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# Isolation and Purification of CNF1 (Cytotoxic necrosis factor 1) produced from bacteria *Escherichia coli* and study its role against apoptosis *in vitro*

Muthanna H. Hassan[1], May T. Flayih[1], Nahi Y. Yaseen[2]

1 Biology Departmen\ College of Science\ University of Baghdad
2 Iraqi Center of Cancer & Medical Genetics Researches\ University of Al-Mustansiriyah

## Abstract:

Through the period April - December 2012, urine specimens were collected from186 patiants attending five hospitals. Seventy six isolates of uropathogenic E. coli were obtained from these samples. These isolates were identified as E. coli according to the cultural and morphological characteristics on MacConkey agar, eosin methylene blue agar, biochemical test (vitek 2 system), and API 20 E system. The results showed that only four E. coli isolates (E4, E8, E23, E36) produced cytotoxic necrosis factor 1(CNF1), and E23 was the more efficient isolate in CNF1 production and have high activity on tissue culture. CNF1 produced from this isolate was purified by two steps; the first was by ion exchange chromatography using DEAE-Sephadex, and the second step was gel filtration by Sephacryl S-100. Also study Polymerase Chain Reaction (PCR) for cnf1 gene and used primer cnf1. The size of amplified gene was 543pb. The cytotoxic effect of different concentrations of CNF1 on cancer lines (RD, AMN3, , Hela, PC3 and HePG2) at 72 hr were examined .The effect was manifested multinucleated and enlargement in cell line. The low IC50 was 104µg/ml against HePG2cancer cells after 72 hr. Also study multiparameter cytotoxic assay. The results revealed low concentration of cytochrome c, total nuclear intensity, cell membrane permeability in comparative positive control. The mitochondria concentration was high compared to the positive control on of cell lines in the concentration (100,25, 12.5,6.25).

***keywords:*** *CNF1, Escherichia coli, apoptosis, in vitro*

## Introduction:

Urinary tract infections (UTIs) are among the most common bacterial infections of humans. More than 80% of UTIs are caused by Uropathogenic E. coli (UPEC) strains [1]. The virulence factors in UPEC strains are P fim-briae (pap), a fimbrial adhesin I (afaI), hemolysin (hly), cytotoxic necrotizing factor 1(cnf 1), and S fimbriae (sfa) [2]. CNF1 a protein toxin, first described in 1983 by Caprioli and coworkers as a toxin capable of causing multinucleation (cytotoxic) in cultured cells and necrosis in rabbit skin (necrotizing) [3], This toxin is an AB-type toxin which cause urinary tract infections and neonatal meningitis, it has a 115 kDa single-chain molecule comprising an N-terminal receptor binding domain and a C-terminal catalytic domain, which contains deamidase activity [4].The PCR studies showed that the gene for CNF1 is more common than that for CNF2, CNF1 and CNF3, which are chromosomally encoded while CNF2 is located on a transmissible plasmid [5]. All CNFs are identical in length (1013/1014 aa) and comprise a modular structure with an N-terminal receptor binding domain , in conjunction with the central translocation domain, it mediates cellular entry . The C-terminal part of CNF1 (720 to 1014 amino acid) harbours the full catalytic activity [6]. The aim of study Isolation and Purification of CNF1(Cytotoxic necrosis factor 1) produced from bacteria E.coli and study it's role against apoptosis in vitro.

**Corresponding address:**
**Muthanna H. Hassan**
Biology Departmen\ College of Science\ University of Baghdad
**Email:** muthanna_477_aliaqi@yahoo.com

## Materials and methods:

**Patients:**

Through the period extending from April 2012 till December 2012, 186 samples of urine were collected from patients with urinary tract infections and prostate cancer in sterilized containers from five hospitals in Baghdad and Anbar.

