

ANTI-ARTHRITIC AND ANTI-OXIDANT ACTIVITY OF CRAB SHELL CHITIN AND BEE VENOM MELITTIN BY PAPAINE INDUCED OSTEOARTHRITIS IN RABBITS

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ABSTRACT

Osteoarthritis (OA) is a common joint degenerative disease associated with ageing and excessive weight as important risk factors. As Matrix metalloproteinase's (MMPs) are the prominent biomarkers in degenerative diseases. Our objective was to investigate whether the bee venom and chitin has inhibitory activity on MMPs on papain induced osteoarthritis in rabbits. Bee venom was collected by electric stimulation method and chitin was extracted from crab shells by fermentation. To assess the activities, MMP-3 levels in synovial fluids are estimated by zymography method. Treatment with bee venom (1.2mg/kg s.c) and chitin (80mg/kg i.a) reduced MMP-3 levels in synovial fluid. Our data shows that bee venom and chitin has MMP-inhibition when compared with prednisolone treated group and control group the data obtained were significant. The anti-oxidant activity of Bee venom and Chitin was evaluated by free radical scavenging activity by DPPH (1,1-Diphenyl-2-picrylhydrazyl) method.

KEYWORDS: Bee venom, Melittin, Chitin, Matrix metalloproteinase, Osteoarthritis.

1. INTRODUCTION

Osteoarthritis (OA) is the most common form of arthritis seen clinically. This disease is thought of as the "wear and tear" or primary degenerative form of arthritis. Osteoarthritis (OA), It is the most prevalent disorder of the musculoskeletal system that is believed to be a consequence of mechanical and biological events that destabilize the normal coupling of degradation and synthesis within articular joint tissues. Episodic inflammation at the clinical stage is a well documented phenomenon and believed to be involved in the disease progression in (OA).^[1] The term arthritis implies an inflammatory process, which in fact may not necessarily be involved in many of the cases of osteoarthritis. It is for this reason that many use the term arthrosis or degenerative joint disease (DJD) for this condition. Unlike rheumatoid arthritis, which usually effects the respective joints symmetrically (both knees, both hands etc.), OA often occurs in one joint without similar pathology in its symmetrical equivalent. Osteoarthritis (OA) is characterized by a slow and gradual onset, usually starting with morning stiffness in a few weight-bearing joints (especially the knees). Eventually, pain is associated with movement leading to loss of joint function.^[2]

Bee venom(BV) is a poison which is secreted from stings of *Apis mellifera L* to defend themselves from prey, the venom of honey bees contains a large number of therapeutic substances which have prominent pharmacological applications in folkloric medicine of china. Bee venom has been used since ancient times in traditional medicine to treat various diseases which is known as "APITHERAPY", the origin of Apitherapy can be traced back to ancient Egypt, Greece and has been practiced in China for 3-5000 years and healing properties are included in many religious texts including the Vedas, Bible and Quran.³ BV contains a variety of peptides including melittin, apamin, Adolapin, and the MCD peptide. It also contains enzymes (e.g.,PLA2), biologically active amines (e.g., histamine and epinephrine) and non-peptide components (including lipids, carbohydrates and free amino acids.⁴ Bee venom has a wide pharmacological activities like Anti-inflammatory, Anti-arthritic, Anti-Nociceptive/ Analgesic, Anti cancer, Anti oxidant activity, anti bacterial, Multiple sclerosis, Lupus, Sciatica, Low back pain and tennis elbow.^[4,5]

Chitin is rich in marine products like shells of crustaceans, crab shells. The Mud crab *Scylla Serrata* contains more than 60% of chitin in their shells which can be easily extracted by utilizing micro-organisms and

enzymes.^[6] chitin is translucent, pliable, resilient, and quite tough. It is slightly soluble in water and hygroscopic and forms a soft and gel like slimy texture when placed in long contact with water. The medicinal properties and uses of chitin include Anti-microbial activity, Anti-Oxidant activity, Anti-cancer activity, Anti-Inflammatory activity, Anti-fungal activity, Immuno-Stimulating activity.^[7,8]

Based on the above information it could be hypothesized that Bee venom melittin and chitin would inhibit the MMP's produced in the osteoarthritis, which could reduce the further degradation of cartilage and have protective activity. The present study was aimed at investigating the anti arthritic activity of melittin from bee venom, and chitin from crab shell against papain induced arthritic model in rabbits.

