

Evaluation of Sardine Fish Extract Meal on Papain Induced Osteoarthritis in Experimental Rats

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| Received: 27.03.2019 | Accepted: 07.04.2019 | Published: 30.04.2019

DOI: [10.21276/sjimps.2019.5.4.3](https://doi.org/10.21276/sjimps.2019.5.4.3)

Abstract

Aim: The aim of the present study was to evaluate the effect of sardine (*Sardinella gibbosa*) fish extract on papain induced osteoarthritis in rats. **Methodology:** In this study 30 wistar rats were randomly selected and divided into five groups. Group-1 (-ve Control), Group 2 (+ve control), Group 3 (Calcium 75 mg/kg as standard), Group 4 (Sardine extract 30 gms/kg), Group 5 (15 gms/kg sardine extract). Osteoarthritis was induced in the right knees of all the groups of rats except negative control by injecting 0.2 ml (4%) of papain solution with 0.1 ml (0.03M) of cystein as activator. All groups were left for development of osteoarthritis. Rats were sacrificed on day 1, 2, 7, 14, 21 & 28 days of post papain injection. **Results:** Estimation of calcium and phosphorus in fish extract was carried out by Atomic absorption spectrophotometer. The percentage inhibition of knee thickness of sardine fish extract and Calcium groups were found to be 68% and 77% respectively. Various haematological parameters like RBC count and Hb, WBC count and ESR were recorded. Parameters like blood glucose, urea, globulin, creatinine, total protein, and albumin were calculated. Histopathological studies of knee joints of rats were also carried out. **Conclusion:** *Sardinella gibbosa* fish extract at a dose level of 30 gm/kg p.o., exhibited reduction in osteoarthritis due to the rich source of Ca & selenium and maintained the normal haematological and biochemical parameters in papain induced osteoarthritic rats.

Keywords: *Sardinella gibbosa*, Sardines, Papain, Osteo arthritis, Selenium.

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INTRODUCTION

Osteoarthritis (OA) is a disorder affecting 250 million people worldwide [1-3]. Even though there is a development in the field of OA treatment many people still suffer and we are still in need of efficient treatment [4]. In India Natural compounds are commonly used for the treatment of Osteoarthritis and the World Health Organization (WHO) recommends the international community to use traditional ayurvedic system of medicine in the management of OA [5].

Marine resources give us good number of specific and potent bioactive substances like proteins, enzymes, polyether, fatty acids, polysaccharides and lectins. Proteins that are obtained from marine sources possess unique properties like foaming capacity, gelling nature, film formation and antimicrobial activity [6]. Bioactive peptides generally contain 3–20 amino acid residues and based on their amino acid composition and sequence its activity differs. Some of the reported peptides have different biological activities like antioxidant, antimicrobial, antihypertensive, immunomodulatory, antithrombotic, anticancer activities, along with nutritional value [7].

In general fish is the major source of various bioactive substances like antioxidants, Vitamins, polyunsaturated fatty acids, polysaccharides, minerals, enzymes etc. Fish is a rich source of lipids, protein, vitamins and minerals. ¹⁵These important source of substances exert different pharmacological activities, and that can be added in diet for the treatment of various ailments [8].

Sardines (*Sardinella gibbosa*) are small variety of oily fishes that belongs to the family Clupeidea. They are known as pilchard in some areas. They are generally packed in cans and are commonly identified as canned sardines. There are 21 types of fish that can come under the Sardines category. Sardina, sardinops, sardinella and dussumieria are the familiar species of Sardines [9].

The chemical constituents of *Sardinella gibbosa* [10] consists of rich source of vitamins. It contains high concentration of Vitamin-A, D, B₁₂, C, K, E, B₆, thiamine, niacin, pantothenic acid, riboflavin, folate, choline, and betaine. *Sardinella gibbosa* has high

contents of fats and fatty acids such as omega-3 fatty acids, polyunsaturated, saturated, monounsaturated omega-6 fatty acids etc. *Sardinella gibbosa* is one of the rich sources for proteins; it contains approximately about 36.7% of proteins. *Sardinella gibbosa* contains various minerals like calcium, phosphorous, potassium, magnesium, sodium, iron, zinc, copper, manganese, selenium and fluoride. Along with these above constituents it contains other contents like sterols, water, ash etc.

