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Research Article
Myricetin Exerts Anti-osteoarthritic Effects in IL-1β Stimulated SW1353 Cells via Regulating Matrix Metalloproteinases and Modulating JNK/P38MAPK/Ap-1/c-Fos and JAK/STAT Signalling

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Abstract

In arthritis, osteoarthritis (OA) is very common and is a progressive chronic illness of the joint impairing its function and thereby eventually affects the quality of patient's life. Currently employed therapeutic drugs aim to alleviate OA symptoms, however it poses adverse effects. Hence, identification of novel compounds that could effectively block the progression of OA and as well reduce symptoms is of high clinical value. This investigation was carried out to assess the effects a flavonoid, myricetin on the IL-1β induced SW1353 cells. The SW1353 cells were subjected to myricetin treatment at 75, 150 or 300 μg for 3 h before IL-1β stimulation at 5 ng mL^{-1} for 24 h and the treatment with myricetin significantly improved the cell viability of IL-1β-induced SW1353 cells and reduced the levels of PGE2 and NO. The results suggest the anti-inflammatory effects of myricetin. In addition, myricetin effectively supressed matrix metalloproteinases (MMPs-MMP1, 3 and 13) expression and also the expression of mitogen activated protein kinases (ERK, JNK and p38 kinase). The activation of NF-κB and AP-1 (transcription factors) were regulated by myricetin. Further, JAK2/STAT1 signalling was modulated effectively on myricetin treatment at all the three tested doses in a concentration dependent manner. Myricetin thus by targeting major pathways in pathogenesis of OA could be explored further for its therapeutic potential in OA treatment.

Key words: JAK/STAT signalling pathways, matrix metalloproteinases, mitogen activated protein kinases, myricetin, osteoarthritis

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

In arthritis, osteoarthritis (OA) is very common degenerative musculoskeletal disease with multifactorial etiology. The OA is a progressive degradation of the components of extracellular matrix (ECM), osteophyte formation, chondrocyte destruction, subchondral bone remodelling and synovial inflammation eventually leading to pain and disability (Ghosh and Smith, 2002; Felson, 2006; Riddle and Stratford, 2014). Articular cartilage metabolism imbalance has been reported to be critically involved in pathogenesis of OA (Iannone and Lapadula, 2003).

Chondrocytes of the articular cartilage plays crucial role by potentially expressing and as well responding to TNF-α and IL-1β, the major cytokines involved in inflammation (Daheshia and Yao, 2008; Kato *et al*., 2014). It has also been documented that upregulation of matrix metalloproteinases (MMPs): MMP-1 and 13 induced by IL-1β is associated with cessation of cartilage matrix via digestion of type II collagen, which is irreversible (Mix *et al*., 2001; Ahmed *et al*., 2004). Cartilage damage in OA is meticulously correlated to chondrocyte dedifferentiation that is potentially modified by ERK-1/2, JNK and p38 the various mitogen activated protein kinases (MAPK). The IL-1β induced activation of NF-κB and MAPK signalling pathways in the chondrocytes is also reported and this leads to the enhanced expression and release of inflammatory mediators-NO and PGE_2 (Zhang *et al*., 2014a, b) via up-regulation of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) enzyme (Chabane *et al*., 2008). Further, NO escalates the expression of MMPs and inflammatory cytokines (Salvemini *et al*., 1993; Sasaki *et al*., 1998).

Several researchers have confirmed JAK/STAT signalling in chondrocytes (Behera *et al*., 2004; Wen *et al*., 2006; El-Mabrouk *et al*., 2007). Lim and Kim (2011) reported involvement of JAK/STAT pathway in MMP-13 induction in IL-1β-stimulated SW1353 cells. Further, expression of MMP-13 induced by IL-1β involves NF-κB and AP-1 the transcription factors and also c-Fos activation (Lim and Kim, 2011).

A better understanding of IL-1β signalling pathway of chondrocytes aids in development of more effective drugs that could specifically target the molecular events in OA. Owing to the adverse effects of currently used drugs, much of recent researches are focussing on plant-derived compounds in OA therapy. Flavonoids are much explored due to their greater medicinal properties and possess wide range of biological activities that include anti-cancer, anti-inflammatory, anti-oxidant and anti-ulcer properties (Yoshida *et al*., 2005; Hong *et al*., 2006; Padhye *et al*., 2009; Panda *et al*., 2012; Yu *et al*., 2014). Additionally, flavonoids have been found to exert anabolic effect over various components of the bone (Horcajada-Molteni *et al*., 2000). Flavonoids including genistein, kaempferol and quercetin are reported to exert protective effects against bone injury or loss (Morris *et al*., 2006). In the IL-1β treated articular chondrocytes, epigallocatechin-3-gallate (EGCG) was observed to down-regulate MMP-1, 3 and 13 induction.

