Research Paper

Crude extract of Pineapple stem: In-vitro assessment of anti-inflammatory properties

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ABSTRACT

Rheumatoid arthritis (RA) is a chronic autoimmune inflammatory disorder, which affects joints and causes synovial inflammation. Tumor necrosis factor (TNF-α) is the major cytokine involved in pathogenesis. In our study, we tried to investigate the anti-inflammatory response of crude extract of pineapple stem using SW982 as synovitis model induced by TNF-α. Cell survivability was measured using SW982 cells treated with 10 ng/ml TNF-α with and without different concentrations of the extract. The expression level of different pro-inflammatory cytokotkines was analyzed by qPCR and protein expression levels were measured by western blot. Crude extract effectively reduced the levels of IL6, IL-1β, and P65 (Rel A) in SW982 cells. It works as an anti-inflammatory agent to put a barrier in the induction of inflammation. Pineapple stem extract effectively decreases the inflammation in SW982 cells and pro-inflammatory cytokine level. It may be considered as a medicinal plant extract to reduce inflammation in RA disease.

Key words: Pineapple crude extract, SW982, TNF-α, Rheumatoid arthritis, pro-inflammatory cytokine.

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic autoimmune, inflammatory, symmetrical disease of joints. The disease characteristics involved synovial membrane inflammation (synovitis), which ultimately causes cartilage destruction (Smolen et al., 2016). About 0.5-1% of the world population has been known to be affected with RA, women being affected three times more than men (WHO report. 2019). Though RA pathogenesis involved multiple source or etiological factors, the molecular mechanism that induces inflammation in synoviocytes is unclear. It has been observed that hyper plasticity depends upon the change in protein binding characteristics of synovium which causes synoviocytes adhesion and invasion, leading to bone deformation (Tobón et al., 2010). Inflammation is the major cause of disease pathogenesis which is incurred by dysfunction of the immune system and there are negligible/limited drugs available for curing the disease. Therapeutic possibilities for the treatment of RA are Disease-Modifying Anti-Rheumatic Drugs (DMARDs), Nonsteroidal anti-inflammatory drugs (NSAIDs) and steroids. But the high cost and adverse effects of drugs make them unaffordable and less popular (Benjamin et al., 2020). So, there is an intense need for alternate medicine to overcome the drug side effects and disease severity.

To date, the origin of the disease is vague though its development starts with the inflammation of the synovial membrane around the affected joints due to the accumulation of several immune cells. Consequently, numerous amounts of signaling molecules like cytokines and chemokines are released around affected joints. Major Cytokines that are involved in inflammation is tumor necrosis factor (TNF-α) (Gorman and Cope, 2008) and plays multiple roles in RA progression and development. It has been investigated that, it not only works as proinflammatory cytokotkines but also induces the production of plenty of other inflammatory cytokotkines like IL-6, IL-8, IL-1β in synovial fibroblast and neighboring cells. It is mainly produced by monocytes and macrophages, lymphocytes...
(B-cells, and T-cells) and the fibroblast. It has been shown that TNF-α induction causes nuclear factor kappa light chain enhancer of activated B cells (NF-κB) via Mitogen-activated protein kinases (MAPK) pathway (Suzuki et al., 2000; Brennan and McInnes, 2008). Therefore, the study was aimed to understand the pathophysiological effect of NF-κB by investigating pro-inflammatory cytokines stimulated by TNF-α in RA mimicking cell model.

In our investigation, we have used synovitis cell line SW982 (Chang et al., 2014), followed by treatment with crude extract of pineapple stem. Earlier, it has been reported that pineapples (Ananas comosus) are used as a medicinal fruit. Crude extract of fruit stem is rich in proteins, fibers, minerals, polyphenol compounds and can be used as anti-inflammatory activities (Debnath et al., 2021). We therefore used crude extract of pineapple stem and evaluated its anti-inflammatory effect by analyzing the expression level of pro-inflammatory cytokines and proteins. Thus, we determined its therapeutic potential for effective reduction of inflammation and decrease in progression of RA.