Sardinella gibbosa has important traditional uses [9] like Prevention of blood clots, Strengthens bones (osteoporosis), maintains healthy immune system, Anti-cancer properties, Antioxidant properties [11-13].

In the present investigation a scientific attempt was made to estimate bio chemical parameters of the

extracted bioactive meal obtained from sardine fish and it was tested for osteoarthritis induced by papain in experimental rats.

MATERIALS AND METHODS

Collection & Extraction

Sardinella gibbosa were collected from Kakinada, Andhra Pradesh fish merchants and further subjected to extraction process. Pressing is a process that removes oil and water content from the preparation and the solid obtained is known as press cake. Cooking is the critical stage in preparing the fish meal, incomplete cooking and excess cooking makes the preparation unsuitable for pressing. No drying should occur while cooking. Pressing eliminates 70% to 50% of water content and oil is reduced to 4%. Finally the meal was dried in sunlight. The grinding is the last step which helps to break the lumps or particles of bones.



Fig-1: Collection & extraction of *Sardinella gibbosa*

Experimental Protocol

In this study 30 wistar rats with weight range of 200-250 gm were randomly selected and separated into five groups each group consists of 6 animals. Group 1 (-ve Control), Group 2 (+ve control), Group 3 (Calcium 75 mg/kg as standard), Group 4 (high dose of sardine extract 30 gm/kg), Group 5 (low dose of 15 gm/kg sardine extract).

Induction of Osteoarthritis

Osteoarthritis was induced by injecting papain 0.2 ml of 4% with 0.1 ml of 0.03M cystein as activator in the right knee of all groups of rats except negative control. All the groups of rats were left for development and assessment of osteoarthritis. Rats were sacrificed on 1st day, 2nd, 7th, 14th, 21st and 28th days of post papain injection. Same amount of saline was injected to the first group. Femoro-tibial joints of rats were dissected out on each day of sampling. Ligaments and tendons were also separated and then stored in 10% formalin solution with pH 7.4. Decalcification of joints were done by immersing in 5% formic acid for a period

of one week, Later they were processed for examination by paraffin embedding; front organs were sectioned and stained with haematoxylin-eosin solution and examined under light microscope at 100x magnifications.

Estimation of calcium and phosphorous content in sardine fish extract

Estimation of calcium and phosphorus in sardine fish extract was carried out by Atomic absorption spectrophotometer [14-16]. Mineral content like concentrations of calcium and phosphorous were measured in the sardine fish muscle by this method. 5gms of dry fish muscle powder was weighed and placed in digestion tube. 5ml of concentrated nitric acid and 5ml of perchloric acid were added to two separate samples for estimation of Calcium and phosphorus. The reaction was slowed down by placing the tube in hot plate and heated up to 60°C for 30 minutes. The tubes were allowed to cool down and then 10ml of concentrated nitric acid was added and again samples were subjected to digestion. The digested sample was allowed to cool down and deionised water was used for

dilution and hydrogen peroxide additions were repeated until the samples were clear. Finally it was filtered and made volume up to 50 ml using deionised water in volumetric flask. Concentrations of calcium and phosphorous were measured using atomic absorption spectroscopy.

Estimation of Calcium in Rat Serum [17]

2ml sample of serum was taken in a 250 ml Erlenmeyer flask. The sample was made up to 50 ml volume using calcium-free distilled water. To that 0.4ml of 9N NaOH solution and 1 drop of ammonium purpurate indicator were added. The sample was immediately titrated with constant swirling until purple end-point is reached.

Estimation of Phosphorus in Rat Serum

The protein free filtrate was prepared by addition of 0.4ml of serum to 3.5ml of water; 1ml of 15% trichloro acetic acid was added and mixed well, after 5 minutes samples were centrifuged and filtered. The standards of different concentrations of phosphorous were made from the solutions of KH_2PO_4 . 0.5ml of supernatant fluid (standard or test) was added

to 50ml of 10% trichloroacetic acid in a colorimeter tube and mixed well. To this 0.5ml of 1% ammonium molybdate was added. It was mixed well and left for 5 minutes. To this 0.5ml of 3gms of stannous chloride was added. After 15 minutes readings were recorded at 660nm in colorimeter.