Myricetin (3, 3′, 4′, 4, 5, 5′, 7-hexahydroxy-flavone) flavonoid extracted from the bark, fruit and also the leaves of the Chinese bayberry and other edible plants (Shimosaki *et al*., 2011; Chen *et al*., 2013; Kim *et al*., 2013) possesses anti-cancer, anti-inflammatory, anti-microbial, anti-oxidant and anti-proliferative activities (Ong and Khoo, 1997; Wang and Mazza, 2002; Yanez *et al*., 2004). Considering the bioactive effects of myricetin, it was investigated whether myricetin could down-regulate IL-1β-induced cartilage destruction and inflammatory responses via targeting major pathways as MAPK, c-Fos/AP-1 and JAK/STAT signalling.

MATERIALS AND METHODS

Chemicals and antibodies: Myricetin was procured from Sigma-Aldrich (St., Louis, MO, USA). DMEM (Dulbecco's modified Eagle's medium), FBS (fetal bovine serum) and penicillin-streptomycin were procured from Gibco-BRL (Grand Island, NY, USA). Recombinant human IL-1β, ELISA kit of PGE2, MMP-3 and 13 were procured from R and D systems (Minneapolis, MN, USA). From Beyotime Institute of Biotechnology (Shanghai, China) Griess reagent was procured. Antibodies against p38, p-p38, ERK, p-ERK, JNK, p-JNK, NF-κB p65, JAK2, p-JAK2, STAT1, p-STAT1 (Cell Signalling Technology Inc. Beverly, MA, USA) c-Jun, c-Fos, AP-1, MMP-1, MMP-3 and MMP-13 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used for expression analysis.

SW1353 cell culture: The SW1353 cells (human chondrosarcoma cell line) were procured from ATCC (Manassas, VA, USA) and were retained in DMEM that was supplemented with 10% FBS, glutamine, 100 U mL^{-1} of penicillin and 100 mg mL^{-1} of streptomycin. The cells were cultured at 37°C with 5% CO_2 and after attaining 80% confluence, the cells were incubated with myricetin at 75, 150 or 300 μg mL^{-1} for 3 h and were subsequently treated with IL-1β (5 ng mL^{-1}) for 24 h.

Annexin V-FITC/ PI staining: Cell apoptosis was measured by annexin V-FITC/PI staining. Briefly, SW1353 cells processed

with myricetin (3 h), stimulated using IL-1β (24 h) were inoculated at 2×10^5 cells per each well in a 24 well plate and cultured overnight at 37°C. Apoptosis was detected using apoptosis detection kit (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The cells were then washed in Phosphate Buffered Saline (PBS) and treated with FITC-conjugated anti-annexin V antibody and Propidium Iodide (PI) at room temperature for 15 min and washed further and with flow cytometer (FACS Calibur, BD Biosciences) the cells were examined for fluorescence.

ELISA: After treatment with myricetin and IL-1β, the culture medium was analyzed by ELISA for MMP-3, 13 and PGE2 (R and D systems, Minneapolis, MN, USA) as per manufacturer's procedure.

NO assay: The NO concentration in the culture medium of chondrocytes treated with different concentrations of myricetin and IL-1β were determined using Griess reagent as described by Au *et al.* (2007).

RT-PCR analysis: Using RNeasy mini kit (Qiagen) total RNA was isolated from cells treated with myricetin and IL-1β and by determining the absorbance at 260 and 280 nm the RNA concentration was determined. Using Gene Cycler thermal cycler (Bio-Rad) cDNA was synthesized. Expression of MMP-3 and 13 genes was assessed using the following primer sequences: MMP-3: 5'-TGC-GTG-GCA-GTT-TGC-TCA-GCC-3' (sense) and 5'-GAA-TGT-GAG-TGG-AGT-CACCTC-3' (antisense); MMP-13: 5'-GCT TAG AGG TGA CTG GCA AC-3' (sense), 5'- CCG GTG TAG GTG TAG ATA GGA AC-3' (antisense). The relative expression of MMP-3 and 13 were normalized with that of GADPH -5'-TCT CCTCTG ACT TCA ACA GCG AC-3' (sense), 5' CCC TGT TGC TGT AGC CAA ATT C-3' (antisense). The reaction mixture (5 µL) was separated on agarose gel (1.5%) electrophoresis and the bands were visualized after ethidium bromide staining.

Western blott analysis: The SW1353 cells treated with myricetin (75, 150 or 300 µg mL^{-1}) and stimulated with IL-1β were subjected to protein expression analysis by western blotting. Following the treatments, cells were lysed and the contents (nuclear and cytoplasmic proteins) were extracted using NE-PER mammalian protein extraction reagent (Thermo). Concentrations of the protein isolates were determined using BCA protein assay kit (Bio-Rad Laboratories, USA). Equal amount (40 µg) of the protein samples were separated on SDS-polyacrylamide gel (12%) and transferred to PVDF membranes which were blocked with non-fat dry milk (5%) and was treated with primary antibodies and left overnight at 4°C after which it was incubated with peroxidase-conjugated secondary antibodies at room temperature for 60 min. The immunoreactive bands were visualized using enhanced chemiluminescence reagents (ECL) and analysed by densitometry using a FluorChem Q System (Alpha Innotech, CA, USA).

