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Expression, Isolation, and Characterization of Tag-less Human Interleukin 6 in Salt inducible Bacterial System; GJ-1158

Diksha Waghuji Kanake[1], Priyankar Sen[1*]

[1]Centre for Bio-Separation Technology, Vellore Institute of Technology, Vellore 632014, Tamil Nadu, India - 632014.
[2]Shri Shivaji College of Arts, Commerce and science, Kandhar, Nanded, Maharashtra. India – 431714
Email: dikshakanake2017@gmail.com
Correspondence:
Dr Priyankar Sen, Centre for Bio-Separation Technology, Vellore Institute of Technology, Vellore 632014, Tamilnadu, India - 632014.
*Email: priyankar.sen@vit.ac.in, priyankarjsr@gmail.com
ORCID: 0000-0003-4729-7297

ABSTRACT

Background: hIL-6 is a cytokine that is produced in response to inflammation and tissue injury. It has pleiotropic effect and is composed of 212 amino acids. Recently, in COVID-19, breast cancer, gastric cancer etc hIL-6 became a target for chemotherapy. It is also used as a biomarker for various autoimmune and viral diseases. It is already expressed in, many systems but E.coli, GJ1158 is suitable for the expression of hIL-6. Furthermore, we performed an in silico docking study in the presence of plant-derived anti-inflammatory alkaloid Mandindoline A to probe the potential hIL-6 interaction. This can have implications for the next anti-IL-6 treatments.

Objective: Here in this work the hIL-6 gene was subcloned into pRSET-B from the mammalian expression vector pCDNA 3.1 and expressed in a salt inducible expression system GJ1158 with help of sodium chloride instead of IPTG as an inducer.

Methodology: Protein is getting expressed in soluble as well as aggregate form. The soluble fraction was obtained through freeze-thaw cycles and subsequently lyophilized. Those soluble parts of protein get loaded on the 12% SDS-gel. The lyophilized sample used for the purification and MALDI characterization.

Results: Human interleukin-6 was expressed on a 12% SDS gel at nearly 23.7 kDa and get confirmed by Western blotting by using anti-interleukin-6 antibody.

Conclusion: hIL-6 is successfully produced from a salt inducible system GJ1158. As per best of our knowledge, till now there is no record found for the expression of tagless hIL-6 in GJ1158.

KEYWORDS: human interleukin-6, Salt Induction, GJ1158 Expression Host, IL-6- a Biomarker, Characterization of hIL-6 and Target for various disorders.

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INTRODUCTION

Human interleukin-6 is secreted by T cells, B cells, macrophages, monocytes, fibroblasts, keratinocytes, endothelial cells, mesangial cells, adipocytes, and some tumor cells [1]. It acts as both a pro-inflammatory mediator and an anti-inflammatory mediator. IL6 has pleiotropic effects which are involved in a wide range of biological processes. IL-6 helps the differentiation of T-helper (Th) and T-regulatory cells (Tr) from naïve CD4 and CD8 cells respectively. It aids in the maturation and differentiation of B-cells into antibody-producing cells also it helps in the hematopoiesis process but, on the other hand, at the first stage of infection, IL-6 comes into the blood and reaches up to the liver. The liver then activates hepatocytes, which release large amounts of C-reactive protein (CRP), Serum amyloid A (SAA), fibrinogen, and hepcidin and decrease the release of albumin which causes AA amyloidosis, cardiovascular events, edema, anemia, etc. it can cause osteoporosis due to synovial fibroblast and sclerosis due to collagen increment from IL-6. This indicates that IL-6 is associated with a wide range of severe inflammatory disorders, including sepsis and rheumatoid arthritis [2].

For these reasons, the use of small molecule modulators, including ones such as Mandindoline A, may open new opportunities in IL-6 target therapy, particularly in the treatment of autoimmune-mediated and inflammatory diseases. Mandindoline A is also a natural product with demonstrated anti-inflammatory action. The interaction of hIL-6 with Mandindoline A was assessed by using molecular docking to investigate the potency of Mandindoline A as an IL-6 modulator.

In a recent event, in COVID-19, IL-6 became a major target. The Chinese National Health Commission approved tocilizumab, an interleukin (IL)-6 inhibitor, in March 2020 for the treatment of COVID-19 ARDS (acute respiratory distress syndrome brought on by a coronavirus infection in 2019). Due to the absence of effective treatments and to reduce the high pandemic mortality,

several IL-6 inhibitors, including sarilumab and siltuximab, have since been used off-label against COVID-19 [3]. In cases of gastric cancer as well as breast cancer, using cisplatin, an IL-6 inhibitor, can control STAT-3 signaling pathways, which helps control gastric cancer [4, 5].

Pilot investigations showed that IL6 targeting is an effective alternative to current antibiotic-based treatments for these conditions and also a desirable clinical target for cancer therapy. Today, it is used as a target and marker for various autoimmune and viral diseases, as well as for the detection and treatment of cancer. Demand for hIL-6 is increasing. It is challenging to extract IL6 from human tissues, and because of that, it results in limited protein yields. Functionally active recombinant production of IL6 in large amounts is required [6].