RESULTS

Knee Thickness

Effect of *Sardinella gibbosa* fish extract (15 gm/kg & 30 gm/kg) and calcium (75 mg/kg) on papain induced osteoarthritis in rat is shown in table 1. The thickness of knee in calcium treated group of animals had significantly reduced ($p < 0.001$) to normal when compared to control group of animals. Extract of sardine fish (30 gm/kg) administered group of animals exhibited significant reduction to normal after 14th day when compared to standard and normal control group. The effect of (15 gm/kg) fish extract on knee thickness was less significant as compared to 30gm/kg. The percentage inhibition of knee thickness of sardine fish extract (30 mg/kg) and calcium (75 mg/kg) was 73% and 81% respectively shown in Table-1.

Table-1: Effect of *Sardinella gibbosa* fish extract in reducing the knee thickness of rats

Groups	Dose	Knee thickness in mm				Percentage inhibition
		7 th day	14 th day	21 st day	28 th day	
Group 1 Negative control	Saline	2.1±0.2	2.1±0.2	2.05±0.2	2.0±0.2	Nil
Group 2 Positive control	Papain 0.2ml 4%	2.2±0.2	2.15±0.2	2.1±0.2	2.1±0.2	Nil
Group 3 Standard	Calcium (75 gm/kg)	2.2±0.2	2.1±0.2	1.9±0.2	1.6±0.2	81%
Group 4 Sardine extract	15 gm/kg	2.5±0.2	2.4±0.2	2.25±0.2	2.05±0.2	32%
Group 5 Sardine extract	30 gm/kg	2.3±0.2	2.1±0.2	2.0±0.2	1.85±0.2	73%

Values expressed as mean \pm SEM (n=6), (***) $P < 0.001$, (*) $P < 0.01$, (*) $P < 0.05$) as comparable to control group.

Haematological Parameters

The haematological parameters of papain induced osteoarthritis in rats are shown in table 2. RBC count and haemoglobin level was significantly decreased; WBC count and ESR of osteoarthritic rats

were increased, as compared to control group of animals. The sardine meal treatment was found to control the altered haematological parameters on different phases of papain induced osteoarthritis.

Table-2: Haematological parameters of Papain induced osteoarthritic rats

Animal Groups	RBC (millions/mm ³)	WBC (Thousands/mm ³)	Hb (g/dl)	ESR (mm/hr)
Group I	5.81±0.25	7.86±0.32	14.20±0.62	3.96±0.05
Group II	4.93±0.15 *	12.94±0.25 *	11.54±0.48 *	10.56±0.10 *
Group III	5.78±0.45 *	8.75±0.45 ***	14.34±0.58 *	5.32±0.08 **
Group IV	5.79±0.16 *	10.65±0.42 ***	13.98±0.34 *	6.85±0.08 **
Group V	5.81±0.21 *	9.14±0.81 *	14.65±0.45 *	5.70±0.09 **

Values mentioned as Mean \pm S.E.M, n=6 (***) $P < 0.001$, (*) $P < 0.01$, (*) $P < 0.05$)

Urea, Serum Creatinine and blood glucose

Table-3 shows significant increase in urea, creatinine levels of blood in papain induced osteoarthritic rats as compared to control group of

animals. *Sardinella gibbosa* fish extract and calcium preparations were found to reduce the changes occurred due to papain induction by means of treatment.

