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Cloning, Expression and Bioactivity of Human Tumor Necrosis Factor Alpha

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ABSTRACT
A dramatic increase in the amount of recombinant proteins that used in therapeutic applications. TNF-α is one of over 750 recombinant proteins available for use in a broad range of therapeutic applications, including neutrophil chemotaxis, anticoagulatory inhibition, cytolysis and cytostasis of many tumor cell lines. Therefore, in this research Human TNF-α gene has been amplified from the PHA-stimulated peripheral mononuclear blood cells (PBMCs) cDNA pool isolated from healthy volunteers. The amplified human *TNF-α* gene was combined and was cloned to pPIC9 Plasmid. The fusion gene was expressed in the host strain *p.pastoris GS115* using the electroporation technique and inserted under the *AOX1*promoter's control. Tricine-SDS–PAGE and Western blotting were achieved for methanol-induced expression strain to confirm the successfully secretion of fusion TNF-α protein into the culture medium. Ni-NTA resin was used to purify the recombinant protein. (MTT) assay was used for screening the biological activity of the purified human TNF-α results showed that the Fusion TNF-α reduced the viability of HEP2 cells as long as metabolic activity in dose-dependent manner. Our findings show that TNF-α-gene expressed in the present study is active and had the same pharmacokinetic and biochemical properties as the standard TNF-α and recommended to be used therapeutically as treatment for human diseases, and could be helpful to develop new strategies to cancer therapy.

Keywords: hTNF alpha, cloning, Expression, P.Pastoris, biological activity, MTT assay

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INTRODUCTION
A dramatic increase in the amount of recombinant proteins that used in therapeutic applications. Recombinant Human Tumor Necrosis Factor-α is one of more than 750 available heterologous proteins that used for wide spectrum of therapeutic applications including ,chemotaxis of neutrophils, inhibition of anticoagulatory mechanisms, cytolysis and cytostasis of many tumor cell lines (Katharina *et al,* 2015). Human tumor necrosis factor α (hTNFα), a pleiotropic cytokine pro-inflammatory with many biological impacts that participates in the regulation covers numerous operations ranging from host immune defense mechanisms against multiple infections and injuries to serious septic shock poisoning or other associated illnesses and injury restoration. Is a promising drug discovery target (B*enoît et al,* 2015), (Michał *et al,* 2018).

The human TNF-a gene is situated on the short arm of chromosome 6, 21 within the Class III region of the human major histocompatibility complex (MHC) Contribute to the pathogenesis of a broad spectrum of autoimmune diseases and infectious diseases. Genetic changes in the locus of TNF-α are now known to be directly engaged in elevated manufacturing of TNF-α (Harishankar *et al,* 2019).TNF plays a contradictory role in pathological impacts through the proliferation, differentiation or apoptosis of cellular signals by its two receptors for transmembrane. TNF features are in fact complicated. Indeed, TNF functions are complex, where on one side it confers resistance to disease and On the other hand, pathological complications can occur (Li *et al,* 2018), (Sujuan *et al,* 2018).

The human tumor necrosis factor plays a significant role in the growth of various illnesses of pathophysiology. Recent studies have shown that in vitro aberration in TNF manufacturing leads to a big amount of human illnesses, such as lupus erythematous (Wfaa *et al,* 2016). Risk for male infertility (Taymour , and Mai 2016) Inflammation of multiple organs, inflammatory intestinal disease and cancer (Mujeeb *et al,* 2016). Several kinds of tumor cells constitutively express TNF-including ovarian cancer, breast cancer, and other influences TNF were involved in an Immune System cancer such as lymphoma (Eugene and Avi 2004). TNF-'s anticancer activity is similar through its capacity to boost NF-B's pro-inflammatory transcription factor, which activates gene expression associated with tumor cell invasion, survival, angiogenesis, metastasis. and proliferation (Calip *et al,* 2018) Thirty six Several types of tumor cells constitutively express TNF — including ovarian cancer, breast cancer and others thirty seven The majority of tumor cells expressing TNF- have constitutive activation of NF-B. These tumor cells are "addicted" to NF-B because they are highly dependent on this factor for their survival (Dalia *et al,* 2015).

