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ANTI-INFLAMMATORY AND ANTI- HYPERURICEMIC EFFECT OF URICASE FROM *Bacillus subtilis* MM13 AS A POTENTIAL THERAPEUTIC AGENT FOR REDEMPTION FROM GOUT ISOLATED FROM BIRDS FECAL CONTAMINATED SOIL

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ABSTRACT

Gout, the most common inflammatory arthritis is associated with a persistently raised plasma uric acid concentration. When these levels rise above the solubility limit it results in crystal deposition in joints, acute inflammation in response to those crystals causes severe pain. Treatment for severe gout includes injection of non-human uricase and anti-hyperuricemic and anti-inflammatory agents to reduce uric acid levels and inflammation. In the present study, the anti-inflammatory and the anti-hyperuricemic activity of the uricase produced by *Bacillus subtilis* MM13, isolated from poultry soil was evaluated in LPS-induced RAW 264.7 cells. To measure the effects of the uricase produced by *Bacillus subtilis* MM13, MTT assay, Trypan blue assay, xanthine oxidase activity assay, uric acid production and Gene expression analysis were performed. MTT and trypan blue assay demonstrated that uricase suppressed cell proliferation in a dose-dependent manner. The *in vitro* anti-hyperuricemic effect of uricase was identified due to the inhibition of Xanthine oxidase in LPS-induced RAW cells. Gene expression studies showed that Uricase suppressed the expression of inflammatory cytokine,

tumour necrosis factor α (TNF- α), and increased production of IL-10, a potent inhibitor of TNF- α. Considering the appreciable *in vitro* anti-hyperuricemic and anti-inflammatory activities, it is proposed that this preparation can be further studied for safety and efficacy under *in vivo* conditions which can be subsequently used for the prophylactic treatment of gout.

Keywords: Uricase, hyperuricemic, MTT assay, Trypan blue assay, prophylaxis

INTRODUCTION

Gout, the most common inflammatory arthritis with a prevalence ranging from 1–4% worldwide is associated with a persistently raised uric acid concentration. In India, approximately 0.12-0.19% population is affected by gout and is been reported as a disproportionate burden of disease in men, the elderly and racial/ethnic minorities **[1]**. When the serum uric acid levels rise above 7.0 mg/L, hyperuricemia occurs where the tissues are supersaturated with uric acid; subsequently, monosodium urate crystals accumulate in and around the joints where they are phagocytosed by neutrophils, macrophages, mast cells and dendritic cells within the synovium, which release inflammatory cytokines such as tumour necrosis factor (TNF-α) and can cause severe pain **[2]** affecting the individual's normal life routine.

Developed countries have seen a surge in the prevalence of gout and hyperuricemia over the past two decades, necessitating more intensive research **[3]**. It has been reported that about 80–90% of people with gout are hyperuricemic, and 10% of the patients with hyperuricemias develop gout **[4]**. Chronic hyperuricemia may lead to symptoms of gout, chronic kidney disease, hypertension, and cardiovascular diseases **[5, 4]**. Hyperuricemia and its associated diseases can be treated by an agent that upholds urate concentrations from precipitating and eliminate prevailing urate crystals. Uric acid-lowering agents (ULTs) classified into three main classes: xanthine oxidase inhibitors that reduce uric acid synthesis, uricosurics that inhibit URAT1 thereby increasing uric acid excretion, urolytics/ uricase that enhance metabolic hydrolysis of uric acid **[3]** have been used conventionally in the treatment of hyperuricemia **[6]**.

Owing to this, uricase from microbial sources can be represented as an alternative approach that can be more effective than the modified uricase formulations. In arthritic gout and renal disease, microbial uricase has emerged as a potential source of therapy for hyperuricemia **[7]**. Fermented extracts from microbes with substantial XOD inhibition activity and high uricase activity have also been

reported **[6]**.