2. MATERIALS AND METHODS

2.1. Collection of Bee Venom^[9]

Bee venom was collected by using bee venom collector by electric stimulation method. On an average about 1-2 mg per day was collected and the collected bee venom was dried and stored at 4°C for further usage.

2.2. Extraction of Chitin from Crab Shells^[10]

Mud crab *Scylla serrata* shells were collected, washed, crushed and subjected to fermentation by using demineralization and deproteination microorganisms and the residual chitin was collected mixed with organic mixture of chloroform, methanol and water(1:2:4) for separation. The extracted chitin was used for study.

2.3. Animals

New Zealand white, rabbits weighing 2 to 3 kg purchased from Acharya N.G. Ranga Veterinary university rabbit research division, Rajendranagar,

Hyderabad. The animals were brought in the transport cages and were housed in quarantine conditions for seven days to be monitored for normalcy and to allow time for acclimation to the new environment and handling. The rabbits were placed individually into 4 square foot stainless iron cages and all housed in the same area with an ambient temperature of 21-22°C and a relative humidity of 50-70%. A 12:12h light: dark (photoperiod) was maintained. All the precautions were taken to avoid the contamination due to materials and humans. The rabbits are fed on standard 14% protein Diet obtained from National centre for laboratory animal sciences NIN, Hyderabad. All the Experimental procedures and protocols used in the study were reviewed by institutional Animals Ethics Committee (Regd No: 1662/PO/a/CPCSEA, 2013) and were in accordance with the guidelines of CPCSEA.

2.4. Preliminary analysis of drugs

2.4.1. Analysis of Bee venom by HPLC method^[11,12]

The method developed by *Helena et al.*^[12] was followed by using C18 column with pore size 180Å and a flow rate of 2ml/min and eluents like 0.1% trifluoro acetic acid and Acetonitril:water (80:20) were used. The compounds were identified by using UV detectors at 220nm wavelength.^[11]

2.4.2. FTIR Analysis of Crab shell Chitin

The Crab shell chitin samples were characterized in potassium bromide (KBr) pellet by Infrared Spectrophotometer in the range of 400 to 4000 cm⁻¹ which was according to *limam et al.*^[7]

2.5. Experimental Methodology

2.5.1. Grouping of animals

The rabbits were divided into 6 groups in equal number randomly (n=6) with both sexes.

Table 1: Grouping of animals.

S. No	Group number	Treatment received
1	Group I	Normal control received Normal saline(0.3 ml/joint i.a)
2	Group II	Papain induced osteoarthritic control.
3	Group III	Papain induced osteoarthritis animals received Standard drug prednisolone (10mg/kg, s.c).
4	Group IV	Papain induced osteoarthritis animals received Bee venom (1.2mg/kg s.c) and chitin (80mg/kg, i.a).
5	Group V	Papain induced osteoarthritis animals received Bee venom 1.2mg/kg s.c)
6	Group VI	Papain induced osteoarthritis animals received Chitin (80mg/kg, i.a)

2.5.2. Osteoarthritis induction by Papain^[13-18]

Osteoarthritis was induced in both ankles in all groups except the normal control group by injecting 0.2 ml of 4% papain solution^[13] with 0.1 ml of 0.03 M cystein as activator intra-Articularly.^[14,15] Same amount of saline was injected into the ankles of the first group. To facilitate easy intra articular injection^[16] the rabbits are to be anaesthetized with Ketamine and Xylazine (10/3.0mg/kg IM). The induction of osteoarthritis starts on day 8th after the acclimatization of seven days and injection (i.a) was repeated on the day 11, 14 and 15th

day of study for perfect cartilage degradation.^[17,18] The treatment was started on 16th day and the synovial fluid was collected from tibio-calcaneal or ankle joint on 20,28 and 35th day.