MATERIALS AND METHOD
Ethical Issues
The ethical issues in the present study are based on The acceptance of the Scientific Committee of Genetic Engineering Department at Institute of Genetic Engineering and Biotechnology for Post Graduate Studies / University of Baghdad .Iraq .All persons participate in this study were understood the objectives of study and signed an informed consent.

Strains, vectors and Reagents
Standard strains of *E.coli DH5α* competent strain. *pPic9k* plasmid vector ,*Pi pastoris GS115* were bought From Takara Biotechnology Co., Ltd. and then maintained in our laboratory. The primers oligonucleotide sequence and prob were supplied by Shanghai Gene Core Biotechnology Co., Ltd. to amplify the target DNA used in this research.

General molecular biology reagents including *EcoRI* and *Not* 1 Restriction enzymes and *T4* DNA ligase were purchased from Bioneer/South Korea.

Methods

Amount of five ml of venous blood was withdrawn from healthy volunteer aged 35 years in anticoagulant tube under septic conditions were separated by using Histopaque1.007 (Sigma) in density gradient in cold centrifugation and washed three times with phosphate-buffered saline (PBS). PBMCs were diluted to 5×100 cells/ml in RPMI 1640 (Gibco) containing 10% fetal calf serum (Gibco). Phytohemagglutinin (PHA) Lectin has been added to the 5 ug / ml cell suspension and incubated for 24 hours at 37'C in a 5% CO2 humidified incubator. The activated PBMCs have been purified with PBS phosphate buffer saline.

RNA isolation and PCR amplification

Total RNA has been extracted from purified PBMCs using Trizol LS (LifeTechnologies). From the isolated total RNA , cDNA was synthesized using oligo (dT) primer and stored at –20°C until used for PCR.The amplification of the human TNF-a gene was carried out by using the first-strand cDNA as a template and two sets of specific primers ,the forward primer was 5' CTGGTTCAGAACTCAGGTCCT -3'and the reverse primer was 5'GAGGTAAAGCCCGTCAGCA -3', Primers contain EcoR1 and NotI restriction sites on the 5`ends for amplification of human TNF-a fragment. PCR product was purified (Purification kit AXy Gene miniprepkit),

The PCR reaction was carried out in a complete quantity of 25 µl with a DNA template, of 2, µl, 1.5 µl dNTP mixture, 2.5 µl PCR buffer, 1.5 µl forward primers, 1 µl reverse primer, 1 µl Ex Taq polymerase and 16 µl D.D water. The initial denaturation of PCR conditions was 95 C for 3 min, followed by 30 cycles at 95 C for 1 min, Annealing for 1 min at 55 C and extending for 1 min at 73 C, and lastly extending the PCR product for 5 min at 73 C. The PCR amplified product was analyzed on 1.5 % agarose gel. TNF gene was removed from the gel and purified using the purification kit for DNA.

Construction of recombinant protein

The amplified human TNF-a gene fragment and *pPic9k* cloning vector were double digested with *EcoR1* and *NotI* restriction enzymes .Both the restricted products were cleaned up using (Axygen) gel clean up kit. The product obtained was checked on 1.5 % agarose gel, and ligation reactions was carried out by the TNF-a gene into the *EcoRI* and *NotI* sites *of pPIC9K* to produce the expression plasmid, The gene was inserted at the *EcoRI* site of *pPIC9K* and used to generate an in-frame protein fusion of the inserted DNA with the *Saccharomyces cerevisiae* α-mating factor signal sequence to enable the secretion of recombinant protein.

Transformation of competent cells with pPic9k

The recombinant *(TNF- pPic9k*) was transformed into the heat-shocked *E. coli DH5a* competent cells as described by Hanahan (Hanahan *et al,* 1995), positively clones were cultivated at 37 C overnight on LB plates containing 100 mg / ml of ampicillin. To ensure that Positive bacterial cells contain the pPic9k vector with the TNF insert gene plasmid Expression vector was isolated from Positive bacterial clones using Plasmid Miniprep Kit (Axygen company), Then recombinant plasmids were first digested with EcoRI restriction enzymes then double digestion reaction was accomplished by using EcoRI and NotI enzyme.