Statistical analysis: The results obtained from all the experiments are represented as Mean±SD, taken from three or six individual experiments. Statistical significance between means of various experimental groups were assessed by ANOVA (one-way analysis of variance) followed by Duncan's Multiple Range Test (DMRT) *post hoc* analysis. Statistical differences at $p<0.05$ between the values are considered significant.

RESULTS

Myricetin inhibited apoptosis in IL-1β-stimulated SW1353 cells: Chondrocytes pre-treated with myricetin (75, 150 or 300 µg) and stimulated with IL-1β were subjected to annexin V/PI staining to assess apoptosis. Significantly raised ($p<0.05$) apoptosis of SW1353 cells was observed upon treatment with IL-1β (Fig. 1), however, pretreatment with myricetin significantly ($p<0.05$) reduced the apoptotic cell counts in a dose-dependent way.

Myricetin reduced NO and PGE2 levels: Inflammatory mediators such as NO and PGE2 play important roles in

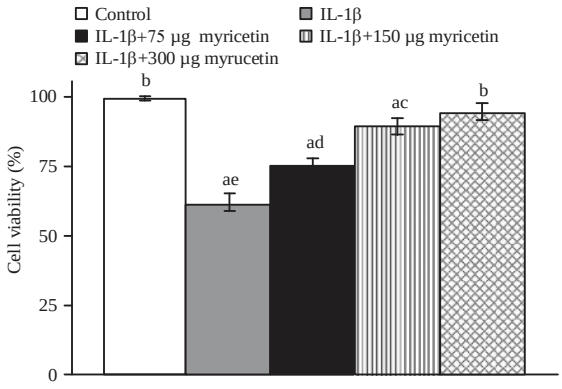

Fig. 1: Myricetin inhibited IL-1β-induced apoptosis of chondrocytes, results are expressed as Mean±SD with n = 6. At $p<0.05$, the statistical significance is related against control[a], [b-e]Denotes mean values within the same group that differ from each other at $p<0.05$ as derived from ANOVA (one-way) and DMRT analysis

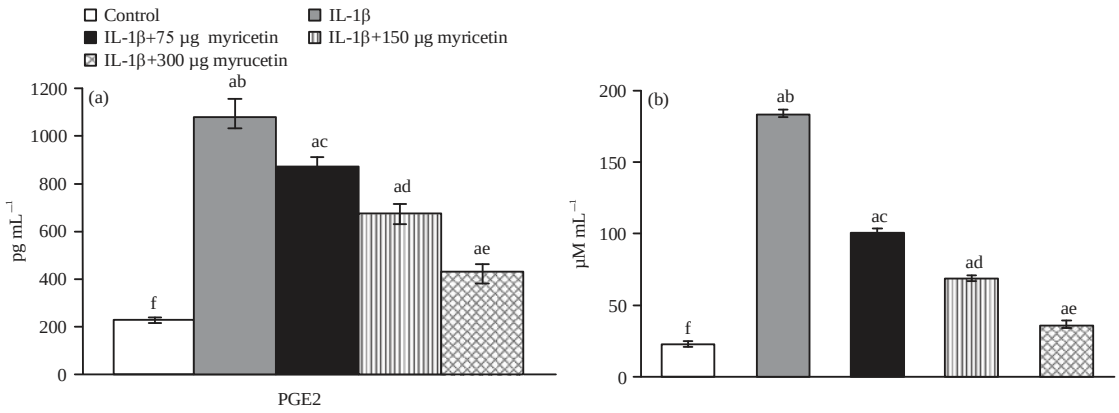

Fig. 2(a-b): Myricetin effectively reduced, (a) PGE2 and IL-6 levels and (b) NO levels, results are expressed as Mean±SD with n = 6. At $p<0.05$, the statistical significance is related against control[a], [b-f]Denotes mean values within the same group that differ from each other at $p<0.05$ as derived from ANOVA (one-way) and DMR analysis

progression of OA (Li *et al*., 2014). In this study, myricetin effect on the levels of PGE2 and NO released by the IL-1β-stimulated SW1353 cells was determined. Significantly increased ($p<0.05$) NO and PGE2 levels in the culture medium was observed on IL-1β induction, while myricetin was found to significantly reduce the levels. Myricetin at 300 µg concentration effectively decreased the levels compared to lower doses (Fig. 2).

Myricetin down-regulates matrix metalloproteinases in the IL-1β-stimulated SW1353 cells: The MMPs up-regulated expression is considered to be critical in degrading ECM in OA. Thus, suppression of their expression would benefit in treating OA. The expression levels of MMP-1, 3 and 13 in SW1353 cells stimulated by IL-1β was determined. Significantly higher ($p<0.05$) levels of MMP-3 and 13 was observed in the medium of IL-1β-induced chondrocytes as determined by ELISA (Fig. 3). However, exposure to myricetin at 75, 150 or 300 µg markedly ($p<0.05$) resulted in down-regulation. Further, expression of MMPs at molecular (gene and protein) levels was also analysed. The RT-PCR and western blotting analysis revealed that MMP expression was higher in cells stimulated with IL-1β and not treated with myricetin (Fig. 3). Prior exposure to myricetin was found to down-regulate the expression with 300 µg dose exhibiting maximum effects in comparison to lower doses of 75 or 150 µg. The observations suggest that myricetin could aid in inhibiting ECM degradation in OA.