E. coli has benefits over other therapeutic protein production platforms, including the capacity to grow on cheap carbon sources, accumulate biomass quickly, ferment materials at high cell densities, and scale up production with ease. Similarly, the genetics and molecular biology of E. coli are well-known [7]. Salt-induced overexpression of genes cloned downstream of the phage T7 f10 promoter was demonstrated in an Escherichia coli strain (GJ1158), which carries a single chromosomally integrated copy of the gene for phage T7 RNA polymerase under the transcriptional control of the cis-regulatory elements of the osmoresponsive proU operon [8]. IL6 has been expressed in E. coli by numerous research teams, but the recombinant proteins that are produced have a propensity to aggregate. This may be because human IL6 naturally arises not just as monomers but also as multimeric aggregates with various molecular sizes [6].

By employing NaCl induction rather than the more expensive, more hazardous, and toxic isopropyl-D-thiogalactopyranoside (IPTG) induction, we have investigated a novel method of purification and an affordable strategy to create a tag-less recombinant hIL-6 [9,10]. This study's Escherichia coli strain (GJ1158) has been described as a salt-inducible system. It is made up of the Osmoresponsive proU operon, which is in charge of the phage T7 RNA polymerase gene. When NaCl is present, cells begin to produce T7 RNA polymerase, which causes the desired protein to be produced under the control of the T7 promoter [9, 11].

MATERIALS AND METHODS

2.1 Strain, plasmid, and culture growth conditions

The cloning vector pRSET-B and the host bacteria *E. coli* GJ1158 were used in this study. GJ1158 was cultured in Luria-Bertani (LB) broth (consisting of 10 g/L peptone and 5 g/L yeast extract) devoid of NaCl and supplemented with 100 µg/mL ampicillin. The culture was then incubated at 37 °C with continuous shaking at 160 rpm.

2.2 Competent Cell Preparation:

Competent cells were prepared using a cold 100 mM calcium chloride solution, followed by heat-shock transformation through the use of heat (42.5 °C) and ice-cold shock. Colonies get selected on the ampicillin-containing agar plates.

2.3 Cloning of hIL-6 gene

The hIL-6 gene (639 bp) was amplified by PCR using the primers 5' GGATCC ATGAACTCCTTCTCCACAAGCG 3' (forward primer with the *BamHI* restriction site) having Tm of 70.870 c and 5' AAGCTT CATTTGCCGAAGAGAGCCC 3' (reverse primer with the *Hind III* restriction site) having Tm of 64.97° C. The PCR conditions include initial denaturation at 95 °C, annealing at 57.5 °C, followed by extension at 72 °C for 30 cycles. Cohesive ends were fabricated by exploiting the specific restriction sites at the ends of the amplicons.

2.4 Sub-cloning of hIL-6 with pRSET B:

The hIL-6 gene and the pRSET-B vector were double-digested with the help of Bam HI and Hind III (50µl reaction) and this has been kept at 21°C for 12 hr. After that, this restricted product ligated with the help of the *T4 DNA Ligase* enzyme (30µl reaction). This recombinant pRSET B was transformed into GJ1158 competent cells by heat shock method, and then the transformed colony was screened on ampicillin (100µg) containing agar plates. Then the colony PCR was done for the selection of recombinant insert [13]. 1% agarose gel electrophoresis (BioRad) was done to analyze the insert throughout the process. Then the positive colonies were used for the further expression studies.

2.5 Expression of hIL-6:

The transformed colony was inoculated for 4 hours. When the OD reached 0.6, induction was performed with the help of NaCl (0.1M and 0.2M) as well as IPTG (0.02mM and 0.05mM) at 2 different temperatures, 25°C and 37°C. After induction, cells were harvested at 12000 RPM and suspended in lysis buffer (Tris-HCL 50Mm, pH- 8). Then freezing and thawing have been followed by sonication (Pulse on for 5 sec, pulse off for 10 sec). Centrifugation was done after the sonication. The supernatant contained the soluble protein, while the pellets consisted of inclusion bodies..

2.5.: SDS-PAGE:

Cultures at 3, 5, and 12 hours were centrifuged at 12,000g, for 10 min. at 4°C temperature and the palate resuspended with the help of lysis buffer, 50mM Tris buffer, pH-8. This resuspended palate has been kept at -80°C (freeze and thaw) for cell lysis. The supernatant was taken and concentrated up to 30-50µl. This product has been loaded on 12% SDS gel.

2.6: Western Blot:

SDS-PAGE is done first; the gel is kept in a transfer buffer. Nitrocellulose membrane is used as a blotting paper which is kept in on the SDS-Gel to transfer the bands by using electrical voltage at 90V for 90 min. after running, nitrocellulose paper is

incubated with 5% BSA/Skimmed milk for 1hr. After washing with 1%PBS, the primary antibody was used then the membrane was incubated with a secondary antibody.

Western blot confirmed the presence of the recombinant/purified hIL-6 protein. After an hour and a half of SDS-PAGE, the protein bands got transferred out of the polyacrylamide gel and onto a nitrocellulose membrane. This setting was applied on a Bio-Rad transblot apparatus, at 90 V. After transfer, the protein bands were visualized on the membrane upon staining with Ponceau. Blockage of the remaining surface of the membrane was carried out with skimmed milk to ensure that no nonspecific binding took place that would alter the results. The stain used was washed off before we put the membrane to incubate with TNF-α protein (40 ng/mL) in PBS buffer of pH 7 for the whole night. Later, it was treated with the primary monoclonal hIL-6 antibody, following which washing with PBST was done. Afterwards, the secondary antibody conjugate followed by washing with PBST was applied. DAB (3,3′-Diaminobenzidine) staining was performed finally to develop the protein bands.