Though various bacteria such as *Bacillus* species **[8]**, *Microbacterium* sp. **[9]** and *Streptomyces* **[10]**, have been reported as uricase producers, not all bacterial uricase are thermos table and their biocompatibility and anti-inflammatory and the anti-hyperuricemic activity are unknown. Uricase enzymes with these properties will have the advantage for mass production, transportation, storage and clinical application. Among the other bacteria's, urate oxidase from thermophilic *Bacillus* sp. TB-90 has been reported to be used for diagnostic purposes since it reveals high activity and thermo stability in an extensive range of pH **[11]**. Based on this theory, *Bacillus subtilis* was isolated from soil samples in and around Hosur area, Krishnagiri District, Tamil Nadu and an isolate that produced the highest uricase activity was identified, designated as *Bacillus subtilis* MM13 **[12]** and its efficacy was evaluated. The main objective of this study is to evaluate the anti-inflammatory and the anti-hyperuricemic activity of the potent local uricase produced by *Bacillus subtilis* MM13 and it was evaluated in LPS-induced RAW 264.7 cells.

MATERIALS AND METHODS

Chemicals and Reagents

Chemicals and reagents used in the present study were procured from standard chemical manufacturers and distributors (Sigma Aldrich Chemicals Pvt. Ltd).

Cell culture

RAW 264.7 cells were procured from the Cell repository of ATCC. These cell lines are cultured in 1X Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (PenStrep), and maintained in a humidified atmosphere of 5% CO_2 at 37 °C. When confluent (80% confluence), cells were passaged and used for further experiments in an exponential growth stage.

Cytotoxicity Evaluation

Effects of uricase was evaluated using a modified MTT assay **[13]**, a mitochondrial-dependent reduction of 3-(4,5-dimethylthizaol-2yl)2,5-diphenyl tetrazolium bromide (MTT) to formazan. The plates were kept on a shaker for 10 min at room temperature and then the absorbance was measured using a microplate reader at a wavelength of 490 nm and 630 nm. The control group consisting of untreated cells was considered as 100% of viable cells after carrying out the assay, the inhibition rate was calculated using the following formula:.

% Inhibition Rate = {1- [A_{490} - A_{630} (Treated) / A_{490} – A_{630} (control)] x 100

Trypan Blue Exclusion Assay

Based on the principle that living cells possess

intact cell membranes, the dye exclusion test is used to determine the number of viable cells present in a cell suspension **[14]**. Viable (unstained) and nonviable (blue-stained) cells were counted using a hemocytometer and calculated as given below.

The average number of cells per field = Sum of cells per field /Number of fields

Total number of cells per mL ($x* 10^4$/mL) = Average number of cells per field × dilution factor.

(The dilution factor is 2 because the cells were with equal volumes of trypan blue)

%Viability = Number of colourless cells/Total number of cells × 100

Morphological Analysis - Phase Contrast Microscopy

LPS induced RAW264.7 Cells (5×10^5 cells/well) were incubated for 24 h with selected four concentrations of uricase (0, 30, 50, and 100 μg/ml) in tissue culture dishes (60 mm diameter). After incubation, the medium was discarded and cells were washed once with Phosphate Buffer Saline (1X PBS). The morphological changes observed were viewed at 200x magnification **[15]**.

Xanthine oxidase (XO) activity assay

The Xanthine oxidase activity was carried out by **[16]**. Xanthine oxidase levels were measured, an increase in the absorbance indicates the formation of uric acid from xanthine. The treated cell pellets were mixed with assay buffer and the supernatants were isolated and pre-incubated at 37 °C for 40 min. Blanks contained the same reaction mixture without xanthine. A volume of 0.1 mL of 100% (w/v) trichloroacetic acid (TCA) was added to the working solution and the mixture was centrifuged at 5000 x g for 10 min and a second absorbance (A2) was measured again at 293 nm. The absorbance change (A) is calculated as follows:

A = [(A2) assay - (A2) blank] – [(A1) assay - (A1) blank]

Note: A/min =A/30

Uric acid Estimation

Uric acid was estimated using a commercial Uric Acid Kit (Uricase - PAP method, *Tulip Diagnostics*) according to the manufacturer's instruction. Briefly, RAW264.7 cells were plated at 5×10^5 cells/well. The contents were mixed well and incubated for 5 min at 37 °C and the absorbance was measured at 520 nm within 30 min after incubation and calculated as follows:

Uric acid in mg/dL = (Absorbance at T / Absorbance at S) X 8

Gene Expression Analysis

RAW264.7 cells were seeded in culture flasks induced with 500 ng/mL LPS for 4 h and treated with 30, 50, and 60 µg/mL of uricase for 24 h. DNA was isolated using a Nucleospin