Expression vector, (*pPic9k- TNF*) which was propagated and checked in the positive cells, was isolated and linearized by *SalI* restriction to increase integration into the *P.pastoris* genome through recombination events. Prior to the transformation process. *P pastoris GS115* host strain was induced into a competent state for transformation with linerised plasmid by using the electroporation method as described by (Scorer *et al,* 1994) .This strain has a mutation in histidinol dehydrogenase (his4) that prevents it from synthesizing histidine(LinCereghino *et al,* 2005).

Expression of human TNF- α gene in *P. pastoris*

By performing small scale expression studies TNF-a expression was assessed by using a single colony able to grow without histidine supplement on MD plates. The culture supernatant was purified by ultrafiltration as the first step to concentrate the supernatant and remove small impurities. The ultrafiltrate was resuspended in lysis buffer (8 M urea, 0.1 MNaH2PO4, pH 8.0) and mixed with Ni-NTA (Qiagen).

SDS-PAGE analysis

Tricine- SDS-PAGE technique was employed to follow the expression of TNF- α as described by Schagger and von Jagow, (Schagger H and Von G. 1987)).Samples were prepared by mixing 5 µl of 2X Tricine SDS loading buffer with 10 µl of protein solution followed by boiling in water bath at 100°C for 5 minutes then loading on gel, after that centrifugation (15000 rpm for 1min) to remove insoluble material) then samples were loaded immediately. The gel was pre-electrophoresed for 1 hr at 30 V, until the gel front progressed past the stacker and into the separating gel, at which point it was increased to about 150 V for to 5 hrs. until the gel front reached the bottom.

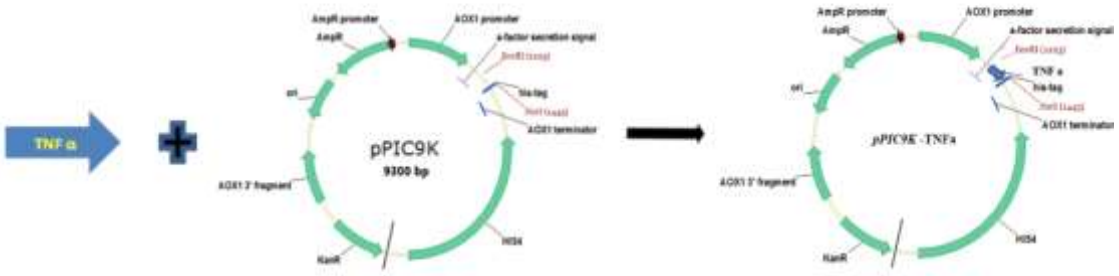
Western blot analysis

Western blot analysis was performed according to the procedure described by Bjerrum (Bjerrum O and Heegaard H. 2001) .the gel was transferred to nitrocellulose membrane and placed in transfer buffer, then were electro-blotted onto a polyvinylidenedifluoride (PVDF) membrane (Millipore Immobilon-P, USA. After blocking the membrane with 5% non-fat dried milk in TBST at room temperature for 1 hr, the membrane was exposed to the primary antibody at 1:1000 to 10 ml block solution and then incubated at room temperature for 1 hrs. With gentle shake .After incubation of the membrane treated with a solution of horseradish peroxidase conjugated secondary drug at dilution (1:5000), the membrane was then cleaned three times in 10 ml of TBST in 5% non-fat TBST milk for 1 hour with mild shaking. And put in a film x-ray cassette The

Image of western blots was scanned using a canoscan 8000F scanner, (USA).

Screening of Recombinant TNF- pPic9k in P. pastoris strains

To confirm whether transformants *P.pastoris* contain TNF-pPic9k plasmid expression vector or not ,genomic DNA was extracted from transformants His+ cells and used as template for PCR analysis according to Linder 1996.PCR amplification was confirmed by 1.5% agarose gel electrophoresis (Linder *et al,* 1996).



Schematic diagram illustration of the steps of Construction human TNF- α into pPIC9K expression vector.