2.7: MALDI-TOF mass spectrometry

The protein was analyzed in a commercial MALDI mass spectrometer (ULtrafleXtreme, Bruker Daltonics) by the Indian Institute of Science, Bangalore. 25 KV voltage was used along with 2000 Hz of laser frequency [12]. Data were collected in the mass range of 500 Da to 6000 Da from a sample loaded in 5µl of a mixture of water and 50% acetonitrile with 0.1% TFA [20].

2.8: In Silico Study

Human IL-6 crystal structure (PDB entry [insert ID]) was obtained from the RCSB Protein Data Bank. A 3D model of Mandindoline A was obtained from PubChem from the PubChem database and energy-minimized with MMFF94 force field in Avogadro. Docking was performed using Autodock Vina (grid box at the IL-6 receptor binding site). The scores of binding affinity and the contact residues were calculated and visualized using PyMOL and Discovery Studio.

RESULTS:

Using the pRSET-B vector, the hIL-6 gene was successfully cloned into E. coli GJ1158. Three techniques were used to confirm the ligation of the insert into the vector: (a) the clone was amplified using PCR using the designated primers. (b) Sanger sequencing of the constructed recombinant plasmid the presence of the correct gene sequence of hIL-6 was confirmed.

3.1. Gradient PCR:

6 different temperatures have been taken to check the annealing temperature of the primer 50.2°C, 53°C, 57.5°C, 59.9°C, 64.5°C, and 66.2 °C. From that, 57.5°C is used as a standard annealing temperature. (Fig. 1)

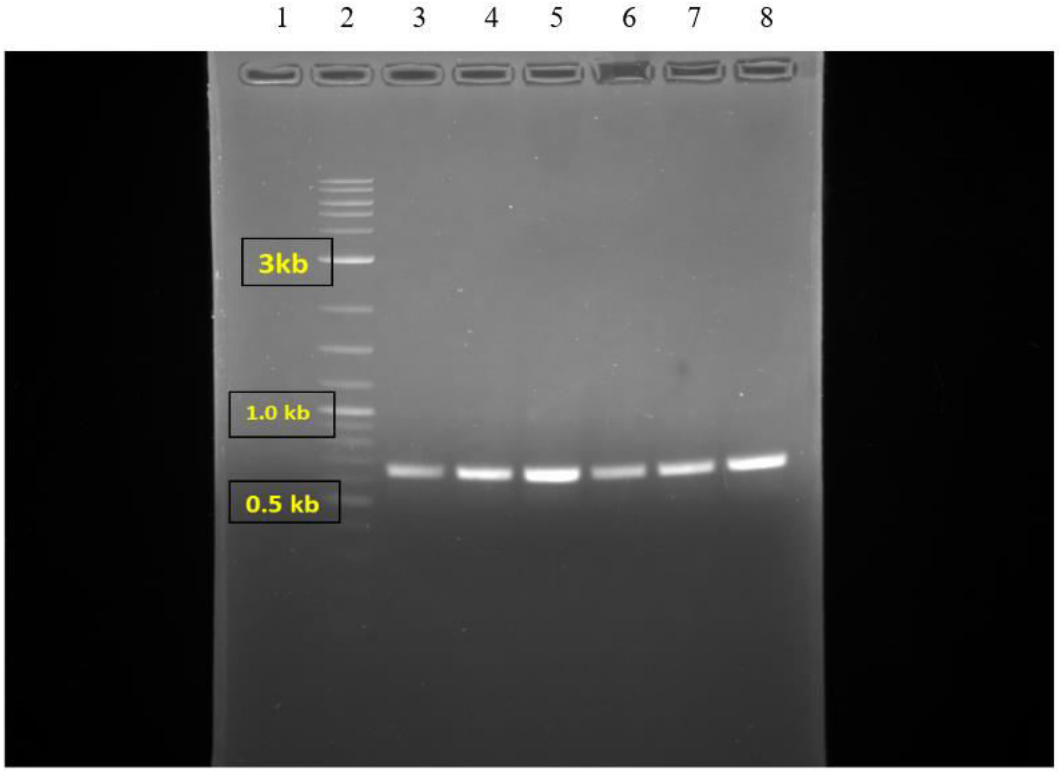


Fig. 1: Confirmation of hIL-6 gene (Gradient PCR): 2nd lane - 2kb ladder, 3: 50.2°C, 4: 53°C, 5: 57.5°C, 6: 59.9°C, 7: 64.5°C, 8: 66.2 °C Gradient PCR for hIL-6 (3µg/well)

3.2. Sub-Cloning:

hIL-6 was present in the pCDNA3.1 construct, a mammalian expression vector. After designing the primers including restriction sites against the hIL-6 gene, PCR was done and then restriction digestion was done with the help of Bam HI and Hind III (as chosen same restriction site of sub-cloned vector pRSET-B). Similarly, the vector pRSET-B was also digested by the same

restriction enzymes to produce compatible and cohesive ends. T4 DNA Ligase was used to ligate the insert and vector, and the recombinant plasmid (pRSET-B + hIL-6) was transformed into *E. coli* DH5-alpha (the maintenance host) and *E. coli* GJ1158 (the expression host) at the same time. The transformed cells were selected using 100 µg/mL ampicillin, and the colony PCR was performed to select the clones with an appropriate insert. Agarose gel electrophoresis (BioRad) using 1% agarose was used to analyze the DNA throughout the process. The positive clone selected was subjected to expression studies (Fig. 2 B1 and B2).