2.5.3. Synovial fluid Aspiration

Synovial fluid was collected from Tibio-calcaneal or ankle joint. The rabbits were anesthetized by combination of Ketamine hydrochloride and Xylazine Hydrochloride (10/3.0mg/kg IM).¹⁹ before anaesthetizing as a preanaesthetic medication the rabbits were

Atropinized with Atropine sulphate (0.05mg/kg IM).^[20] both stifles were shaved, aseptically prepared, and the ankle joints were lavaged with sterile saline. A 22 gauge, 1 inch needle was inserted adjacent to the lateral aspect of the tibia into the ankle joint.^[21-25] A 1 ml syringe was used to inject 200µl of sterile saline that was immediately aspirated back. In the Intra-Anesthetic period the body Temperature was maintained at 37°C with heating pads which also helps in fast recovery from Anesthesia. To avoid respiratory inconvenience Resuscitation tube was used.

2.5.4. MMP estimation by Zymography

2.5.4.1. Measurement of protein concentration

The protein concentration in synovial samples was estimated by using BCA (bicinchoninic acid) method and following the instruction of Pierce® BCA Protein Assay Kit. After adding the reagents A and B measure the absorbance at 562nm and calculate the amount of protein in the samples.^[26]

2.5.4.2. Zymography methodology^[27-31]

The gelatinolytic activity of MMPs was measured essentially as described *Fernandez-Patron et al.* The samples of conditioned were mixed with electrophoresis loading buffer (10:1, vol: vol, 20µl per well) and subjected to electrophoresis, which was carried out on a 7.5% SDS-PAGE co-polymerized with gelatin (2 mg/ml).²⁷⁻³⁰ Gelatin was added from a 10 fold concentrated stock containing pre-boiled gelatin type A (20 mg/ml) and 1% SDS. Following electrophoresis, gels were washed with 2.5% Triton X-100 for 1 hr (3 times, 20 min each) and incubated for 48 hrs in incubation buffer (25 mM Tris base, pH 7.5, 5 mM CaCl₂, 0.9% NaCl, 0.05% NaN₃) for the development of enzyme activity bands. After incubation, the gels were stained with 0.05% Coomassie brilliant blue G-250 in a mixture of methanol: acetic acid: water (2.5: 1: 6.5) and destained in 4% methanol with 8% acetic acid. The gelatinolytic activities were detected as transparent bands against the background of Coomassie brilliant blue-stained gelatin. If more sensitivity was necessary, the gels were further destained in a solution of 1% Triton x-100 (1–2 hrs). To measure the activities of the detected enzymes, zymograms were scanned and saved as jpg/png image file.^[31] The intensity of the bands was expressed as arbitrary units and analyzed using Myimage analysis V2.0 (Thermo scientific, 3747N.Meridian Road Rockford, IL 61105 USA).

2.5. In vitro Anti-oxidant activity of Bee venom and Chitin by DPPH method^[32]

The free-radical scavenging capacity of Bee venom and chitin was evaluated according to *Brand Williams et al*³². The percentage of anti oxidant activity (AA %) was assessed by DPPH free radical assay. A 0.1mM alcoholic solution of DPPH in methanol was prepared and 2mL of this solution was added to 0.3mL of different Bee venom and chitin concentrations (20-100 µg/ml) and allowed to

react at room temperature. After 30min, the absorbance values were measured at 517nm.

The reaction mixture of 2ml of 0.1mM alcoholic DPPH solution with 3ml of different concentrations (20-100µg) of L-Ascorbic acid acts as standard, the reaction mixture without sample or standard drug acts as blank. The radical scavenging activity (AA %) was expressed as percentage of DPPH radical elimination calculated according to the following equation:

$$AA\% = 100 - \frac{(ABS_{sample} - ABS_{blank})}{ABS_{control}} \times 100$$

2.6. STATISTICAL ANALYSIS

Statistical analyses were performed by using Student's t-tests and one-way analysis of variance (one way ANOVA). A value of P<0.05 was considered significant. Data are presented as means ± S.D.