Biological activity of TNF alpha

The purified human TNF-α was tested for its biological activity, using the (MTT) assay, (3-[4, 5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide. Briefly, 100 µL per well of HEP2 cells were seeded in triplicate at 5 × 103 cells mL into a 96-well microtiter plate and cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and incubated for 18 h at 37°C with 5% CO2. After that recombinant human (rTNF-α) were added to the cells at different concentrations 20,30 and 50 µg/ml and further incubated for 24, 48 and 72 hrs. Then, , 20 µl of a 5 mg/ml MTT solution (Sigma, USA) was added to each well and incubation was continued for 3 h at 37°C with 5% CO2 to allow MTT metabolization. The resultant formazan crystals were dissolved by adding 100 µl of isopropyl alcohol with 0.4 N HCl per well. Cell viability was analyzed using a ELISA plate reader (Invitrogen.UK,) and optical density measured at 540 nm. The optical density of wells containing cells cultured without rTNF-α was assumed to be 100% of cell viability.

RESULTS

Recombinant DNA technology is playing a central role in generate and improving a large amounts of a pharmaceuticals protein such as Insulin that used for diabetes mellitus treatment (Rafid ,2019) TNF-alpha is now a well-established pharmacological target for diseases therapy, and provides many pharmaceuticals benefits (Alain *et al,* 2019) the results of isolated mRNA are shown in Fig (1). mRNA of TNF-a was found in freshly isolated this may be the result of activation caused by Phytohemagglutinin (PHA) lectin .Treatment of monocytes with (PHA) led to accumulation of mRNA of TNF-a (Neetu *et al,* 2018) .

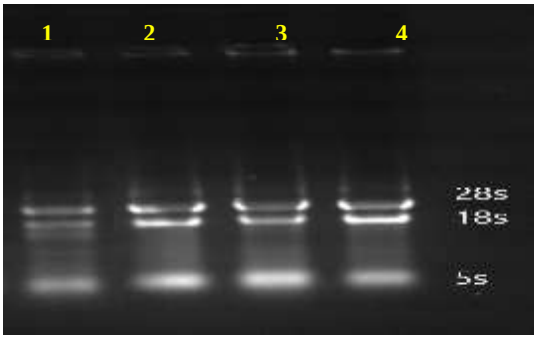


Figure 1: Total RNA isolated from (PBMCs)was fractionated on 1% agarose gel electrophoresis .lane 1,2,3and ,4 the bands of 28S and 18S rRNA and smear of faint bands which represent mRNA.

The cDNA prepared from total RNA isolated from PHA - stimulated PBMCs revealed the presence 249 bp fragments on 2% agarose gel, obtained by using TNF – α gene specific primer in RT-PCR amplification (Fig. 2).

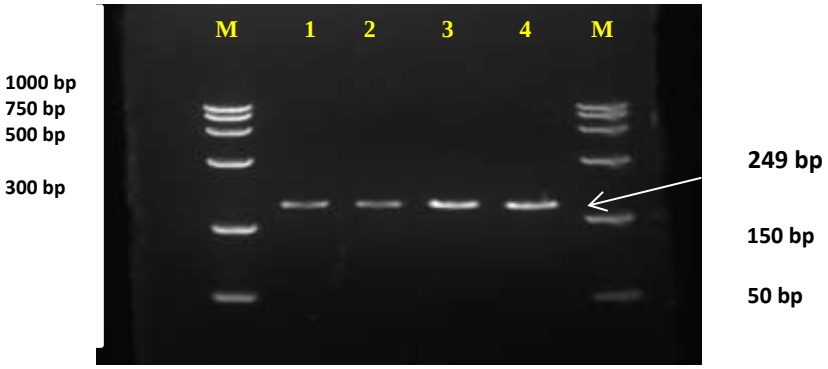


Figure 2: Amplification of hTNF – α gene by RT-PCR. The products were fractionated on 2% agarose gel electrophoresis. Lane M : DNA marker 1000 bp. molecular weight standard (1000, 750 , 500 , 300,150 , 50bp) Lane 1-4 : human TNF – α gene.

The amplified TNF α gene was inserted into the *pPIC9* vector to construct the *P. pastoris* expression vector (*pPIC9-TNFα*) which was identified by double enzymatic digestion with the *EcoRI and NotI*. The corresponding bands were observed by 1.5% gel electrophoresis (Fig. 3).