DNA isolation kit according to the manufacturer's instruction and PCR was performed with the below-given primers:

β-Actin

5' TCAAGGTGGGTGTCTTTCCTG 3' (F)

5' ATTTGCGGTGGACGATGGAG 3' (R)

TNF- alpha

5'ACACAGAAGACACTCAGGGA 3' (F)

5' CCGGTACTTAACCCTACCCCC 3'(R)

IL10

5'AATCACGGCTCAGTTCTCCC 3'(F)

5' GGTCACACCATTTGCTGGGT 3'(R)

The amplification sequence protocol was conducted for 35 cycles: Initial denaturation - 1 cycle at 95 °C for 2 min, DNA denaturation - 95 °C for 1 min, Annealing Temperatures - 54 °C (β-Actin), 54.6 °C (IL-10) and 55.4 °C (TNF- alpha) for 30 s and Extension - 72 °C for 1 min, Final extension - 72 °C for 5 min.

RESULTS:

MTT ASSAY

The inhibitory effect of uricase was assessed on RAW264.7 mouse macrophage cells using the MTT assay in **Figure 1**. The cells were treated with uricase at concentrations of 10 - 100 µg/ml for

24 h. Uricase mediated cell proliferation in a dose-dependent manner suggests that uricase inhibits the growth of RAW264.7 cells. The LC50 was determined to be 50 µg/ml concentrations with 54.26% inhibition.

Trypan Blue Exclusion Assay

Trypan blue exclusion assay, the most common and earliest method was used for cell viability measurement. Cells that are compromised by uricase treatment become permeable and it binds to the intracellular proteins and render them bluish colour and the cell death rate was determined. Dose-dependent cell death was observed, cell viability decreased with the increasing concentration shown in **Figure 2**.

Morphological changes by Phase Contrast Inverted Microscope

Changes in the cellular morphology under uricase treatment were observed in different concentrations in **Figure 3**. Similar to the cell proliferation and cell inhibition assays the cells shrank and subsequently detached in a dose-dependent manner when compared with the control. Untreated RAW264.7 cells appeared normal (circular shaped).

Xanthine Oxidase assay.

Figure 4 illustrates the effect of three different concentrations of uricase (30 µg/mL, 50 µg/mL and 60 µg/mL) as compared to LPS induced RAW cells and control (untreated cells), in inhibition of xanthine oxidase enzyme. All three concentrations of uricase showed similar XO inhibitory activity. Results were subjected to one-way ANOVA (Tukey multiple comparison tests).

Uric acid Estimation

In vitro effects of uricase, uric acid concentration in the cells are illustrated in **Figure 5**. The uricacid concentration was significantly reduced by uricase in all three concentrations (30 µg/mL, 50 µg/mL and 60 µg/mL) after pretreatment with LPS compared with the non-treated LPS induced group ($p < 0.0001$).

Gene Expression Analysis

The roles of pro-inflammatory cytokine (TNF-α) and anti-inflammatory cytokines (IL-10) were investigated. Down-regulated gene expression levels were observed for TNF- α in uricase treated cells and an upregulated expression was observed for IL- 10 gene, in a dose-dependent manner in **Figure 6**. IL-10 is a potent inhibitor of TNF-α and administration of uricase enzyme at a defined concentration can help to reduce the pro-inflammatory cytokine TNF-α, thereby reducing cell damage.

DISCUSSION

The most frequent inflammatory arthritis, characterized by prevalence was higher than 1% in both occidental and oriental countries and was found to be gout **[17]**. It should be viewed seriously, due to its manifestation with various other complications including hypertension, cardiovascular risk, chronic kidney disorder, obesity, and metabolic syndrome **[18, 19]**.

MTT and trypan blue assay revealed that *Bacillus subtilis* MM13 uricase had strong inhibitory activity against LPS induced RAW cells in a dose-dependent manner (Fig. 1). At 100 µg/mL concentration more than 80% of cell growth was inhibited. The IC50 was determined to be 50 µg/ml by the MTT assay which was further confirmed by trypan blue assay. Hence, a concentration one below and one above the IC50 was selected for further studies along with the concentration which exhibited the IC50 range. Morphological evaluation on the concentrations 30 µg/mL, 50 µg/mL and 60 µg/mL were in line with the MTT and trypan blue assay results. Changes indicative of cell inhibition, where LPS induced RAW cells deteriorated, in a dose-dependent manner and parallel morphological changes including shrinkage, blebbing and distorted cell membrane followed by cellular detachment were observed in response to *Bacillus subtilis* MM13 uricase treatment (Fig. 3).