Myricetin modulated expression of MAPKs: Studies have shown MAPKs such as ERK, p38 MAPK and JNK, playing important role in expression of MMPs and destruction of cartilage (Thalhamer *et al*., 2008). Higher expression of MAPKs on IL-1β stimulation was observed significantly ($p<0.05$). Myricetin exposure for 3 h prior to IL-1β stimulation of SW1353 interestingly down-regulated the expression in a dose-dependent way (Fig. 4). Thus, suppression of MAPKs by myricetin could in part effectively have reduced expression of MMPs.

Influence of myricetin on JAK2/STAT1 signalling pathways: The IL-1β treatment was observed to rise JAK2 expression and STAT1 activation was down-streamed as seen by enhanced phosphorylated STAT1 (Fig. 5). It has been reported that the STAT activation pathway is associated with MMP-13 expressions in chondrocytes treated with IL-1β (Litherland *et al*., 2010). While, IL-1β caused significant JAK2, STAT1 activation, myricetin treatment considerably inhibited the activation and also reduced the expression of total STAT1 and JAK2 as well in a dose-dependent manner thus leading to suppression of MMP-13 expression.

Myricetin modulated expression of NF-κB and AP-1 transcription factors: As observed, myricetin treatment caused a significant ($p<0.05$) decrease in NF-κB p65 and AP-1 levels, suggesting decreased nuclear translocation and essentially suppression of NF-κB and AP-1 signalling (Fig. 6). Further, c-Jun and c-Fos levels were also significantly reduced by myricetin. Thus, myricetin was observed to block both at the levels of c-Fos and c-Jun and also AP-1 thereby, down-regulating the signalling.