Table-3: Blood glucose, urea, creatinine and serum protein levels of *Sardinella gibbosa* treated papain induced osteoarthritic rats

Groups	Urea (mg/dl)	Creatinine (mg/dl)	Protein (mg/dl)	Albumin (mg/dl)	Globulin (mg/dl)	A/G Ratio	Cerruloplasmin (mg/dl)	Fibrinogen (mg/dl)
Group I	22.62 ±0.80	1.34±0.04	6.40±0.22	3.46±0.16	2.12±0.04	1.56±0.14	19.43±2.43	42.25 ±2.16
Group II	29.50 ±0.37 a*	2.28±0.02 a*	4.46±0.23 a*	2.16±0.07 a*	2.56±0.04 a*	0.71±0.20 a*	50.60±2.78 a*	159.35 ±4.93 a*
Group III	21.25 ±0.95 b*	1.89±0.06 a**b*	4.42±0.18 a*b*	3.06±0.20 a*b*	2.94±0.06 a*b*	1.27±0.07 a*b*	20.16±1.24 b*	86.06 ±3.42 a*b*
Group IV	24.66 ±0.62 a***b*	1.94±0.08 a*b*	4.24±0.17 a*b*	3.01±0.07 a*b*	2.32±0.05 a*b*	1.74±0.07 a*b*	24.20±3.45 a***b*	94.25 ±6.01 a*b*
Group V	23.75 ±1.75 b**	1.97±0.06 a**b*	4.95±0.25 a*b*	3.02±0.35 a*b*	2.76±0.09 a*b*	1.84±0.07 a*b*	23.55±3.29 a***b*	95.45 ±5.05 a*b*

Values mentioned as Mean ± S.E.M, n=6 (***P<0.001, *p<0.01, *P<0.05)

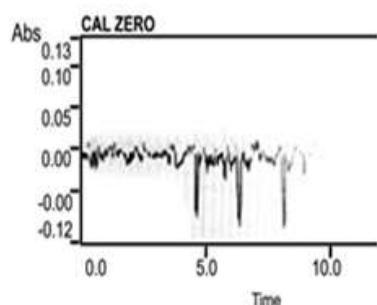
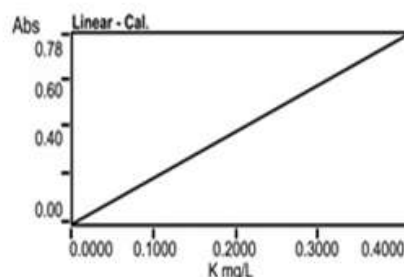
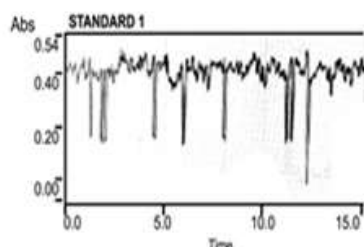
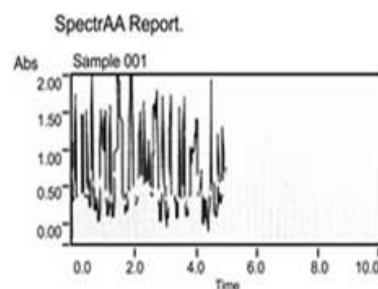
Calcium and Phosphorus Estimation

When compared to low dose, high dose of Sardine extract 30gms/kg exhibited increased serum calcium and phosphorus levels in papain induced

osteoarthritis in rats as compared to standard and control group of animals. The results are exhibited in Table-4.

Table-4: Estimation of Calcium and Phosphorus content in *Salmonella gibbosa* treated rat serum

Groups	Calcium(mg/dl)		Phosphorus(mg/dl)	
	Normal Range	Sample	Normal Range	Sample
Group 1	5.3-13	10.2	3.11-11	8.7
Group 2	5.3-13	4.5	3.11-11	2.6
Group 3	5.3-13	8.9	3.11-11	8.5
Group 4	5.3-13	7.0	3.11-11	6.2
Group 5	5.3-13	8.1	3.11-11	7.6

**Calcium Zero****Linear Graph****Standard Graph****Test Sample Graph****Fig-2: Estimation of Calcium in sardine fish extract treated rats using Atomic absorption Spectrophotometer**