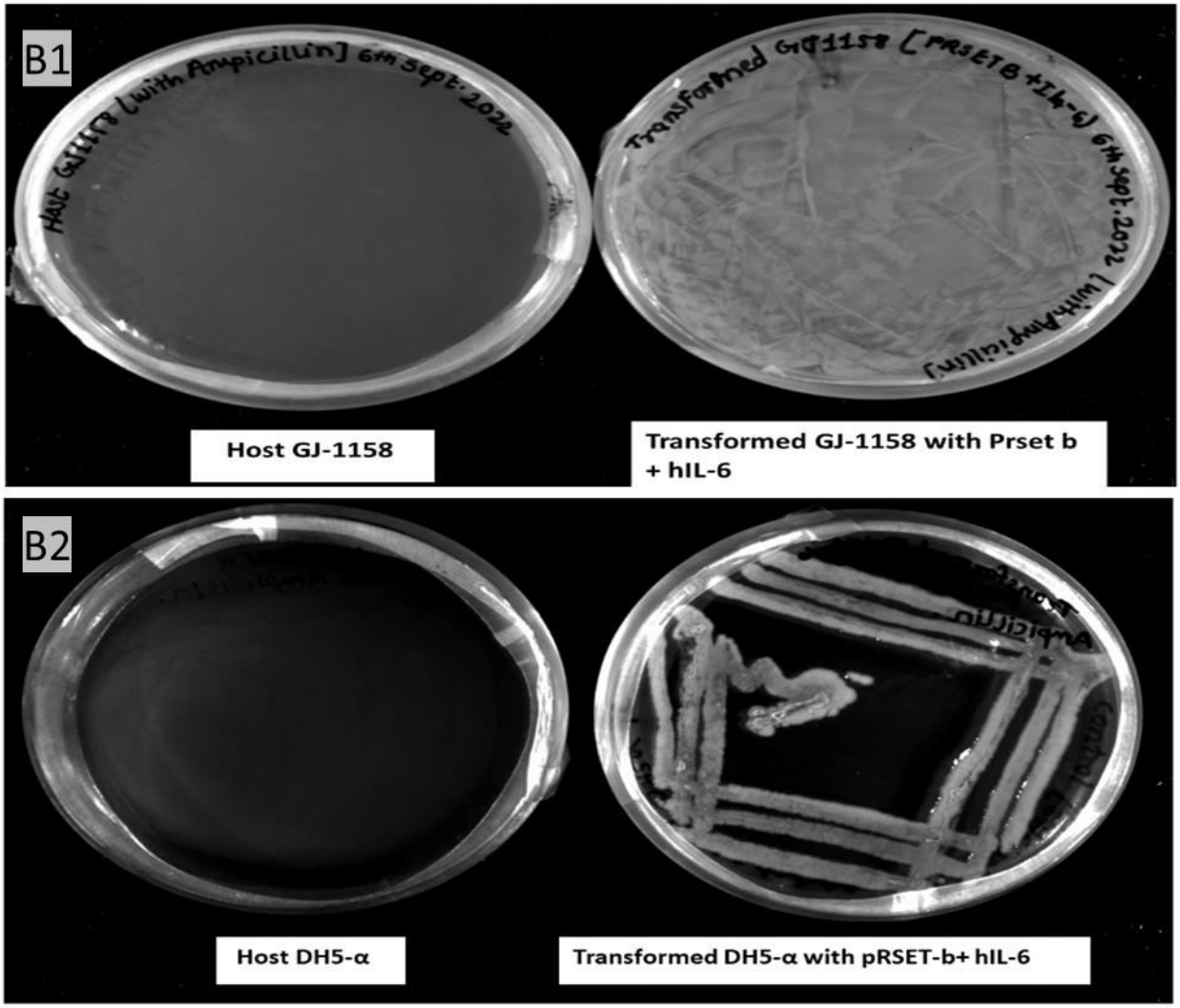


Fig. 2: (B1)-Transformation (DH5α) screening on an ampicillin-containing agar plate. The right plate is transformed DH5α (pRSET-b + hIL-6) and the left plate is DH5α host. And (B2)-Transformation (GJ1158) screening on ampicillin-containing agar plate: The right plate is transformed GJ1158 (pRSET-b + hIL-6) and the left plate is GJ1158 host.