3. RESULTS

3.1. Analysis of Bee venom by HPLC

The HPLC study for development of the method for separation, identification and assay of individual components of honeybee venom shown in Fig-I showed that chromatographic separation obtained using columns packed with C18 material with pore size 180Å have good Separation. Retention time for apamine was 8.617 min, for phospholipase A² 20.467 min and for melittin was 28.075 min. The amount of melittin was found to be 61% and that of apamine 2.09% and Phospholipase A² was 12%.

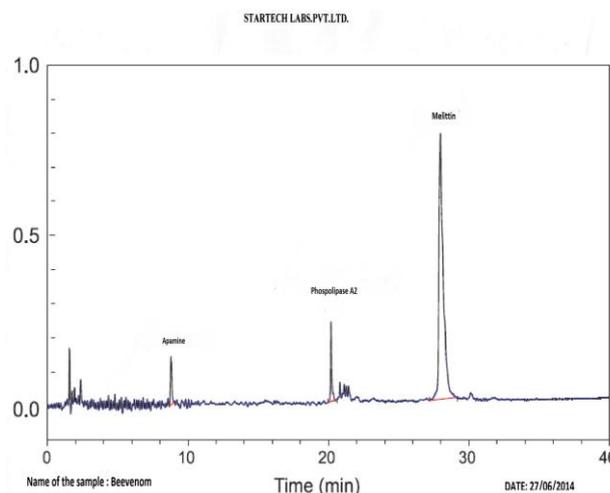


Fig-1: HPLC analysis of Bee venom.

3.2. Analysis of Chitin by FTIR

The crab shell chitin showed an intense peak at 1406 cm⁻¹ which corresponded to the N-H deformation of amide II. The bands at 1516 cm⁻¹ and another at 1583 cm⁻¹ are attributed to the vibrations of the amide I band, and the band at 1627 cm⁻¹ corresponds to the amide I stretching of C=O. The band at 1697 cm⁻¹ could be attributed to the stretching of C-N vibration of the superimposed C=O group, linked to OH group by H bonding. These bands

can be clearly observed in all samples. The sharp band at 1217 cm⁻¹ corresponds to a symmetrical deformation of

the CH₃ group, the results of FTIR spectra of chitin are shown in Fig-2.

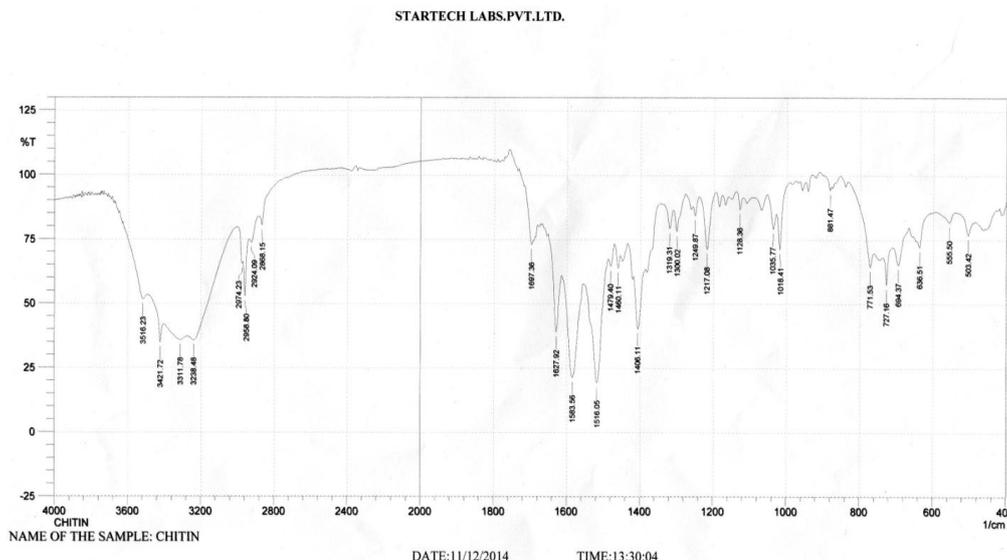


Fig-2: FTIR Analysis of Chitin.