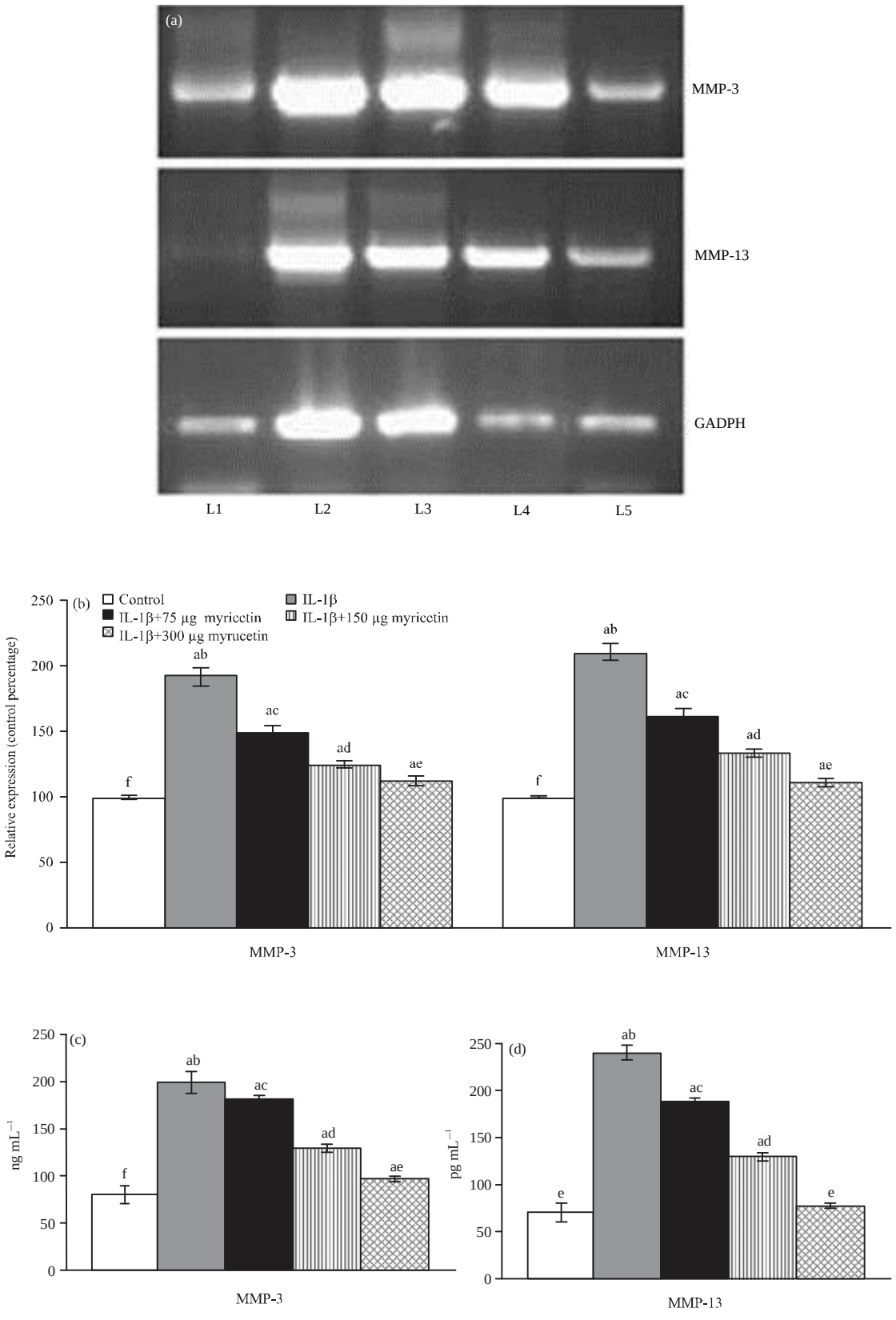

Fig. 3(a-f): Continue

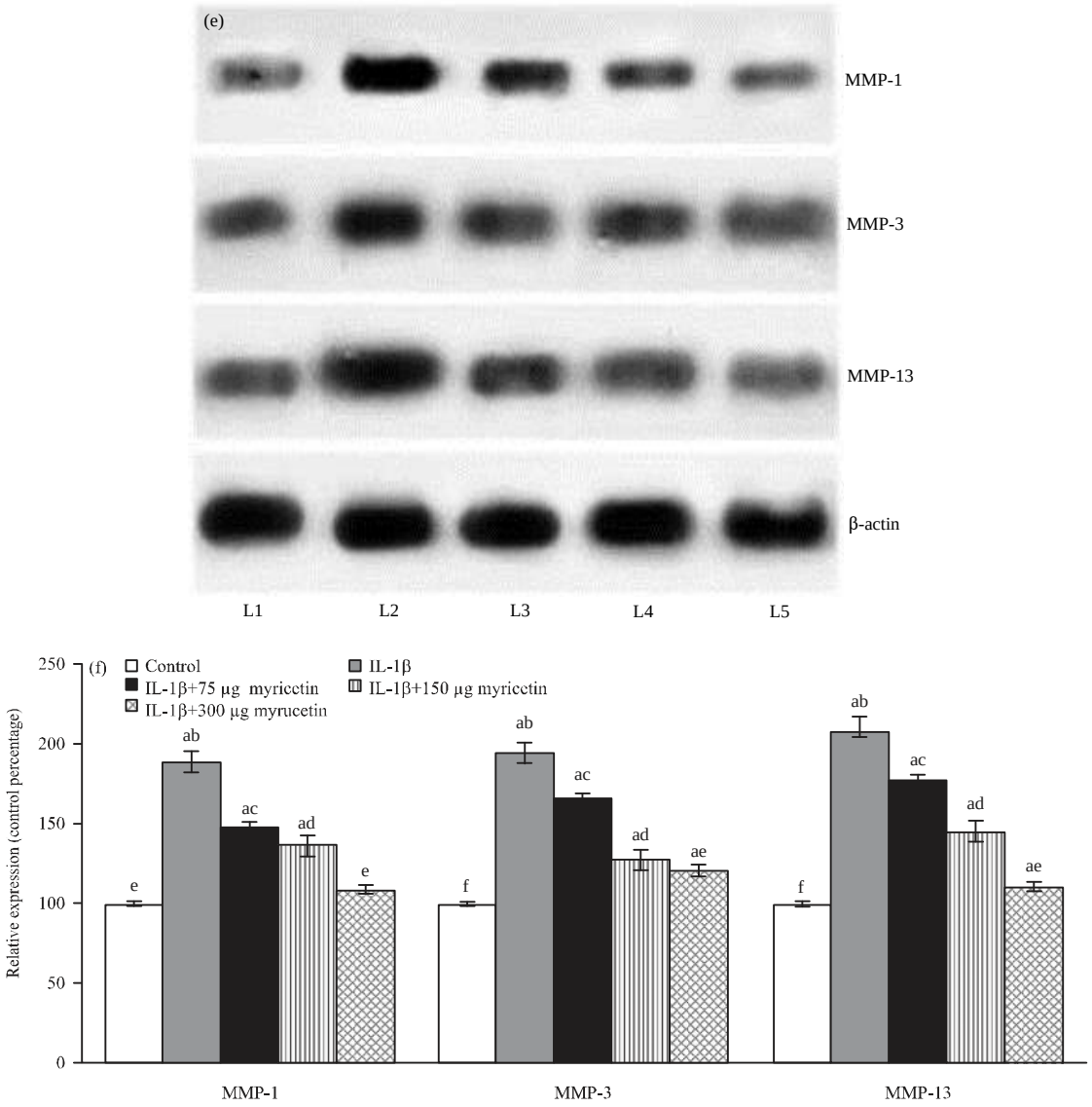

Fig. 3(a-f): Myricetin down-regulated the expressions of matrix metalloproteinases, (a, b) Myricetin effectively suppressed mRNA levels of MMP-3 and 13 as determined by RT-PCR analysis, (c, d) Levels of MMP-3 and 13 in the culture medium (ELISA) was reduced by myricetin, (e, f) Western blotting analysis indicated significantly down-regulated expressions of MMP-1, 3 and 1 3 by myricetin, results are expressed as Mean±SD with n = 6. At $p<0.05$, the statistical significance is related against control[a], [b-f]Denotes mean values within the same group that differ from each other at $p<0.05$ as derived from ANOVA (one-way) and DMRT analysis, L1: Control, L2: IL-1β, L3: IL-1β+75 µg myricetin, L4: IL-1β+150 µg myrcetin, L5: IL-1β+300 µg myricetin