3.3 PCR – Confirmation of Transformed hIL-6 gene:

Following transformation, recombinant pRSET-B with the hIL-6 gene from GJ1158 was isolated for confirmation by PCR amplification with primers against the hIL-6 gene. (Fig.3)

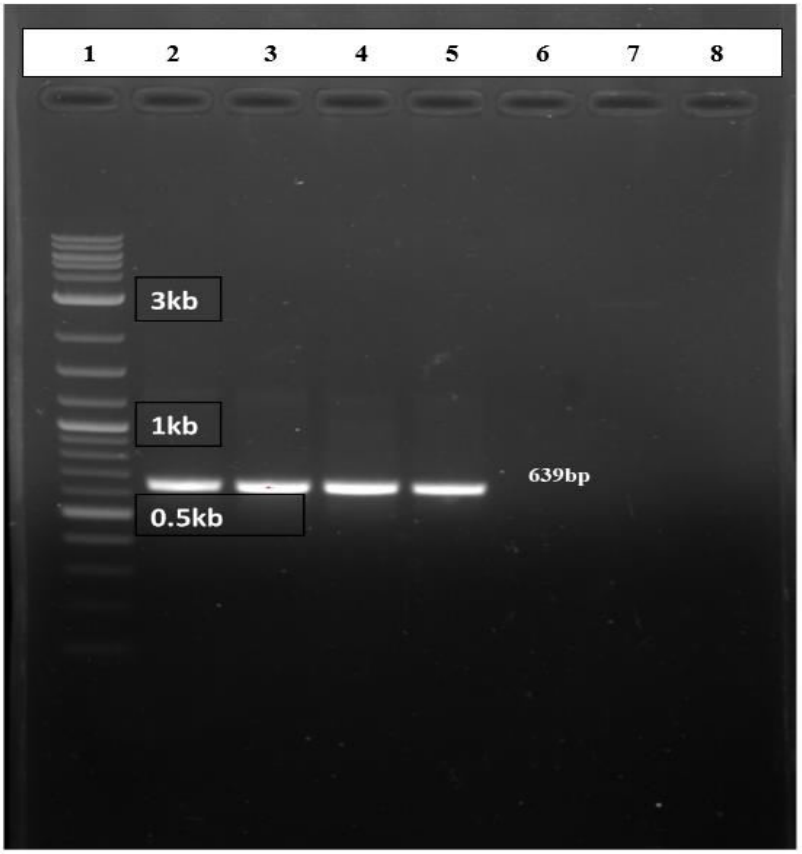


Fig. 3: Confirmation of gene after transformation: 1st lane – 2kb ladder, 2nd, 3rd, 4th, 5th lane – hIL6 gene.

A 2kb ladder was used as a marker. In the figure, between the 1KB and 0.5KB markers, the hIL-6 gene band is clearly visible (in all lanes) at approximately 639BP, which is close to the 0.6KB marker, confirming the presence of the hIL-6 gene.

3.4. Expression:

The E. coli GJ1158 positive clone was grown in LB broth and given an incubation period at 37 °C with 180 rpm orbital shaking until the OD reached 0.4–0.6. The protein expression was then enhanced by inducing the cells with different NaCl concentrations (0.1–0.2 M) and incubating them at different temperatures (25 °C–37 °C). As a negative control, cells with only the pRSET B vector were employed. Additionally, IPTG was used to induce the cells to compare the protein expression.

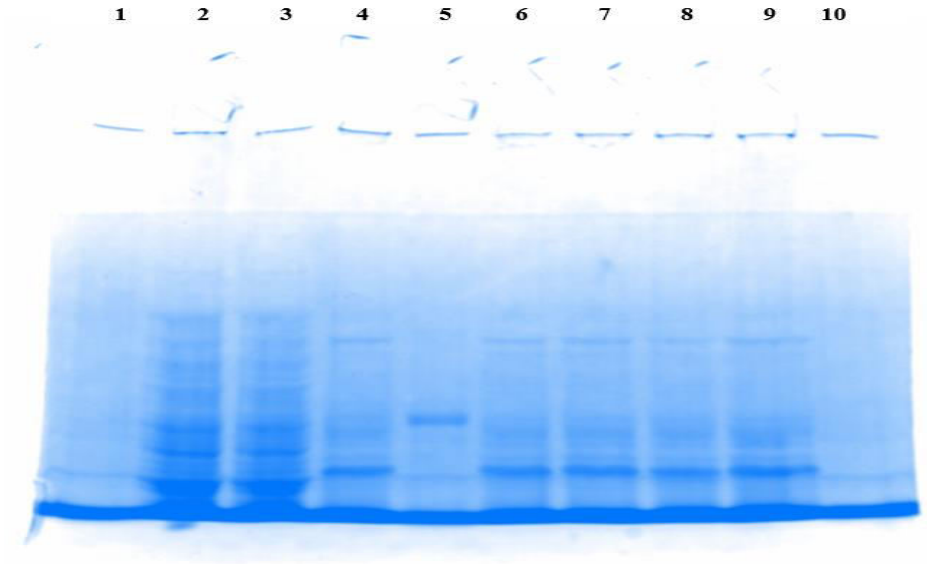


Fig. 4 SDS-PAGE (hIL-6): Lane No.: 1 – Dye, 2 – Host Induce, 3 – Host Un induce, 4 – Host +Vector +Gene Un Induce, 5 – Marker (IgG), 6 – Host +Vector +Gene Induce (NaCl – 0.1M), 7 - Host +Vector +Gene Induce (NaCl – 0.2M), 8 - Host +Vector +Gene Induce (IPTG – 0.2mM), 9 - Host +Vector +Gene Induce (IPTG – 0.5 mM), 10 – Dye

In the 5th lane, the IgG marker shows bands at 50kDa and 25kDa. Human IL-6, which has a molecular weight of approximately 23kDa, is clearly visible in all lanes except for the Host (lane 1, 2) and Un-induced Transformed host (lanes 3, 4).