Xanthine oxidase plays the role of housekeeping and the rate-limiting enzyme during purine catabolism. In the present study, the inhibition of XO activity was found in all uricase treated concentrations (30 µg/mL, 50 µg/mL and 60 µg/mL). LPS induced RAW cells showed higher activity compared to treated and control groups ($p \leq 0.01$). All uricase concentrations showed inhibition of

more than 50%. Xanthine oxidase inhibitors(XOI) are considered the first-line drugs in ULT for gout **[20]**. Further **[21]** reported that hyperuricemic subjects showed that XOIs are more effective in secondary prevention, significantly reducing the occurrence of major adverse cardiovascular events in individuals with previous transient ischemic attacks, stroke, unstable angina, or myocardial infarction. In the present study, all uricase treated RAW cells showed inhibition of more than 50% revealing the anti-hyperuricemic effect of uricase. In the present investigation urate-lowering effects of *Bacillus subtilis* MM13 uricase was evaluated at different concentrations on LPS induced RAW cells in **Figure 5**. Uricase at all three concentrations (30, 50 and 60 µg/mL) was found to be effective in reducing uric acid, which was consistent with the XO inhibition results that suggested that the results of the hypouricemic action paralleled the reduction in XOI activities. As a preliminary evaluation, this study first confirmed that *Bacillus subtilis* MM13 uricase reduced the uric acid levels suggesting that this study shall be further performed under *in vivo* conditions to be confirmed as a therapeutic model.

Further elucidated the molecular mechanism of *Bacillus subtilis* MM13 uricase Gene expression analysis was carried out. Studies have proposed that stimulation of IL- I and TNF production by monosodium crystals maybe a crucial link and the inhibition of these could be a promising strategy **[22-25]**. In the present investigation, gene expression studies with uricase from *Bacillus subtilis* MM13 indicated a dose-dependent suppression of TNF-α. TNF-α was strongly down-regulated by a higher dose of uricase whereas a milder suppression was observable in a low dose of uricase. To further evaluate if uricase triggers cytokine suppression in LPS induced RAW cells, we estimated the expression of the IL-10 gene, a major anti-inflammatory cytokine. From our results, it is evident that IL-10 reduces the effect of TNF-α indicating that the uricase from *Bacillus subtilis* MM13 has significant anti-inflammatory activity. A report on IL-10 suggests that reducing the effect of TNF-α significantly decrease the secretion of PGE2, COX-2, and PLA2 **[26]**. The reduction in the levels of TNF- α in the treated cells indicates that the uricase from *Bacillus subtilis* MM13 has significant anti-inflammatory activity.

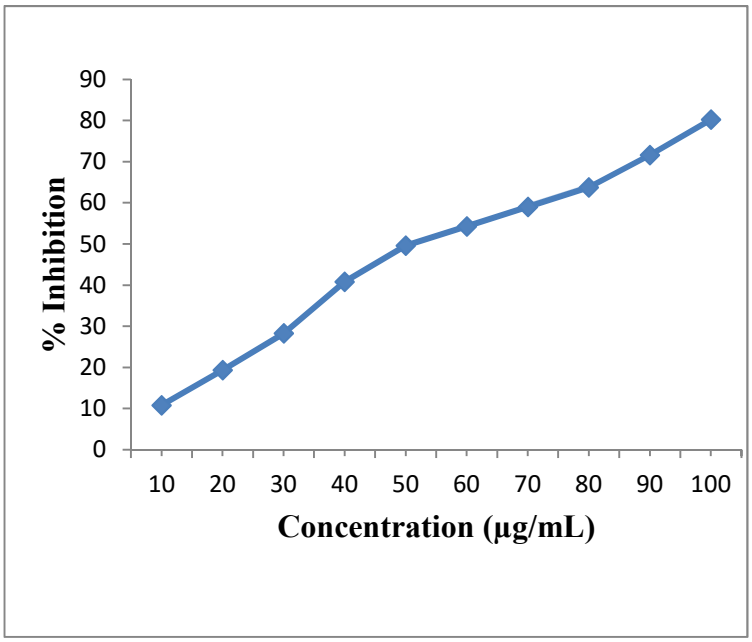

Figure 1: Cytotoxic effect of uricase on Raw 264.7 cells. Different concentrations of uricase (10-100 µg/mL) were used for cell treatment