DISCUSSION

Osteoarthritis (OA) is considered by erosion of articular cartilage and pain (Rosenzweig *et al*., 2014). Anti-inflammatory drugs that are generally prescribed for treating OA are recommended for long-term use thus making the possibility of side effects potentially high (O'Neil *et al*., 2012). Accumulating reports have demonstrated the anti-arthritic effects of plant-derived compounds (Zhao *et al*., 2014). The effectiveness of myricetin on IL-1β-induced SW1353 cells was explored.

The critical catabolic factor in OA is the pro-inflammatory cytokine IL-1β (Imamura *et al*., 2014) that leads to cartilage breakdown (Xu *et al*., 2014). The IL-1β is reported as an effective inducer of MMPs in OA. The IL-1β up-regulates the expression of MMP-1, 3 and 13 ultimately leading to the

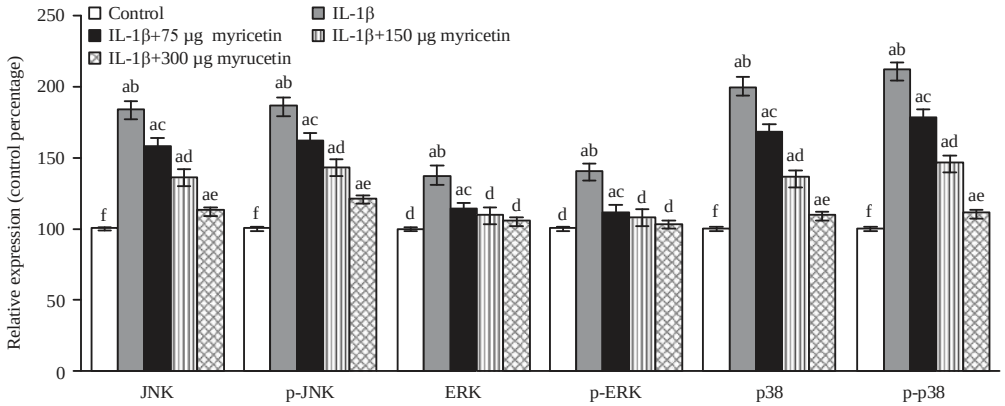

Fig. 4: Myricetin potentially regulates the MAPK signalling pathway, IL-1β-induced activation of the MAPK pathway were markedly ($p<0.05$) inhibited on myricetin treatment, results are expressed as Mean±SD with n = 6. At $p<0.05$, the statistical significance is related against control[a], [b-f]Denotes mean values within the same group that differ from each other at $p<0.05$ as derived from ANOVA (one-way) and DMRT analysis