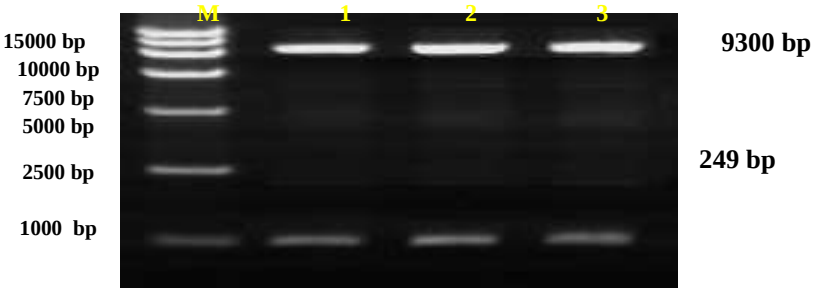


Figure 3: Double digestion reaction of *TNFα - pPic9k* plasmids with EcoRI and NotI restriction enzymes fractionated on 1.5% agarose gel electrophoresi Lane M : DNA marker 15000 bp. molecular weight standard (15000, 10000, 7500, 5000, 2500, 1000, 250). Lanes 1, 2, and 3, the size of *pPIC9K P. pastoris* expression vector with double digestion with EcoRI and NotI which containing human TNFα gene.

Then (*pPIC9- TNFα)* vector was transformed into E. coli DH5a competent cells by the heat-shock method .Recombinant plasmid was confirmed by colony PCR and Restriction analysis Results shown in Figure (4) indicate the presence of band of about 9 549 pb as compared to DNA ladder. Integration of plasmid DNA into the genome of *P. Pastoris* was achieved by homologous recombination, the (*pPIC9- TNF α*) plasmid was linearized with *SalI* restriction enzyme and transformed into electrocompetent *P. pastoris*. Successful integration was confirmed by PCR and sequencing. (Fig4).

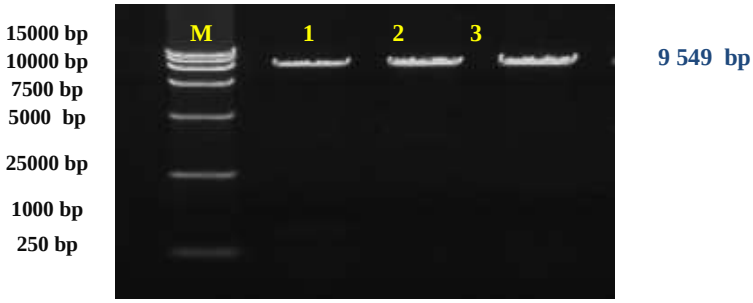


Figure 4: Linearised *TNFα-pPic9k* Plasmid with SalI. DNA was fractionated on 1% agarose gel electrophoresis Lane M: DNA marker 15000 bp. molecular weight standard (15000, 10000, 7500, 5000, 2500, 1000.250 bp). Lanes 1, 2, and -3: band of Linearised *pPIC9- TNF α.*

SDS PAGE and Western blot analysis

In this study the Results recognized by tricine- SDS–PAGE analysis indicate The proteins was expressed successfully with band of approximately the 17.5 KD as shown in figure (5)which mimic the calculated band of human TNFα (17.5 KD). It can be concluded, that recombinant human TNFα had been expressed successfully and subjected to the proteolytic cleavage efficiently in *P. pastoris* and fused gene was translated into protein secreted in the medium and developed on the SDS page gel, as well as detection on western blot membrane.