MATERIALS AND METHODS

Chemicals

Cell culture supplements such as Dulbecco’s Modified Eagle’s Medium (DMEM), antibiotic solution, 1X Trypsin EDTA were purchased from Himedia. Recombinant Human TNF-α was purchased from Genescript. For RNA isolation, Tri-Xtract reagent was obtained from G Biosciences. The cDNA Synthesis Kit was purchased from G Biosciences. For qPCR EvaGreen master mix was purchased from G Biosciences. A real-time PCR (Roche light cycler II 480) machine was used to check mRNA expression. Anti-human GAPDH, anti-p65, goat anti-rabbit IgG conjugated HRP, and horse anti-mouse IgG conjugated HRP were obtained (Jackson). Bradford reagent was obtained from Bio-Rad (Bio-Rad Laboratories). Nitrocellulose (NC) membranes were purchased from Amersham (Amersham Pharmacia Biotech). A semi-dry blot machine Bio-Rad (Bio-Rad Laboratories). A gel documentary system was purchased from Bio-Rad (Bio-Rad Laboratories (Singapore) Pte. Ltd.).

Extract preparation

Pineapple stem was authenticated by CSIR-NICCAIR with voucher soecimen number NISCAIR/RHMD/consult/2021/3833-34 deposited in Raw material herbarium and museum, Delhi (RHMD). Stems of the fruits were taken out, washed, cut into pieces, and crushed using mortar and pestle. Methanol extraction of these pieces was obtained using a shaker at 150 rpm, 45°C for 48 h. The extract was then filtered, kept at 57-58°C in Hot Air Oven until completely dried and stored at -20°C for further use (Gautam et al., 2010). Before the experiments, the extract was further dissolved in water and DMSO in the ratio of 1:2 (concentration = 1 g/ml).

SW982 synovial sarcoma cell line culture and treatment:

SW982 was obtained from National Centre for Cell Science (NCCS). The cells were cultured in a sealed 25 ml T-culture flask in DMEM medium containing antibiotic solution, supplemented with 10% fetal bovine serum (FBS), and kept at 37°C humidified incubator with 5% CO₂.

Cytotoxicity test

SW982 cells were seeded at 10³cells/well in a 96 well plate, incubated at 37°C for 24 h. Cells were exposed to different concentrations of extract, ranges from 25 to 250 μg/ml with a difference of 25 and incubated further for 24 h with and without TNFα (10 ng/ml). Dimethyl sulfoxide was (DMSO) added as toxic control. Freshly prepared 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was incubated for 3 h and observed for formazan crystals formation. DMSO was added to each well, incubated at room temperature (RT) in the dark for 15 minutes and absorbance was measured at 540 nm (Rai et al., 2018).

Real-time polymerase chain reaction (Real-Time PCR) assay

SW982 cells were cultured in a 25 ml T-culture flask and after 70-80% confluency cells were cultured in a serum-free medium for 24 h. The effects of crude extract of pineapple stem on inflammation were investigated at different concentrations 100,125,150 μg/ml for 2 h followed by treatment with and without 10 ng/ml human recombinant TNF-α for 1 h (without TNF-α and extract taken as control). The total RNA was isolated by using Tri-Xtract RNA Isolation Reagent and 0.8 μg of total RNA was used for reverse transcription to produce cDNA by using the cDNA Synthesis Kit. The transcribed cDNA was mixed with SyberGreen Supermix and the level of mRNA expression was evaluated using a real-time PCR detection system using human-specific primer sequences (Table 1). Obtained data were normalized by β-actin with respect to their constitutive gene analyzed quantitatively using the 2-ΔΔCT formula (Livak and Schmittgen, 2001).

Western blot analysis

SW982 cells were cultured in a 25 ml T-culture flask until
Table 1: Synthesized primer sequences of cytokines and proteins required for qPCR analysis.