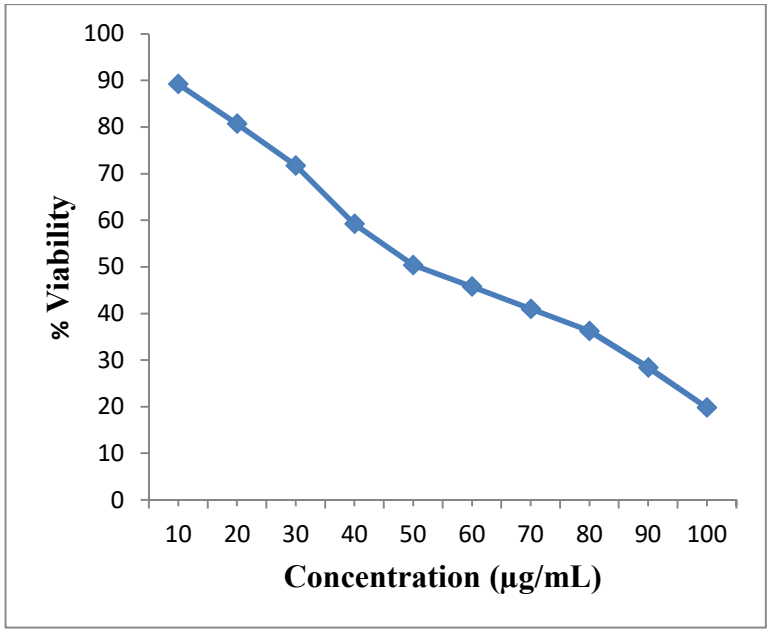

Figure 2: Effect of uricase on cell viability of RAW264.7 cell line - The cell death rate was determined using trypan blue assay. RAW264.7 cells were treated with Uricase (10 – 100 µg/mL) for 24 h. Uricase caused cell death in a dose-dependent manner