3.3. MMP Estimation by zymography
Effect of Bee venom and Chitin on MMP in Synovial Fluid.

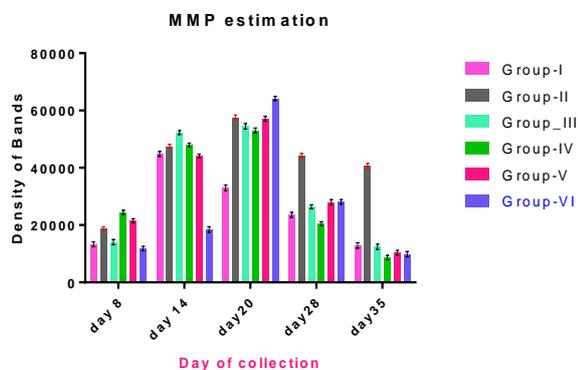
The results shown in Table 2 and Graph-I are the density of bands in zymograms which are expressed as Mean±S.D. The results of treatment Groups shown a

significant effect when compared with osteoarthritic group. The bee venom treatment and chitin treatment groups shown significant results and effective in suppression of MMP levels which can be visualized from Graph-I. The Fig-3 to 7 given the visuals of MMP levels at different time of synovial fluid collection.

Table 2: Effect of Bee venom and Chitin on MMP-3 in Synovial Fluid.

S.No	Group	Density of Bands (Intensity /area)				
		Day-8	Day-14	Day-20	Day-28	Day-35
1.	Group-I	13353±245.37	44846±636.42	32993±159.1	23590±896.4	12896±917.3
2.	Group-II	18243±612.5 ^a	47508±123.0 ^b	57577±870.5 ^a	44300±187.3 ^a	40693±129.7 ^a
3.	Group-III	14061±79.94 ^a	52285±107.5 ^{ns}	54513±915.6 ^b	26421±177.4 ^a	12448±119.5 ^a
4.	Group-IV	24488±716 ^{ns}	47958±127 ^c	53029±132 ^a	20482±108.9 ^a	8669±129.6 ^a
5.	Group-V	21550±154 ^{ns}	44096±143 ^a	57111±111 ^c	27958±100.4 ^a	10374±824.4 ^a
6.	Group-VI	11795±140 ^a	51762±126 ^{ns}	64167±134 ^{ns}	28085±106.9 ^a	2671±137.0 ^a

All the results are expressed as Mean±S.D, n=6. Group I is compared with Groups II. Group III,IV, V and VI compared with Group II. a = p<0.001, b = p<0.01, c = p<0.05, ns = Not significant.



Graph-1: Effect of Bee venom and Chitin on MMP in Synovial Fluid.

All the results are expressed as Mean±S.D, n=6. Group I is compared with Groups II. Group III, IV, V and VI compared with Group II. a = p<0.001, b = p<0.01, c = p<0.05, ns = Not significant.