At 27°C, the expression level appears to be higher than at 37°C. The higher temperature of 37°C tends to promote the formation of inclusion bodies (IBs). Time is also a significant factor in protein expression levels. As time progresses, the formation of IBs increases, typically occurring after the optical density (O.D.) reaches 0.6-0.8, which takes about 5-6 hours.

3.5: Western Blot:

Western blot analysis confirmed the presence of human interleukin-6 (hIL-6) at nearly 23 kDa. A distinct band was observed at this molecular weight when probed with rabbit anti-human IL-6 antibody, indicating successful expression and detection of the target protein. A clear, specific band at ~23 kDa confirms the detection of human IL-6 in the sample.

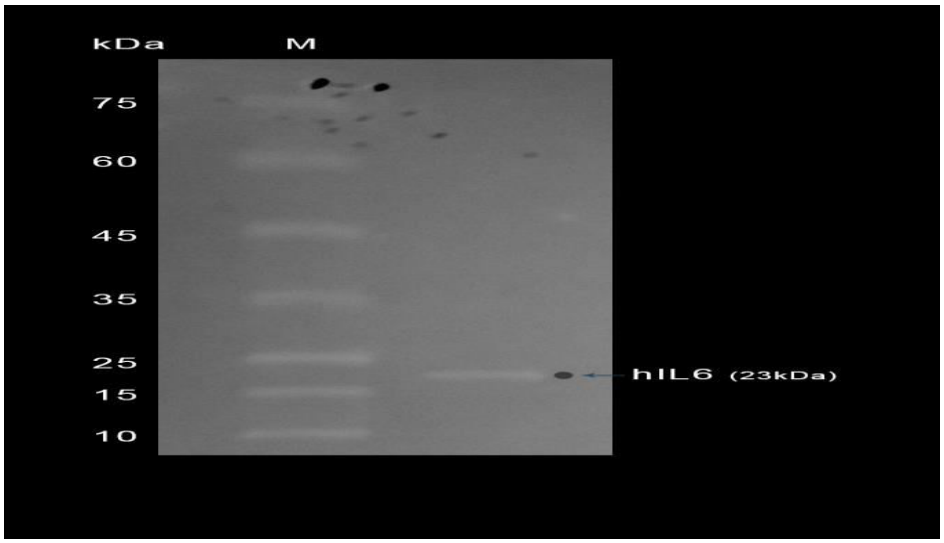
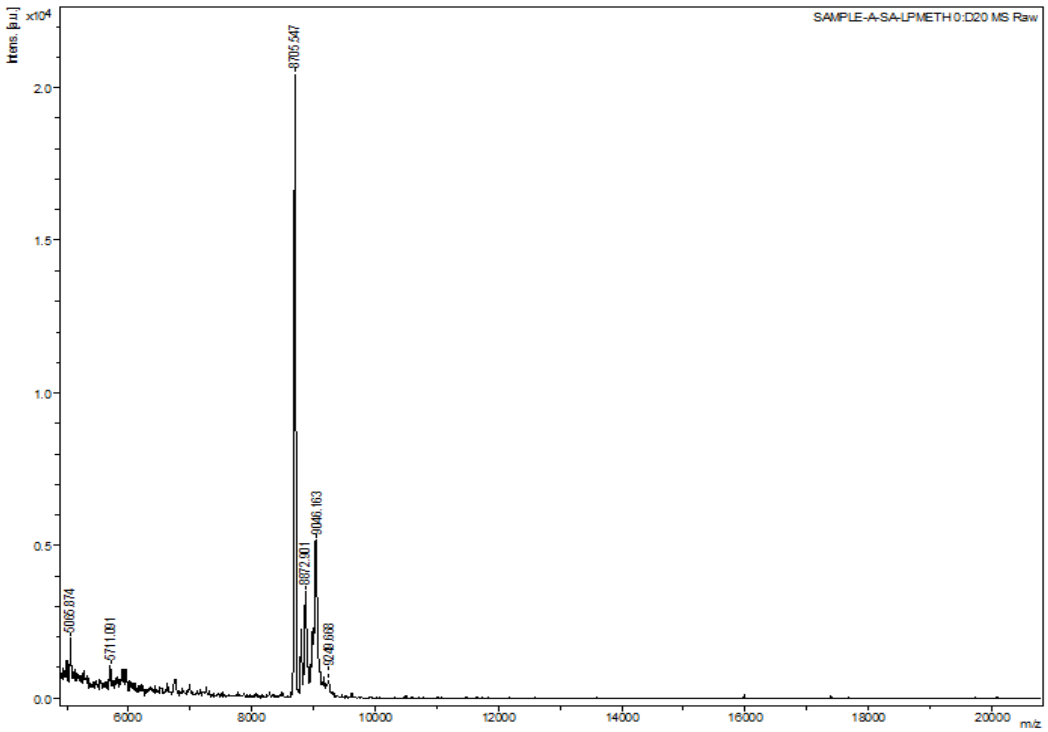


Fig.5 Western Blot:

Lane M: Molecular weight marker (ladder) with bands labeled at ~10, 15, 25, 35, 45, 60, and 75 kDa.
A single sample lane with a band around the 23 kDa mark labeled “hIL6 (23 kDa)”.