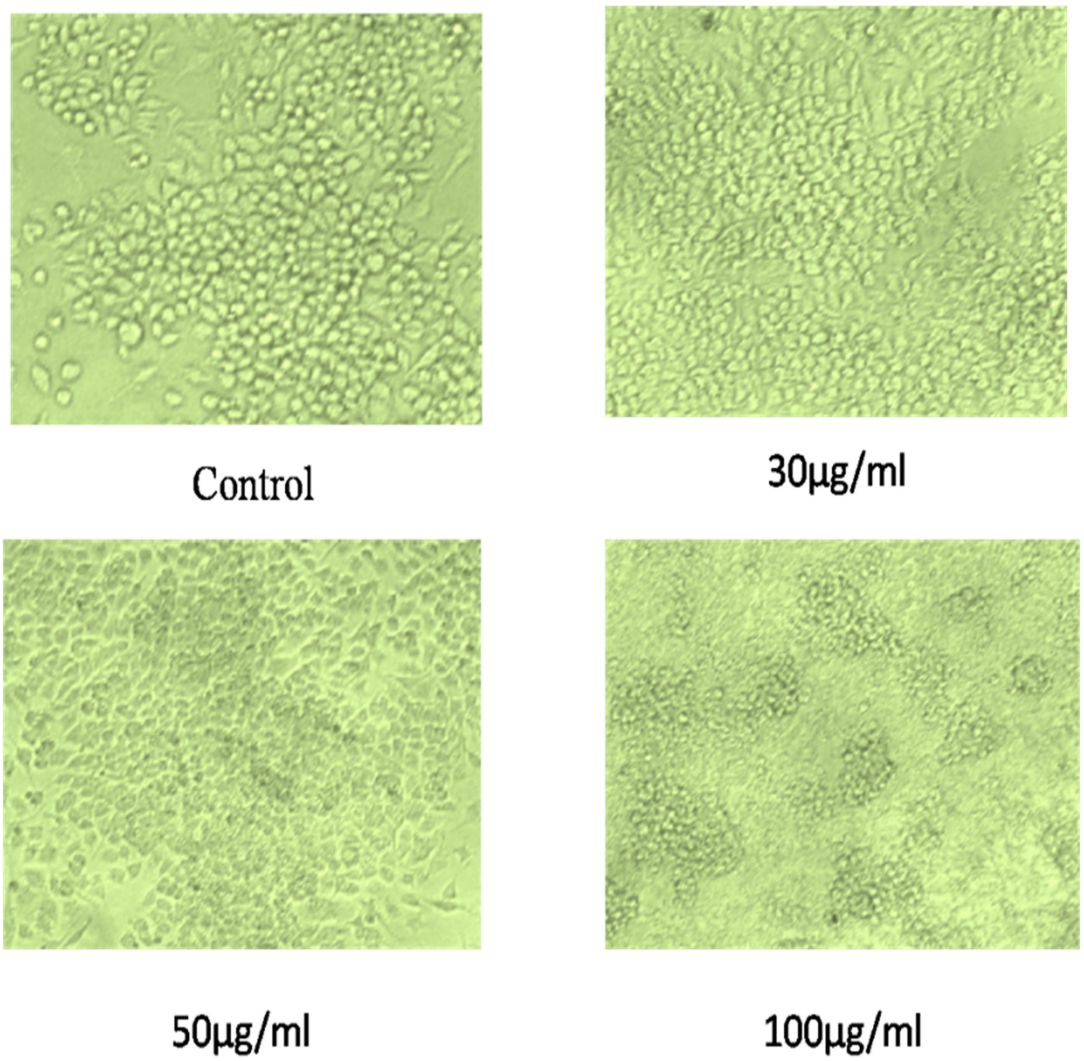

Figure 3. Effect of uricase on RAW 264.7 - Photomicrograph (20 x) represents morphological changes with Shrinkage, detachment, membrane blebbing and distorted shape induced by uricase treatment (30, 50 and 100 µg/ml for 24 h) as compared with control. Control showed normal intact cell morphology and their images were captured by phase contrast microscope.

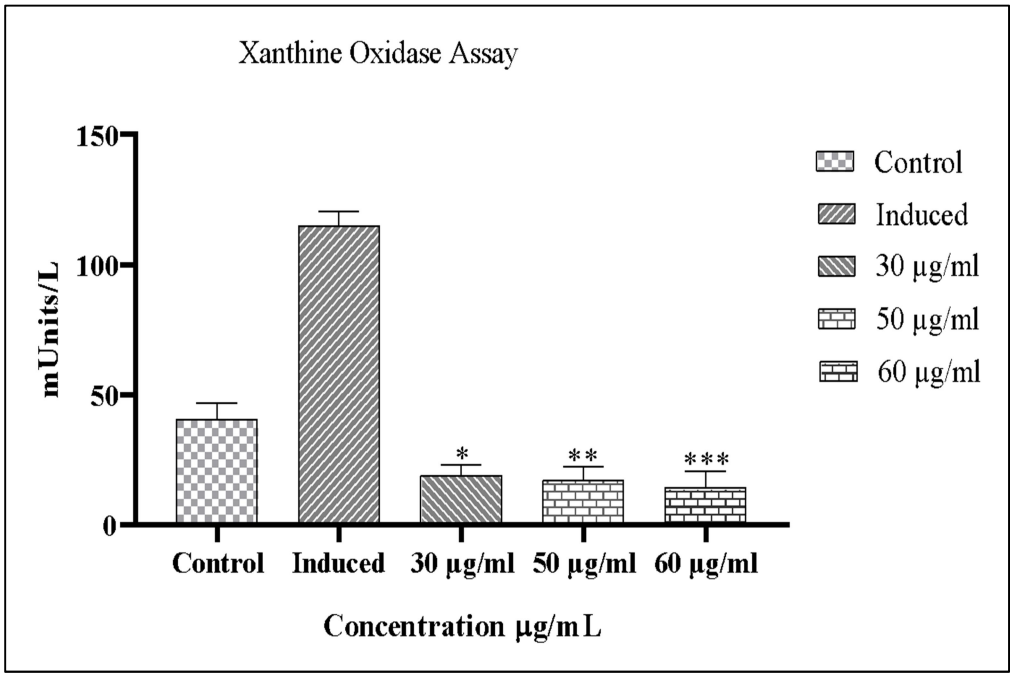

Figure 4: Xanthine oxidase assay - Xanthine Oxidase production of treated groups was significantly decreased when compared to induced group. Results are expressed as mean ± SD. Treated groups showed high significance compared to control (P< 0.001)