MMP Zymographs

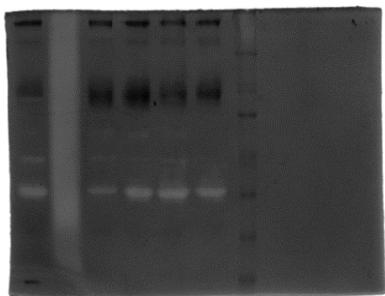


Fig-3: Day 8 MMP Zymography

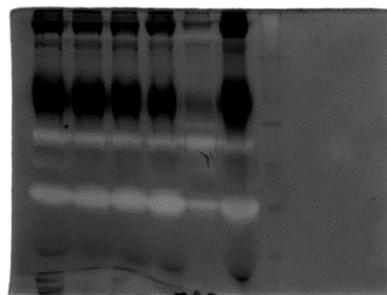


Fig-4: Day 14 MMP Zymography

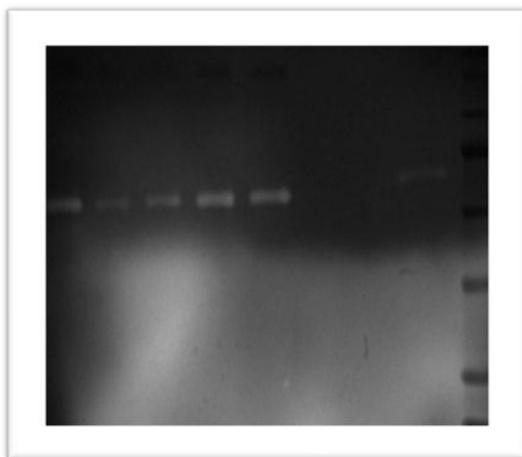


Fig-5: Day 20 MMP Zymography.

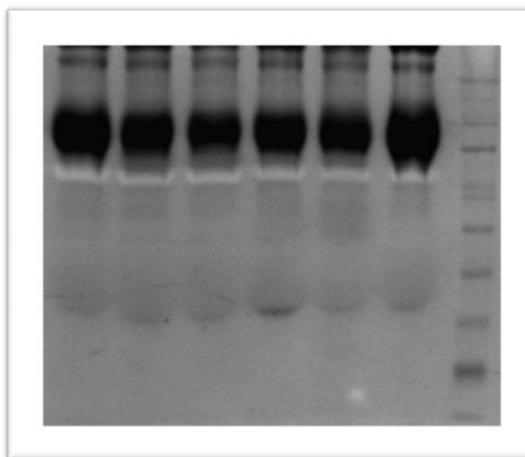


Fig-6 Day 28 MMP Zymography.

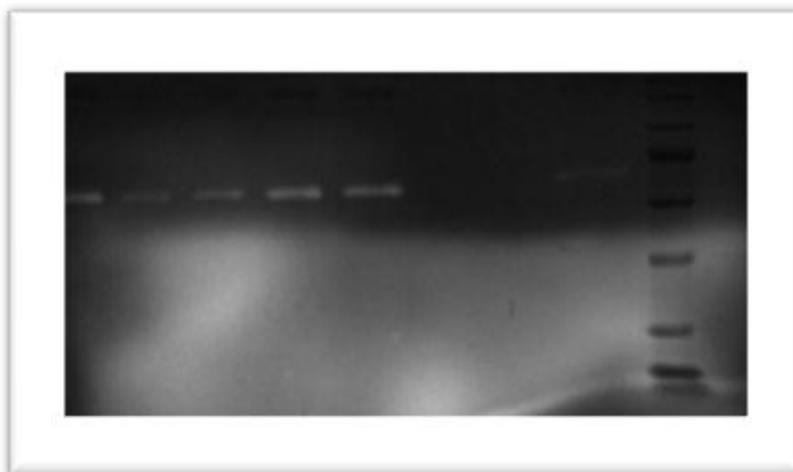


Fig-7: Day 35 MMP Zymography.

3.4. In vitro Anti-oxidant activity of Bee venom and Chitin by DPPH method

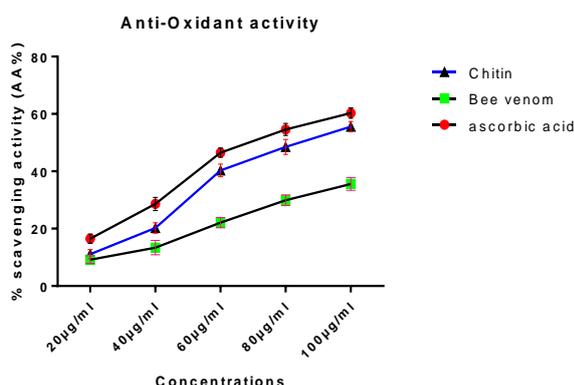
From the results shown in **Table-3** and **Graph-2** Crab shell chitin showed moderate to high antioxidant activities of 48.5-55.6% at 100 μ g/ml concentration but in case of Bee venom it is just 35.6% which make it clear that the chitin has potential Anti-oxidant activity when compared to standard L-Ascorbic acid (60.3%). the

results of Bee venom are not satisfactory as it shown only 35.6% at 100 μ g/ml concentration. The Anti-Oxidant activity of Bee venom and Chitin would help in scavenging the free radicals produced in diseased condition and reduce the incidence of disease.