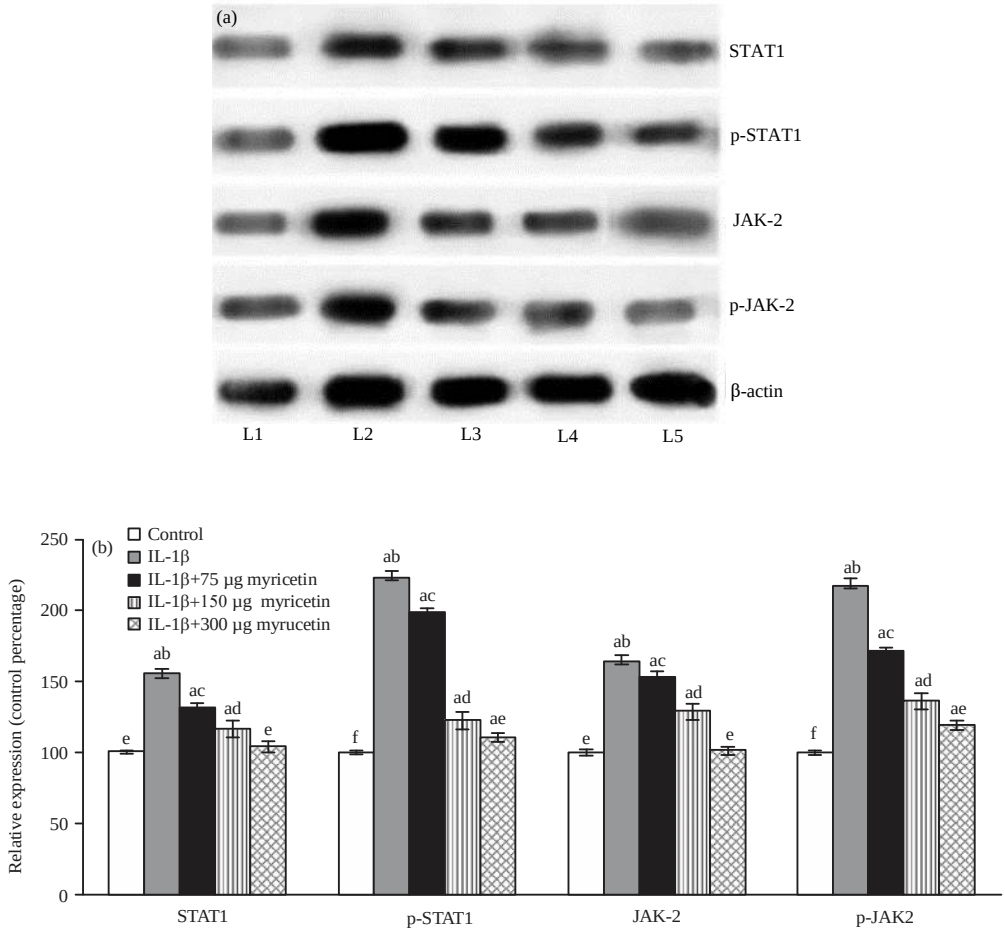

Fig. 5(a-b): Influence of myricetin on STAT1/JAK2 signalling pathway, myricetin effectively down-regulated the STAT1/JAK2 activation in 1L-1β-induced SW1353 cells, results are expressed as Mean±SD with n = 6. At $p<0.05$, the statistical significance is related against control[a], [b-f]Denotes mean values within the same group that differ from each other at $p<0.05$ as derived from ANOVA (one-way) and DMRT analysis, L1: Control, L2: IL-1β, L3: IL-1β+75 µg myricetin, L4: IL-1β+150 µg myrcetin, L5: IL-1β+300 µg myricetin

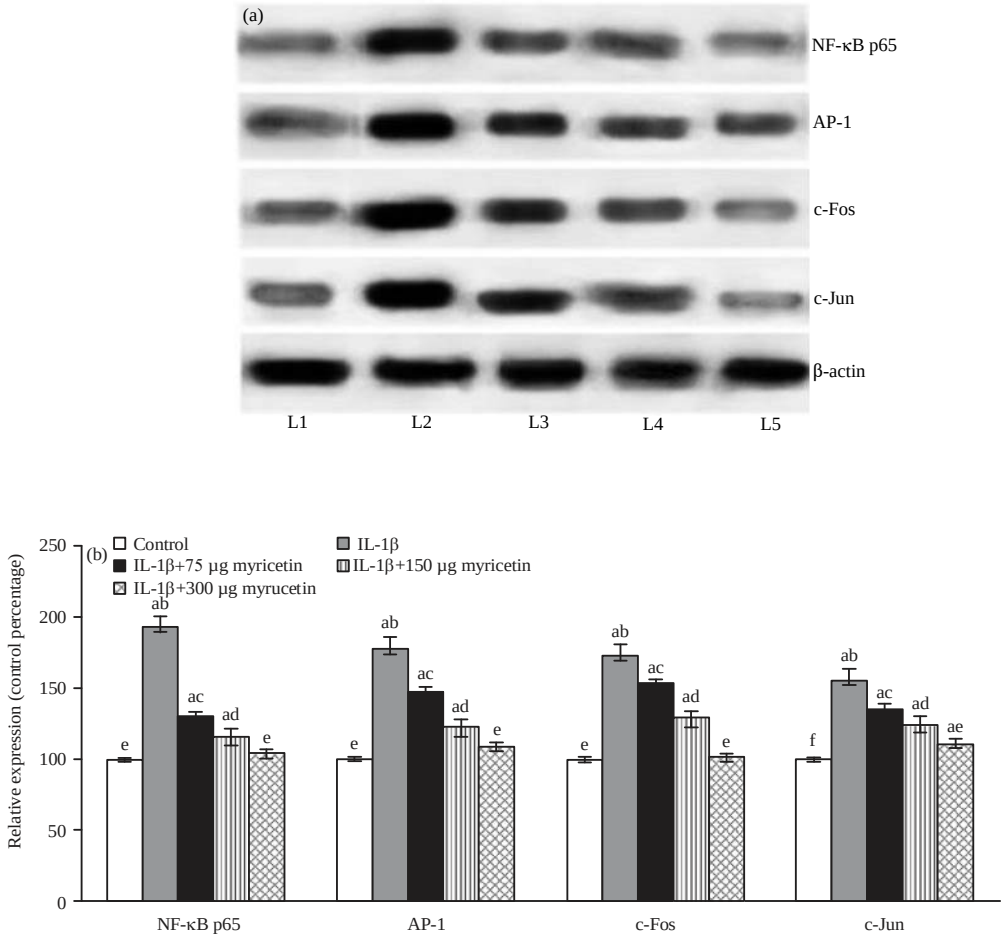

Fig. 6(a-b): Myricetin regulates NF-κB/AP-1 signalling pathway, IL-1β-stimulated NF-κB/AP-1 signalling were significantly supressed by myricetin, results are expressed as Mean±SD with n = 6. At $p<0.05$, the statistical significance is related against control[a], [b-f]Denotes mean values within the same group that differ from each other at $p<0.05$ as derived from ANOVA (one-way) and DMRT analysis, L1: Control, L2: IL-1β, L3: IL-1β+75 μg myricetin, L4: IL-1β+150 μg myrcetin, L5: IL-1β+300 μg myricetin