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Name of Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>IL-1β</td>
<td>5’AAACAGATGAAGTGCTCCTCCAGG3’</td>
<td>5’TGGAGAACCACCTTGTGGCTCAA3’</td>
</tr>
<tr>
<td>2</td>
<td>IL-6</td>
<td>5’GGTACATCCTCGAGGGATCT3’</td>
<td>5’GTGCCTTGGCTGCTTTCAC3’</td>
</tr>
<tr>
<td>3</td>
<td>β-actin</td>
<td>5’ CATCGGAAAGACTGTACG 3’</td>
<td>5’CGCTTGGCTGATCCACATC 3’</td>
</tr>
<tr>
<td>4</td>
<td>P65</td>
<td>5’GAAGGAAGATCCTTCAGCG3’</td>
<td>5’GGGAGGAGTAAAGGATAG3’</td>
</tr>
</tbody>
</table>

they reached 70-80% confluence. The cells were cultured in a serum-free medium for 24 h. Different concentration of pineapple stem crude extract 100,125,150 µg/ml was added 2 h before the treatment of TNFα (10 ng/ml) for 10 min. Cells were harvested in Radioimmunoprecipitation assay buffer (RIPA buffer) containing phosphatase and protease inhibitor. Protein concentration was estimated by Bradford protein assay and the sample was loaded in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Separated proteins were transferred on to NC membrane by the semi-dry method. The membrane was then blocked for 3 h with 5% BSA followed by incubation with primary antibody (P65) overnight. Membrane was then washed with 1XPBS and 0.05% Tween 20, incubated for 1 h with HRP conjugated secondary antibody and developed under the ChemiDoc system using light-sensitive developer (Biswas et al., 2013).

RESULTS

Crude extract of pineapple stem was prepared in methanol. The extract was dissolved in DMSO, stock concentration (1 mg/ml) was made for further use (Figure 1a and 1b). The optimized concentrations of extract were measured by MTT assay. The results suggested the increased survivability of cells with extract at 100, 125, and 150 µg/ml with and without TNFα (Figure 2a and 2b). The effectiveness of extract was confirmed and analyzed via changes in the mRNA expression level of pro-inflammatory cytokine.
Cytokines are IL-1β, IL-6 and mediator proteins p65 (RelA) associated with NF-kB inflammatory pathway. Results showed a significantly decreased level of relative mRNA expression of IL-6, IL-1β, and P65 (Figure 3a, 3b and 3c) compared to cells treated with TNF-α compared with control (cells without TNF and extract was taken as control). It suggests that extract could suppress the mRNA expression of pro-inflammatory cytokines that were induced by TNF-α and the effective dose is 125 μg/ml.

**Western blot analysis**

Cell lysate of SW982 cells with different dosages of extract at 100,125 and 150 μg/ml were analyzed by western blotting for p65 protein. Equal amount of protein was loaded and developed by p65 antibody was a major protein involved in the NF-kB pathway, p65 decreased in all the concentrations but significantly downregulated at 150 μg/ml dose. The level of p65 was downregulated by 0.82-fold in 100 μg/ml extract, 0.466-fold in 125 μg/ml and 0.268-fold in 150 μg/ml extract in comparison to cells with control significantly with p<0.0031 value (Figure 4).

**DISCUSSION**

Pineapple is a tropical plant under the *Bromeliaceae* family which has significant economic importance known for its unique aroma and sweet taste. It consists of protein, fiber, mineral and polyphenol compounds (Alejandra and Emperatriz, 2011; Li et al., 2014). The value of pineapple is increasing due to its attracting aroma/flavor and nutrient content, however, properties of biochemicals present in it may get changed due to climate conditions, maturity level and type of cultivator (Lobo and Paull, 2017). It is useful for human health, based on nutrition and physiochemical composition that helps in improving human health and maintaining the body’s metabolism. Also, the volatile compounds from pineapple flesh are used as natural essence that improves its economic importance (Hossain and Rahman, 2011). Pineapple also possesses antioxidant properties, antimicrobial properties and reduces the risk of diabetes (Pavan et al., 2012). Report shows that bromelain from pineapple is used as an anti-inflammatory agent and helps to strengthen bones (Siow and Hing, 2012; Mohd et al. 2020). Since RA is an inflammatory joint disease and the currently available drugs are causing serious side effects (Oliver and Silman, 2006), we thought of investigating the potential therapeutic value of pineapple for curing RA pathogenesis.