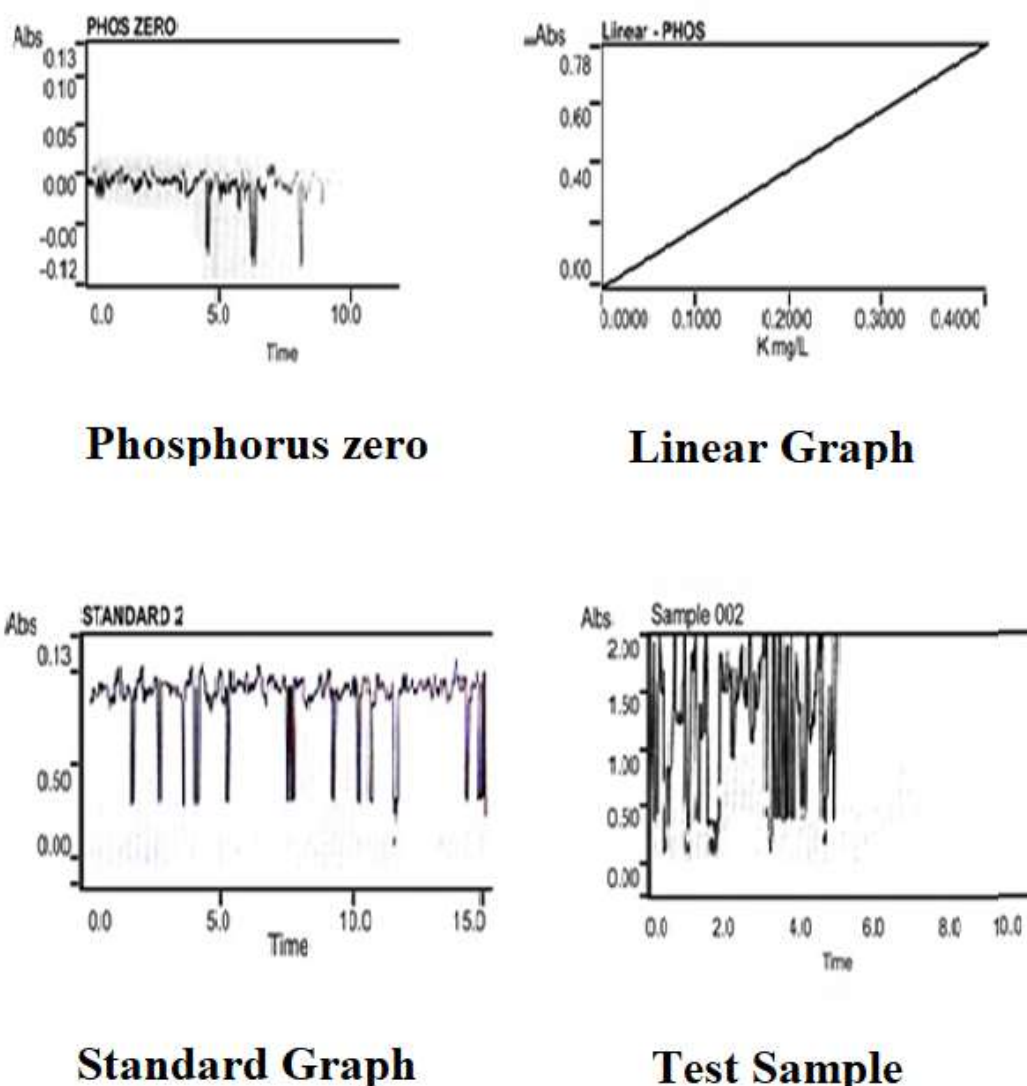


Fig-3: Estimation of Phosphorus in sardine fish extract treated rats using Atomic absorption Spectrophotometer

Albumin, Globulin Total Protein, and A/G Ratio

The individual protein and total protein level changes are shown in Table-3. In papain induced osteoarthritic rats, there was a significant decrease in the levels of albumin, total protein, and A/G ratio, but there was a significant increase in the levels of globulin as compared to control group. Sardine extract treatment and calcium were found to reduce all the changes to normal levels that have occurred due to papain induction.

Acute Phase Proteins

Fibrinogen and ceruloplasmin are known as acute phase proteins. In papain induced osteoarthritic rats, the levels of these two protein levels significantly increased as compared to the osteoarthritic control group of animals (Table-3). It was found that Sardine fish extract treatment has significantly decreased the

acute phase protein levels to normal in osteoarthritic rats.