3.6 MALDI-TOF mass spectrometry:

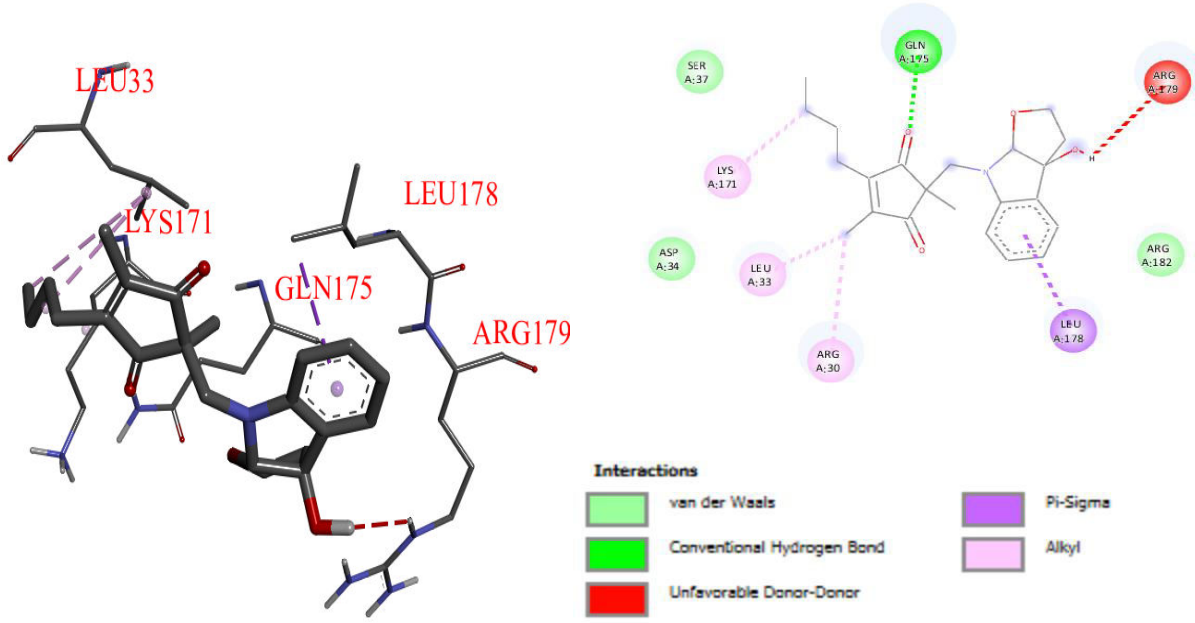


The MALDI-TOF results indicate the molecular weight of the hIL-6 protein. Typically, hIL-6 has a molecular weight of approximately 23 kDa. The presence of a peak at or around this weight in the spectrum confirms the identification of hIL-6. The peaks corresponding to the molecular weight of hIL-6 are essential for confirming the protein's presence. Look for a clear and distinct peak around 23 kDa, which indicates the presence of the hIL-6 protein.

he intensity of the peaks can provide information about the quantity of hIL-6 present in the sample. Higher peak intensity suggests a higher concentration of the protein.

Peaks at different molecular weights may indicate the presence of other proteins or contaminants in the sample. It's crucial to differentiate these from the peak corresponding to hIL-6.

3.7 Molecular Docking of Mandindoline A with hIL-6:



As a measure of the binding interactions, we docked Mandindoline A into human interleukin-6 (hIL-6) with AutoDock Vina. The hIL-6 crystal structure (PDB ID: [insert ID]) was obtained from the RCSB Protein Data Bank. The configuration of mandindoline A was retrieved from PubChem and subjected to an energy minimizing procedure with the MMFF94 force field. Docking of this ligand revealed a binding energy of -8.3 kcal/mol. There were strong hydrogen bonds with Glu172, Lys157, and Arg179, and hydrophobic interactions with Leu178 and Ile153 at the receptor binding pocket of the SARS-CoV-2 RBD. " This indicates that Mandindoline A may inhibit receptor interaction and that it therefore has potential use as a drug.

DISCUSSIONS

A healthy immune system has a perfect balance of anti- and pro-inflammatory chemicals. Inflammation is one of several diseases caused by an imbalance in homeostasis. Interleukin-6 plays an important role in inflammatory diseases, especially viral diseases. Interleukin 6 is a pro-inflammatory as well as an anti-inflammatory mediator [1]. In silico docking of Mandindoline A with hIL-6 demonstrated its potential to bind effectively to IL-6's receptor interface, especially through critical hydrogen and hydrophobic interactions. This supports the hypothesis that Mandindoline A or similar plant-derived compounds may be explored further for anti-inflammatory interventions targeting IL-6. While the docking provides preliminary evidence, experimental validation is necessary to corroborate these findings.