Table: 3: In vitro Anti-Oxidant activity of Bee venom and Chitin by DPPH method.

S.NO	Conc. µg/ml	% of Scavenging activity (AA %)		
		Ascorbic acid	Bee venom	Chitin
1.	20	16.5±0.9	9.2±0.3	11.1±0.2
2.	40	28.6±0.4	13.4±0.8	20.2±0.4
3.	60	46.5±0.6	22.1±0.3	40.4±0.3
4.	80	54.6±0.4	29.9±0.8	48.5±0.4
5.	100	60.3±0.2	35.6±0.8	55.6±0.6

Values are Expressed as Mean ± S.D, n=3.

**Graph-2: In vitro Anti-Oxidant activity of Bee venom and Chitin.**

4. DISCUSSION

Osteoarthritis is a common disorder causing disability in most of geriatric patients and it needs an effective treatment for living of Old age. It is a Degenerative disorder that can be treated by applying Alternative therapies. The aim of the present study was to gain a better understanding of the therapeutic effects of melittin on OA patients. Here in the present study we aimed to inhibit the Disease causing mediators like MMP's. The rabbit model of osteoarthritis with papain induction exactly produces the early stages of OA which make it easy to draw conclusions and extrapolate the results for Humans.

In the pathogenesis of arthritis, the joint destruction is due to the degradation of the extracellular matrix in articular cartilage, which is mainly composed of type II collagen. The remodeling and breakdown of the cartilage matrix were caused by the MMP expression through cleavage to the ECM. Particularly, MMP-3, also known as collagenase 1, is well characterized as degrading cartilage in the progression of arthritis.^[33]

Melittin is a small protein that contains 26 amino acid residues, and represents the principal toxic compound in bee venom. Because it shares its amphipathic properties with a series of peptides, it has been shown to have a detergent-like action. Although melittin is a toxic peptide, it has long been investigated because of its potential therapeutic effects. Especially due to its anti-arthritis property, it continues to attract much interest. Melittin has an anti-inflammatory effect by inhibiting LPS-induced inducible nitric oxide synthase (iNOS) and

cyclooxygenase-2 (COX-2). In addition, melittin inhibits LPS-activated MMP-3 production in human arthritic chondrocytes. Bee venom also induces apoptosis through caspase-3 activation in rheumatoid synovial fibroblasts. Accordingly, the results of this study explained better the clinical effect of bee venom and melittin on arthritis. Thus, we sought to identify the mechanisms involved in the inhibitory effects of melittin on TNF- α -mediated reduction of type II collagen and MMP expression.^[33] In the present study, we demonstrated that melittin specifically inhibits LPS-activated MMP-3 production.

5. CONCLUSION

To the final conclusion from the results obtained by different parameters assessed and the observations made it was clear that Bee venom and Chitin has the ability to inhibit the MMP-3 produced during the Osteoarthritis which was shown in the results of estimation of MMP's by zymography, it is a reflection to the Anti-arthritic activity of Bee venom and chitin. The In vitro Anti-oxidant activity results are clear that chitin possess larger and potential free radical scavenging effect than that of Bee venom when compared with the standard drug L-Ascorbic acid.

So, finally it can be said that from the results obtained the drugs used in the present study has the Anti-Arthritic and Anti-Oxidant activities which still need some more extension of study to get a perfection to make it applicable for human therapies. The present study drugs can also help in the treatment of other diseases like Cancer and cardiovascular studies because they too involve the same biomarkers.

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Conflict of interest

We declare that we have no conflict of interest.

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