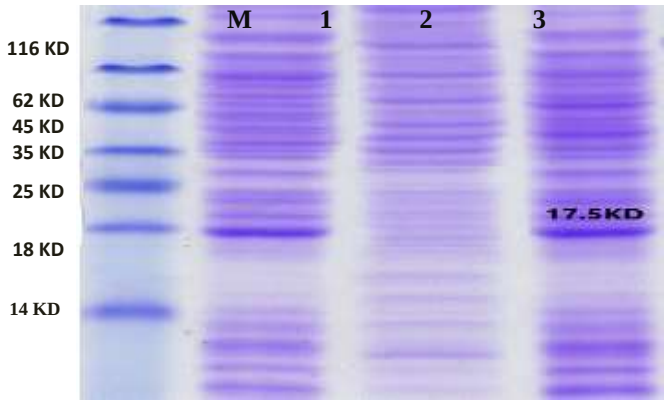


Figure 5: SDS-PAGE of eluted fraction from Ni-NTA agarose bed column .Protein were fractionated on 12% SDS-PAGE.
Lane M Standard molecular weight protein marker 116 KD (marker (116, 62.45, 35 25,18, and 14KD).
Lane 1 Ininduced recombinant Pichia pastoris GS115 Column washed with 50 mM imidazole to remove non-specific binding proteins
Lane 2 Uninduced recombinant Pichia pastoris GS115 Column washed with 50 mM imidazole to remove non-specific binding proteins
Lane 3 Ininduced recombinant Pichia pastoris GS115 eluted of the recombinant proteins with 50mM imidazole

Furthermore, result of SDS–PAGE was synchronized with the similar results of expression patterns of western blot analysis that shown in fig. (6) Which confirms the existence of human TNF α in the medium according to the band size of the secreted protein and established on the SDS page gel .Therefore western blot analysis could be taken as a successfully evidence for human TNF α. Expression. As shown in figure (6) the results in this figure were demonstrated that a band of approximately 17.5 kDa which presence on the SDS page gel was equivalent the calculated band of human TNFα (17.5kDa).

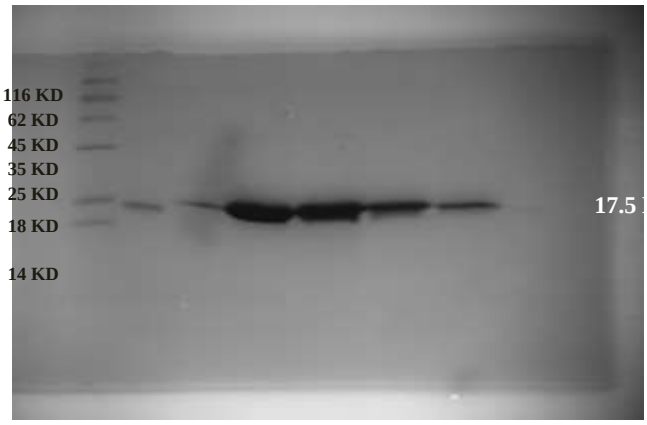


Fig 6: Western blot analysis of separated Protein from recombinant P. pastoris. Lane M: Standard molecular weight protein marker (116, 62.45, 35 25, 18, and 14KD). Lane 1: human TNFα expressed in recombinant strain was about 17.5kDa, which was consistent with the theoretical molecular weight of human TNFα protein.

Biological activity of recombinant human TNF-α

Results shown in figure (7) indicated that recombinant TNF-Reduced HEP2cell viability with a statistically significant p value after 24, 48, 72hrs incubation times. the different tested concentrations of recombinant TNF-α showed inhibitory effect on the metabolic activity as long as HEP2cell viability of the cell after exposure to different concentrations of 20,30 and 50 µg/ml TNF-α give an effect on cell viability of 76.4, 70.53, and 60.14% respectively .

The inhibitory effect of rTNF on HEP2cell growth was dose-dependent manner since the effect of 30 µg/ml TNF-α per day was significantly stronger than the effect of the 20 µg/ml TNF-α -µg dose after 72 incubation times of AMN3cell treatment and maximum decrease (from70.53 to 60.14) was

observed at 50 μg/ml after 72hrs incubation time In compare with The optical density of wells containing cells cultured without TNF-α.