IL-6 is released during the early stages of infection and moves to the liver through the bloodstream to activate the hepatocyte. C-reactive protein, serum amyloid A (SAA), and hepcidin are released by hepatocytes. Long-term SAA causes chronic autoimmune inflammatory diseases, including amyloidosis. Additionally, IL-6 controls the transporters for iron and zinc to modulate serum levels [15]. When hepcidin levels rise, for example, ferroportin is blocked, causing anemia and hypoferremia that are linked to chronic inflammation. The release of RBCs, WBCs, and platelets is caused by the promotion of megakaryocytes by IL-6 once it reaches the bone marrow. Additionally, it stimulates naive CD4+ T cells to differentiate specifically.

The docking outcomes show that Mandindoline A has a positive binding affinity towards hIL-6, and its binding pattern shows potential for inhibitory activity on IL-6 signaling. These findings warrant further in vitro and in vivo confirmation of Mandindoline A as a potential anti-inflammatory compound inhibiting IL-6 for therapeutic intervention of various autoimmune conditions.

In various auto-immune diseases like rheumatoid arthritis, Castleman's disease, and systemic juvenile idiopathic arthritis, as well as viral diseases, IL-6 is the major target to reduce the inflammation by producing anti-IL-6 antibodies or just by blocking the IL-6 receptor with the help of a humanized anti-IL-6 receptor antibody-like tocilizumab [16].

According to numerous studies, the host organism's growth rate drastically decreases when IPTG is added [17]. Thus, intracellular accumulation of glycine betaine has been described as a possible alternative to the inclusion body formation issue [18, 19]. This was accomplished by altering the media composition and supplementing it with choline and glycine betaine precursors. For our analysis, a particular host, E. coli GJ5811, was chosen because it has a metabolic advantage over other hosts. One of the numerous extraordinary characteristics and modifications that GJ5811 is recognized for is its capacity to accumulate glycine betaine in response to a quick disruption of osmotic equilibrium in the relevant enriched media.

Likewise, hIL-6 was neutralized using monoclonal antibodies (mAbs); however, these antibodies have some drawbacks, including limited tissue penetration and an extended half-life in the bloodstream (21). Additionally, mAbs produced in rabbit can be immunogenic in humans, potentially triggering allergic reactions during treatment. Therefore, humanized antibodies are favored since they are less likely to induce such allergic responses (22).
Our lab has already expressed anti-TNFα scfv in GJ1158.

CONCLUSION:

Commercial recombinant protein synthesis always aims to produce proteins as cheaply as possible using simpler, quicker, and safer processes. This work involves the cloning and expression of tagless human interleukin-6 protein in a NaCl-inducible bacterial system. Using low-cost techniques, a tagless recombinant protein was successfully generated in GJ1158. MS studies have verified that E. coli GJ1158 overexpresses the human interleukin-6 protein. It appears that the recommended bacterial expression system and the protein purification techniques are cost-effective, based on the results and the approximate cost estimates.

The Western blot analysis confirmed the presence of human interleukin-6 (hIL-6) at approximately 23 kDa. A prominent band was detected at this molecular weight using the rabbit anti-human IL-6 primary antibody, indicating successful expression and purification of the target protein. The absence of significant non-specific bands demonstrates the specificity of the antibody and the effectiveness of the blocking and washing steps. These results validate the production of recombinant hIL-6 in the tested sample.

Docking results also support the proposition that Mandindoline A might act as a therapeutic modulator of IL-6 activity. The presence of strong hydrogen bonds and hydrophobic contacts at the critical receptor-binding residues allows for the potential Mandindoline A-mediated inhibition of the IL-6-receptor interaction and consequent modulation of downstream inflammatory signaling to occur.

These results provide a solid computational basis for further in vitro and in vivo validation and may pave the way for plant-derived small molecules in drug discovery targeting IL-6.

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CONSENT FOR PUBLICATION
The participants gave informed consent and agreed to participate in the study.

CONFLICT OF INTEREST
The authors declare no conflict of interest, financial or otherwise.

Declaration of Ethics:
The research and publication of this review paper was carried out in accordance with ethical guidelines. There are no conflicts of interest with regard to the publication of this review, according to the authors. This review was conducted without funding. Ethics clearance was not necessary for this review since it does not include any original research involving human or animal participants. Every source of information and reference utilised in this evaluation has been properly cited.

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Legend:
Fig. A Gradient PCR amplification of hIL-6 gene (3µg/well)
Lane no.: 2: Ladder, **3**: 50.2°C, **4**: 53°C, **5**: 57.5°C, **6**: 59.9°C, **7**: 64.5°C, **8**: 66.2 °C Gradient
Annealing temperature get selected by gradient PCR, 57.5°C.
Confirmation of pRSET-B.
Lane No. **3, 5, 7** Digested prSETB with HindIII restriction enzyme(supplementary)
Fig B.1 Confirmation of transformation
Transformed DH5 α only will grow in the presence of ampicillin containing plate on the other hand host DH5 α will not grow in the presence on ampicillin plate.
Fig B.2 Confirmation of transformation
Transformed GJ1158 will grow in the presence of ampicillin containing plate on the other hand host DH5 α will not grow in the presence on ampicillin plate.
Fig. C Confirmation of hIL-6 gene (639Bp):
Lane no. 1: ladder, 2, 3, 4 and 5: hIL-6 gene (2µg)
Fig. D Expression (SDS-Page):
Fig.E Western Blot
Fig. F MALDI TOF Confirmation