degradation of matrix (Klatt *et al*., 2009) and eventually releasing matrix proteoglycans from the cartilage (Sinkov and Cymet, 2003).

Cartilage type II collagen is broken down by MMP-1, while MMP-3 cleaves proteoglycans, gelatin and type II collagen (Ishikawa *et al*., 2005) and MMP-13 majorly cleaves collagen (Neuhold *et al*., 2001). Thus inhibition of MMP expression would potentially aid in OA treatment. Interestingly, myricetin treatment expressively hindered IL-1β-induced MMP-3 and 13 expression both at protein and at mRNA levels. Similar outcomes were also shown by Bang *et al*. (2009), Kim *et al*. (2011) and Ying *et al*. (2013). Myricetin at 300 μg dose more effectively down-regulated MMP-1, 3 and 13 expression compared to the lower doses. The viability of SW1353 cells pre-treated with various concentrations of myricetin (75, 150 or 300 μg) and then with IL-1β was also found to be significantly improved.

In OA, destruction of the cartilage is closely associated with the de-differentiation of chondrocyte and this destroys the biochemical and structural homeostasis in OA and is modulated by MAPKs-ERK-1/-2, JNK and p38 kinase. In addition, it has been shown that MAPKs crucially regulate the expression of MMPs (Mengshol *et al*., 2002; Vincenti and Brinckerhoff, 2002; Sondergaard *et al*., 2010). The IL-1β induces NF-κB and activates MAPK (Akhtar and Haqqi, 2011). The expression of MAPKs-ERK, JNK and p38 MAPK in IL-1β-induced SW1353 cells under the influence of myricetin was assessed. Significantly down-regulated expression of

MAPKs was observed in concentration-dependent manner. Expression of NF-κB p65 was also found to be supressed significantly.

Further, inflammatory mediators NO and PGE2 have been proved to play critical roles in OA development (Li *et al*., 2014). The IL-1β induces iNOS and COX-2, eventually leading to raised levels of NO and PGE2 (Stratz *et al*., 2014). These inflammatory mediators promote joint destruction and cause joint pains (Pecchi *et al*., 2014). Significant reduction in NO and PGE2 levels in the culture medium of SW1353 cells on myricetin pretreatment was observed, suggesting possible down-stream of the expression of iNOS and COX-2. Suppression of inflammatory mediators thus has therapeutic potential in OA treatment. Suppression of NF-κB signalling and MAPKs possibly contributes to the anti-inflammatory responses in OA. These observations suggest the anti-inflammatory effects of myricetin that could help in OA treatment.

The IL-1β has been found to cause activation of NF-κB and AP-1 in chondrocytes and SW1353 cells (Boileau *et al*., 2005; Ho *et al*., 2005; Liacini *et al*., 2005), that eventually leads to protein expression that is involved in inflammatory responses (Vincenti and Brinckerhoff, 2001; Gebauer *et al*., 2005; Fan *et al*., 2006). Lim and Kim (2011) have reported that MAPKs activate AP-1 targetting genes as c-Fos and c-Jun and subsequently induce expression of MMP-13 in chondrocytes (Vincenti and Brinckerhoff, 2001; Boileau *et al*., 2005). The observed higher levels of c-Fos and c-Jun could be due to IL-1β-induced activation of MAPKs and AP-1. Myricetin exposure brought about a noticeable down-regulation of AP-1 as well as that of c-Fos and c-Jun. Inhibition of expression observed are in line with that of ERK, JNK and p38MAPKs. Further JAK/STAT pathway is reported to be associated in the expression of MMP-13 in IL-1β-induced SW1353 cells and chondrocytes (Litherland *et al*., 2010; Lim and Kim, 2011; Lim *et al*., 2011). Thus the suppression of JAK2 and STAT1 observed on myricetin exposure reveal the involvement of JAK2/STAT1 signalling in OA. The supressed levels of MMP 13 may, also be due to decreased expression of AP-1 and down-regulated JAK2/STAT1 signalling.

CONCLUSION

Collectively, the observations of this study indicate that pre-treatment of SW1353 cells with myricetin could effectively inhibit IL-1β induced expression of MMPs (1, 3 and 13), the markers of cartilage degradation and inflammation in OA via down-regulation of MAPKs/AP-1/c-Fos and JAK2/STAT1 signalling. The results suggest myricetin as a potent candidate in treatment of OA. Further, investigations could provide more insights on the other molecular events possibly involved.

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