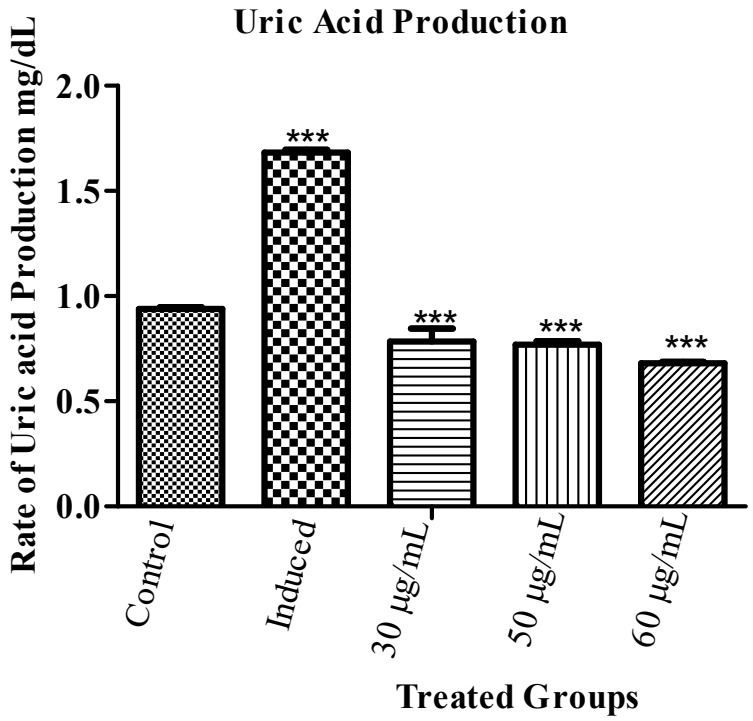

Figure 5: Estimation of Uric acid production - Uric acid production of treated groups was significantly decreased when compared to induced group. Results are expressed as mean ± SD. Treated groups showed high significance compared to control (P< 0.0001)

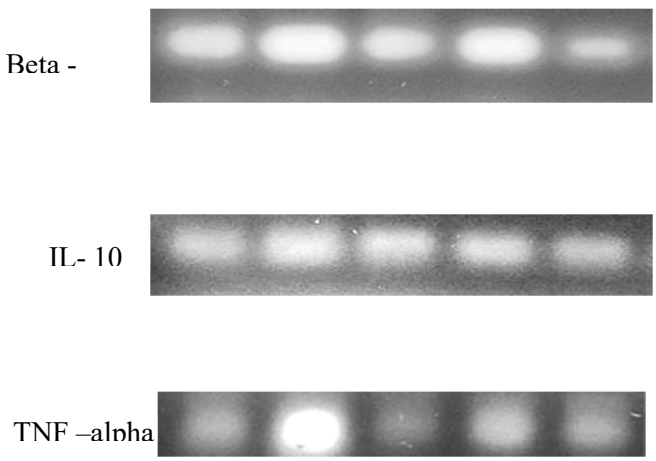

Figure 6: Gene expression analysis of TNF-α and IL-10. Decreased gene expression levels were observed for TNF- alpha in uricase treated cells and increased expression level was observed for IL- 10 gene, with increase in uricase concentration. Housekeeping gene (β – Actin)

CONCLUSION

This study provided conclusive evidence that uricase isolated from *Bacillus subtilis* MM13 has shown anti-inflammatory and anti-hyperuricemic effects under *in vitro*. One of the important properties of uricase is its very high specificity. Considering the appreciable specific anti-hyperuricemic and anti-inflammatory activities, this preparation can be taken further for safety and efficacy testing in vivo and subsequently can be recommended for the prophylactic treatment of gout.

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