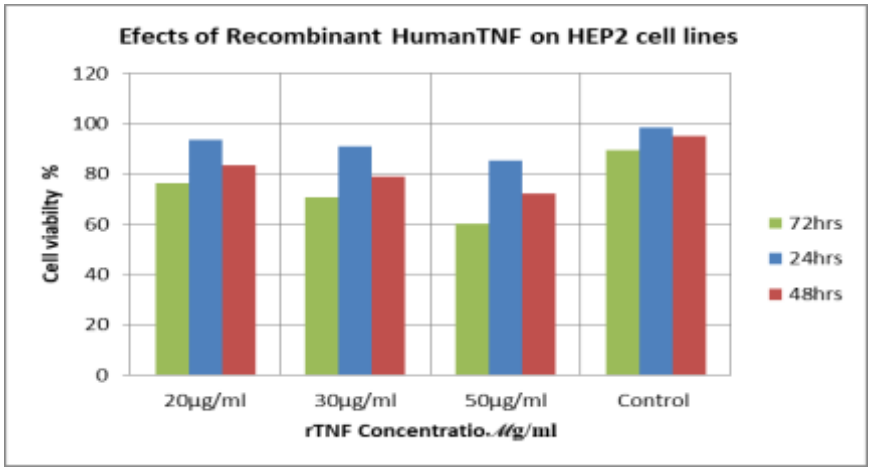


Figure 7: Dose-dependent cytotoxic effect of rTNF-α-mediated in tumor cell lines.

HEP2 Cells were placed into 96-well microtiter plates at a cell density of 5 × 103 cells/well and allowed to incubate for 18 h in culture medium. Culture medium was replaced by RPMI medium containing FBS and Incubation was then continued in the presence of Serial concentrations of rTNF-α After a 24, 48, 72 hrs. cell viability was determined determined using the MTT test.

DISCUSSION

Over 20 years ago With the development of genetic engineering technology Yeast expression systems have been used successfully in the biotechnology industry and basic research for heterologous proteins from human, plant animal, bacterial, and fungal origin Because of the capacity to carry out post-translational, glycosylation, modifications and secretions (Richard *et al,* 2017) .In this research, total RNA from (PBMCs) was effectively isolated with trizol reagent, and the purity and concentration of the total RNA samples were achieved. Moreover, the RNA has been isolated was judged by measuring their absorbance at (A260) and (A280), by spectrophotometer .

The total RNA isolated from (PBMCs with a purity ratio of 1. 88 no extra bands have been observed, and no signs of genomic DNA contamination. RNA integrity is evident on 1% agarose gel electrophoresis was clear as long as three distinct sharp bands.

These bands represented 28S rRNA , 18S and 5s rRNA ,As a result, it was judged that RNA appeared to be comparable in quality (Anjan *et al,* 2013). mRNA isolated (PBMCs) was used as template and converted to (cDNA) using RT-PCR reaction and then (hTNFα), gene was amplified using specific primers contain *EcoRI* and *NotI* restriction sites to be converted latter by these enzymes to sticky ends for cloning of (hTNFα) gene . Results in figure (2) indicated the visualization of PCR product of 249 bp when electrophoresis on 1.5 % agarose gel, which gives good indicator and confirm that the PCR reaction is carried out correctly (Seyyed *et al,* 2018).

Construction of human TNf – α into expression vector

Result shown in Figure (4) indicated the presence of a single sharp band of about 9.549 bp, which is represent the size of (*TNf α- pPIC9K*) *P. pastoris* expression vector which containing (hTNFα) gene.

In this study Several consideration were done in order to obtain successful results, The gene was inserted in the accurate orientation under control of (*AOX*1) inducible promoter which is control methanol-inducible alcohol oxidase in the correct reading frame to allow unlimited expression of human TNF – α gene that is induced from outside by the action of methanol the exogenous inducer (Maryam *et al,* 2017). From other hand our design in construction of the recombinant vector is to insert the human TNF – α gene in the proximity of alpha-factor to facilitate the secretion of the product and allows easy collection. Furthermore the gene was cloned on a vector containing two markers, one for threat of life (auxotroph by histidine) and the other is for selection protection .This design facilitates, secretion expression, protection (Jinjia *et al,* 2017).

