expression of thioredoxin between groups. Thioredoxin fold expression was increased in OA 3 but similar response was also noticed in OA 4. Whereas SOD level was high in OA 4 than that of OA 3. So anti-oxidant status was up regulated in proteoglycans treated groups and it was confirmed by the increased fold expression of thioredoxin and SOD when compared to expression in OA 2. Ostalowska *et al*., (2006) reported the

chondrocyte protective role of anti-oxidant enzyme including SOD during osteoarthritis. They reported that increased SOD can resist cartilage degradation. Guo *et al*., 2008 documented the role of SOD in osteoarthritis by proteomics approach. These results agree with the findings reported above and we assume that proteoglycans can restore cartilage homeostasis by decreasing oxidative stress through activation of SOD and thioredoxin.

7.4 Conclusion

Proteomics approach by the help of peptide based identification and molecular level characterization of osteoarthritic joint protein biomarkers enables better understanding of disease progression and anti-osteoarthric effect of any compound of interest. In the present study, joint proteins of rats of experimental groups were isolated and trypsin digested for LC-MS/MS analysis. The tryptic peptides were separated using a nanoACQUITY ultra performance liquid chromatographic system and MS analysis of eluting peptides was carried out on a SYNAPT® G2 High Definition MS™ System. The acquired ion mobility enhanced MSE spectra was analysed using Progenesis QI for Proteomics V3.0 (Non Linear Dynamics, Waters) for protein identification as well as for the label-free relative protein quantification. The protein identifications were obtained by searching against the *Rattus norvegicus* (Rat) protein database downloaded from UniProt. Above analysis revealed the presence of important protein biomarkers in all the studied groups. Most important structural biomolecules like proteoglycans namely, aggrecan core protein, decorin, biglycan, lumican, fibromodulin and type II collagen fold expression was promisingly high in proteoglycans treated rat groups. Similarly anti-oxidant defense molecule namely, thioredoxin and SOD were up-regulated in the proteoglycans treated groups. Whereas, immunoglobulins, IL-20, cytochrome oxidase, compliment factor H were down regulated in the proteoglycans treated groups. Standard drug indomethacin group and proteoglycans groups shared similarity in terms of decrease in oxidative stress, immunological stress but proteoglycans treated groups showed comparatively better fold expression for proteoglycans and collagen. These results

confirm that proteoglycans can be effective in restoring cartilage structure and in preventing the production of adverse osteoarthric biomarkers which are responsible for the progression of osteoarthritis.

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Chapter 8

Summary

The present study was designed to study isolation, purification and characterization of PGs (proteoglycans) from deep sea shark *Echinorhinus brucus* cartilage and assessment of *in-vitro* anticancer and *in-vivo* anti-osteoarthritic effects were also evaluated. We observed typical chemical composition which is specific to proteoglycans and specific electrophoretic band pattern confirmed the nature of proteoglycans and glycosaminoglycans present. Structural characterization by FTIR and NMR revealed the presence of proteoglycans by obtaining the fuctional groups response corresponding to glycosaminoglycans and proteins. LC MS-MS and subsequent NCBI whole protein database search has confirmed the presence of 3 unique peptide fragments first time in *E. brucus* cartilage which shared sequence similarity with arggrecan core protein, decorin and epiphycan respectively. Also characterization of GAG part of PGs of shark cartilage by tandem mass spectroscopy with ESI revealed that the m/z ion pattern in the 200-1500 range were similar to that of standard GAGs like chrondroitin sulphates A & B and hylauronic acid. Above findings substantiate the reliability of the methods used in the present study to characterize proteoglycans and reveals the purity of isolated proteoglycans. We observed that higher dose of proteoglycans (250µg/ml) isolated from deep sea shark cartilage had greater potential to fight against cervical cancer through the activation of apoptosis and cell cycle arrest. PGs isolated from *E. brucus* also activated apoptotic mediated anti-proliferative activity against MCF-7 cell lines. Apoptosis involvement in anti-proliferative activity of PGs isolated from deep sea shark cartilage was confirmed by caspase 3 & 9 activation, DNA laddering assay, double fluorescent staining with acridine orange/ethedium bromide and gene expression analysis of apoptotic markers such as Bcl-2, BAX and p53. In this experiment, proteoglycans treatment to osteoarthritis induced rats revealed promising anti-osteoarthritic effect. Anti-

oxidant defence system parameters like SOD, catalase and GSH were activated, at the same time lipid peroxidation, CRP and uric acid levels significantly reduced. More importantly inflammatory mediators expression clearly came down and anti-inflammatory cytokine IL-10 level were raised in proteoglycans treated group. Proteomics analysis by LC-MS/MS and subsequent NCBI database search and protein identifications were obtained by searching against the *Rattus norvegicus* (Wistar strain albino rat) protein database downloaded from UniProt. Most important structural biomolecules like proteoglycans namely, aggrecan core protein, decorin, biglycan, lumican, fibromodulin and type II collagen showed enhanced fold expression in proteoglycans treated rat groups. Similarly anti-oxidant defence molecules namely, thioredoxin and SOD were up-regulated in the proteoglycans treated groups. Whereas, immunoglobulins, IL-20, cytochrome oxidase, compliment factor H were down regulated in the proteoglycans treated groups. Results obtained in this study indicate that proteoglycans can be an effective anti-osteoarthritic molecule that acted by restoring cartilage structure and preventing the production of adverse osteoarthric biomarkers.

In conclusion, purity of proteoglycans was confirmed by characterization methods such as chemical, electrophoresis, FTIR, H1-NMR and proteomics-based LC-MS/MS methods. Significant anti-cervical cancer and anti-breast cancer effects of PGs by inducing apoptosis was confirmed. Individual PGs from shark cartilage need to be isolated and further studies have to be done in order to elucidate the effect of each of the PG type. Also detailed investigations are further needed to elucidate the exact mechanisms of action of proteoglycans in apoptosis involved anti-cervical cancer effect. All these results emphasize the importance of marine sector as a tremendous source of anti-cancer and other bioactive agents. In order to strengthen the fight against cancer and other deadly diseases marine sources have to be better exploited. Recently, natural bioactive molecules are being increasingly used to treat arthritis due to less or no side effects. Natural biomolecules like proteoglycans could possibly restore the structural and physiological integrity of joint especially in the articular cartilage by decreasing arthritic progression. Both structural and biochemical results obtained in this

study substantiated the relevance of application of proteoglycans in osteoarthritic treatment. Prolonged usage of this biomolecule can be an ideal option to alleviate the osteoarthritic progression that can offer great relief to patients.

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