Histopathology Studies

Histopathological studies of knee joints of normal rats have intact morphology of synovium. No inflammation to the cells observed, whereas OA induced rats showed cartilage destruction, inflammation to the cells, pannus formation, and fibrin deposition. Calcium treated group of rats showed good protection against cartilage destruction, vascular proliferation and low rate of inflammation to the cells and no pannus formation. Sardine fish extract (30 gm/kg) treated group of rats exhibited good protection against cartilage destruction, vascular proliferation, inflammatory response and on pannus formation. Sardine fish extract (15 gm/kg) treated rats showed moderate destruction.

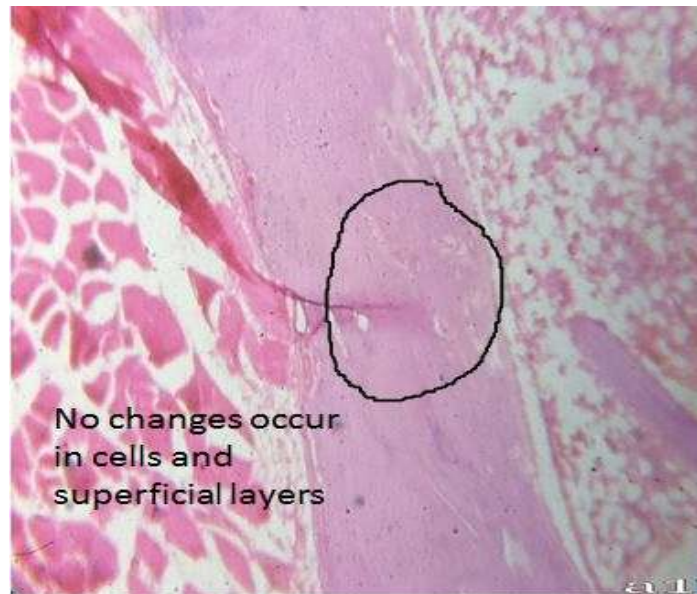


Fig-4a: Rat Cartilage (Normal control)

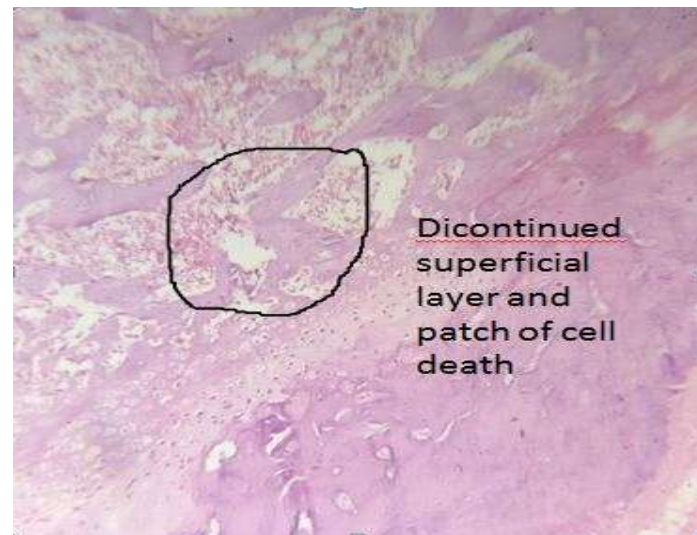


Fig-4b: Rat Cartilage (Disease Control)



Fig-4c: Rat Cartilage (Standard Calcium)



Fig-4d: Rat Cartilage (Sardine low dose)



Fig-4e: Rat Cartilage (Sardine high dose)

DISCUSSION

Osteoarthritis is a chronic inflammatory disease which affects several joints including the tendons, cartilage, and muscles. In the present work, osteoarthritis was induced in rat at multiple joints with an intention to induce inflammation of joints, cartilage and bone destruction. Papain induced arthritis models are widely used in animal experiments of osteoarthritis. This model is widely used due to its strong correlation between efficacy of the therapeutic agents in animal models and also osteoarthritic condition in humans.