Transformation of ***E. coli DH5α*** cells with *pPic9k – hpi*

Our data suggested that *E. coli DH5α* competent cells were successively transformed with *pPIC9K -TNF* by Heat-shock method, since a reasonable number of transformed colonies were appeared on LB agar media supplemented with 100 mg/ml of ampicillin which force the bacteria to harbor the plasmid as a protection strategy (Ahmad *et al*, 2019). The results of Agarose gel electrophoresis in figure (3) of single and double digestion reaction of *TNF - pPic9k* plasmids with *EcoRI* and *NotI* indicate the presence of two sharp band, of about 9 300 bp and 249 bp which is related to the vector and TNF gene respectively . From other hand Competent *P pastoris GS115* host strain were successively transformed with *pPIC9K -TNF* by using electroporation with gene pulser apparatus (Anjan *et al*, 2013). *Pichia pastoris GS115* is auxotrophic strain has a histidinol dehydrogenase gene mutation (his4) that prevents it from histidine synthesis,

The results indicated that a successful integration was occur since a reasonable number of *P. pastoris* colonies had appeared on selective medium (histidine deficient media) which is taken as an indicator for the high level efficiency of transformants with *TNF - pPic9k* linerised Plasmid. We can conclude that the insertion was in the correct orientation and open reading frame and had no errors in insertion of TNF gene into the *pPIC9K* plasmids (LinCereghino *et al,* 2005).

SDS PAGE and Western blot results

Tricine-SDS–PAGE shown in figure (5) indicated the successful production and secretion of the recombinant human TNF-α into the culture supernatants under induction of methanol lane .1, 3 as compared to Unindicted plasmid lane2. In conclusion the expression of TNF-α protein can be attributed to the cloning of target gene in frame and downstream of the α-factor signal sequence in order to facilitate secretion of the human TNF protein into the medium to facilitate purification of recombinant protein (Duan *et al*, 2019).

As long as the TNF-α gene was integrated successfully under the control of methanol-induced alcohol oxidase *(AOX1)* promoter into the genome of *P. pastoris* which expressed the heterologous gene upon induction by methanol. (Cámara *et al*, 2019), (Ching *et al,* 2018). Furthermore tricine-SDS–PAGE result was synchronizing with the identical results of expression patterns analysis of the western blot as appeared in fig. (6) Which emphasize the presence of the expressed TNF-α protein according to protein size that secreted in the culture medium and developed on the gel of tricine-SDS–PAGE. The presence of band of approximately 17.5 kDa in western blot membrane is an evidence for successfully expression and efficient proteolytic cleavage of fusion protein Depend on the alpha-factor prepro-leader in P. pastor cells which secreted the protein in the culture medium and developed on the SDS page as long as on western blot membrane (Rochelle *et al*, 2018), (Nurdian *et al,* 2018).

Biological activity of recombinant human TNF-α

The biological activity of rTNF-α against HEP2cell was estimated in vitro, results in Figure (7) showed that rTNF-α had inhibitory effect on cell viability and metabolic activity versus HEP2cells. Human TNF-α plays a major part in the microenvironment of tumors, and can interact with cells through specific receptors, and trigger Several biological functions such as cell survival, proliferation, differentiation, death and downstream signaling, of the NFκB pathway (Yang *et al,* 2017)Human TNF-α play is an important cytokine that promotes anti-tumor effects by sharing the capability of inducing Cellular apoptosis through inhibiting the major cell survival signal that is anti-apoptotic such as nuclear factor κB (NF-κB) and c-Jun N-terminal kinase (JNK) (Dondossola *et al,* 2016). TNF-α exerts remarkable biological functions through activating diverse cellular events such as promotion and activation of monocytes and neutrophils to active sites for immune responses (Alan and Athena 2019), Furthermore blocked T-Regulator cells that cause immune suppressors by stimulating and activation of macrophage and natural killer cells (Annie *et al,* 2017).

Finally rhTNF-α Contributes to disruption and modulation of new blood microvasculature that supply Tumor cells with nutrients and oxygen via Extracellular signals that induces fibrin accumulation with clotting and increased permeability leading to the Tumor cells necrosis. (Keith *et al,* 2016). Hence our results suggest that TNF-α induced signaling pathways in tumor biology and have cytotoxicity mechanism which involves inhibition of cell viability and metabolic activity of HEP2tumor cells. (Mizuho *et al,* 2018).

In conclusion, our results provide evidence that human TNF-α-gene expressed in research is active and had the same pharmacokinetic and biochemical properties as the standard TNF-α and recommended to be used therapeutically as treatment for human diseases, and could be helpful to develop new strategies to cancer therapy.

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