RA is an autoimmune disease developed in joints symmetrically. Various evidence regarding the anti-inflammatory effect of pineapple from leaf extract has helped in resolving inflammation (Kargutkar and Brijesh, 2018). Extract of pineapples juice shows analgesic and anti-inflammatory effect in *in-vivo* studies provides alternative or safe treatment for inflammatory disease such as osteo arthritis (OA) and RA other than available drugs (Brien et al., 2004; Majeed and Borole, 2015). The combination of pineapple extract and curcumin resulted in the downregulation of inflammation-related transcription factors and pro-inflammatory cytokines (Kritis et al., 2020). Based on available studies, we thought of using pineapple stem extracts as a natural anti-inflammatory component that may alter pro-inflammatory cytokines level by modulating the NF-kB pathway for inflammatory diseases like RA. Previously, it has been demonstrated that TNF-α...
binds to tumor necrosis factor receptor (TNFR), subsequently activates multiple kinases to initiate activation of Inhibitor κB kinase (IKK). Further, phosphorylation of Inhibitor κB (IκB) at specific amino acids activates the proteasomal degradation of IκB which in turn releases dimers of NF-κB (p50/p65) and translocates into the nucleus (Choy and Panayi, 2001). It binds with the enhancer element of target genes and initiates the expression of pro-inflammatory cytokines (IL6, IL-1β) and protein (P65) (Roman-Blas and Jimenez, 2006).

Therefore, the study was aimed to understand the NF-κB pathophysiology by investigating the effect of crude extract of pineapple stem on pro-inflammatory cytokines stimulated by TNF-α in RA mimicking cell model. We used synovitis-like model SW982 cells induced by TNFα and found significantly decreased level of relative gene expression of cytokines IL-1β, IL-6 and mediator protein p65 (Rel A) that are associated with NF-kB inflammatory pathway prominently known for RA pathogenesis. As p65 is a prominent subunit of NF-kB, an important transcription factor of inflammation. Hence checked the extract on the level of protein via western analysis for p65 and found that the level of p65 gets effectively decreased at 150 g/ml compared to mRNA level which is more effectively downregulate at 125 µg/ml. Conclusively this study suggests that TNFα is a prominent cytokine of RA and is

**Figure 3:** Relative expression of IL-6, IL-1β, p65 mRNA expression in SW982 when treated with crude extract pineapple stem at concentration ranges 100,125,150 µg/ml TNF-α for 2 hrs followed by treated with or without 10ng/ml TNF-α for 1 hr and compared to respective controls. C = Control cells without TNF-α and extract, CT = cells with TNF-α10ng/ml for 1hr A) Relative expression of IL-6 gene expression in SW982 cells B) Relative expression of IL-1β gene expression in SW982 cells C) Relative expression of p65 gene expression in SW982 cells. Values are presented as the mean ± SEM (n = 3). **** = < 0.0001, ** = < 0.01 versus normal control or TNF-α treatment (*) by one-way ANOVA.
used in this study to mimic the RA condition. The efficacy of extract as an anti-inflammatory needs further investigation.

CONCLUSION

RA is a lifestyle disease that is to be resolved by various diet interventions. Pineapple is a multipurpose fruit with potential benefits for human health (Figure 5) which can be used as an anti-inflammatory agent against the disease condition. In this study, we have analyzed the molecular mechanism by which crude extract pineapple stem showed anti-inflammatory properties. Further investigations of extract would be needed to assess its potential as
therapeutic agents that are usually associated with inflammatory diseases.

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REFERENCES


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