In the present work the effect of *sardinella gibbosa* extract on papain induced rats exhibited significant inhibitory effect on osteoarthritis. The latent effect which occurred after few days which is characterized by joint swelling and it could be due to the liberation and over production of prostaglandins and bradykinin in knee joints which accompanies leukocyte migration. The inhibition of the knee joint swelling may be associated with inhibition of cell infiltration, neutrophil infiltration and bone erosion. Standard drug calcium (75 gm/kg) and test drug (30 gm/kg) showed

significantly decreased knee thickness. In the present investigation osteoarthritic rat exhibited soft tissue inflammation around the ankle joints during acute phase of osteoarthritis and was due to formation of edema in a particular joint. The inflammation was found to increase in the beginning and later seen constant for 2 weeks. The change in knee volume has been found to associate with an increase in destruction of the cartilage in the joint.

From results it is evident that RBC count and hemoglobin level has decreased indicating the anaemic condition of osteoarthritic rats. The main causes are due to storage of iron in the reticulo-endothelial system and synovial tissue and thus lead to failure of bone marrow to respond anaemic conditions. The significant increase in leukocyte count in papain induced OA is due to the immuno stimulant action and the decrease in leukocyte count for sardine fish extract treated groups indicated its immunomodulatory property. The ESR count that was increased in OA control group animals has been remarkably reduced by sardine fish extract and standard

calcium thereby restoring the normal in osteoarthritic conditions [18].

Creatinine and urea level in the blood was found to increase in the OA control group that indicates the dysfunction of kidneys in osteoarthritic rats. Sardine extract was found to reduce the urea and serum creatinine levels. Blood urea was increased in OA rats and it was assumed that the fraction of urea in the blood of osteoarthritic rats were due to synthesis of arginine in the kidneys.

It was also observed that the papain induced OA has reduced the albumin levels and increased the globulin levels. This change in albumin and globulin levels can occur due to release of inflammatory mediators like histamine, bradykinin and PG's that are tend to increase the permeability of vascular tissues to albumin. Sardine fish extract treatment has significantly increased the albumin level and decreased the globulin level in OA rats thereby confirms the inflammatory suppressive effect in osteoarthritis.

During the tissue injury or inflammatory conditions Ceruloplasmin, a copper containing plasma protein is produced in liver. Its increased serum level during osteoarthritis might be due to joints and bones injury. Whereas Sardine fish extract has considerably reduced the proteins fibrinogen and ceruloplasmin and confirms its role in tissue repair.

The histopathology report of Group I showed the normal architecture of joints. Group II are positive control osteoarthritic animal joints had shown abnormal architecture as compared to the normal joints which show edema formation, degeneration of the cartilage, bone marrow damage and severe inflammatory exudates in the joints. The standard drug treated animal joints showed normality with less inflammatory signs, whereas the sardine fish extract treatment for 14 days had well protected the cartilage from destruction. After 28 days of treatment there was a significant anti-inflammatory symptom in the articular joints of rats as compared to the 14th day of treatment. Degeneration of joint was not found in any treated groups compared to the negative control.

CONCLUSION

Sardinella gibbosa fish extract at a dose level of 30 gm/kg p.o., exhibited marked reduction in inflammation of rat knee joint and it was able to maintain the normal haematological and biochemical parameters in papain induced osteoarthritic rats in papain induced arthritis. The histopathological studies also confirmed the inhibition of osteoarthritis by sardine fish extract in papain induced animal models. The actual mechanism of action of sardine fish extract on papain induced osteoarthritis is not clear with these studies. The actual mechanism of above said properties will be focussed more in future study.

Ethical Issues

The experiment was carried out after the approval from IAEC (Institutional Animal Ethics Committee), GIET School of Pharmacy, Andhra Pradesh, India. (CPCSEA Reg No-1069/PO/ac/07/CPCSEA).

Conflict of Interest

The author has no conflict of interest to declare.

ACKNOWLEDGMENT

The authors are thankful to the management of GIET School of Pharmacy, East Godavari district, Rajahmundry, Andhra Pradesh, India for providing the necessary research facility and support.

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