**Identification of *E. coli***

The samples were processed on MacConkey agar ,Eosin methylene blue agar and blood agar, and then incubated at

370C for 24hrs. The identification of gram negative bacteria was performed by standard biochemical tests (catalase, oxidase, Urease , Indole production, Methyl red, Voges-Proskauer and Citrate utilization test) and confirmed by vitek2 and Api-20 E according to Bergy's Manual for Determinative Bacteriology [7,8].

**Detection of cytotoxic necrosis factor1(CNF1)by PCR:**

Cytotoxic necrotizing factor (cnf1) primer is used for detection of cnf1 in all isolates obtained in this study by using PCR. The primer forward sequence (5'to3') cnf1-F was GAACTTATTAAGGATAGT and the primer reversed sequence (5'to3') cnf1-R was CATTATTTATAACGCTG(alpha /USA). The size amplified product was 543 base pairs. The reaction was performed in a thermal cycler apparatus. After several trials and according to the manufacture's trouble shooting guide, the amplification program is given in table 1

**Table 1** The cycling conditions

| | *Temperature* | *Time* | *Number of cycles* |
|---|---|---|---|
| **Number of cycles** | **94°C** | **4 min** | **1** |
| **Denaturation** | **94°C** | **45 sec** | **35** |
| **Annealing** | **46°C** | **45 sec** | |
| **Extension** | **72°C** | **45 sec** | |
| **Final extension** | **72°C** | **7 min** | **1** |
| **Hold** | **4°C** | **indefinite** | **1** |

**Extraction of CNF1 from bacterial isolates**

To determine the best method for extraction CNF1 was used to disrupt the cells(freeze-thawing( All the E coli isolates were inoculated into 100 ml flasks containing 10 ml of trypticase soy broth. Cultures were incubated at 370C for 24h with shaking (160rpm) and then centrifuged at 10000g for 20 min. The resulting pellets were resuspended in 1 ml of phosphate-buffered saline (PBS) pH 7.2 containing gentamicin (100µg/ml). Bacterial extracts were produced by freeze-thawing twice and were then centrifuged (10000g, 15min) to remove intact cells and debris. Supernatant fluids were then transferred into small Rhesus tube (s) of 1 ml, stored at 40C (Oswald et al., 1994).

**Purification of cytotoxic necrosis factor 1**

Used DEAE-Sephadex column (2.7 x9 cm) for Ion exchange chromatography and equilibrated with 25mM Tris-HCL at pH 7.4 and washed with this buffer after that the CNF1 was eluted by a step gradient 50,100, 250, 500mM NaCl in 25 mM Tris-HCL at pH 8 with stepwise NaCl from 0.1 – 0.5 M .The volume of collected fraction was 5ml in each tube at flow rate 40ml / hr. while Gel filtration chromatography used Sephacryl S-100 poured into column (1.6 x60cm) after packaging, the gel was equilibrated with equilibration buffer (500mM NaCl in 25 mM Tris-HCl at pH7.8). The protein was eluted with the same buffer, then 5mL fractions were collected in each tube with flow rate of 30 ml/hr.

**Cytotoxicity assay**

Used Cytotoxicity MTT stain and High-content screening (HCS) analysis on five cell lines( PC3 , HepG2, Hela cell ,AMN3, RD).(11,12)

## Results and Discussion:

**Isolation and identification of E. coli**

All urine specimens were cultured on MacConkey agar, EMB agar and blood agar plates. According to the growth characteristics, 76 (40.86%) samples were identified as E. coli (figure 1) .

All isolates were negative for oxidase, urease production, Voges Prauskaur and citrate utilization tests, while they gave positive results by catalase, motility test , indole production and methyl red and lactose fermentation tests.(10) Kligler iron agar developed an acidic slant, no change bottom, H2S negative with gas production. Also, when E. coli isolates were cultured on MacConkey agar and incubated at 37 °C for 24h, they appeared as small, pink and lactose fermenter colonies, while on blood agar they appeared as creamy and -hemolytic colonies. On EMB agar they appeared as green metallic shine colonies (13,14).also The identification of E. coli isolates was confirmed by Api-20E system and vitek-2 system .

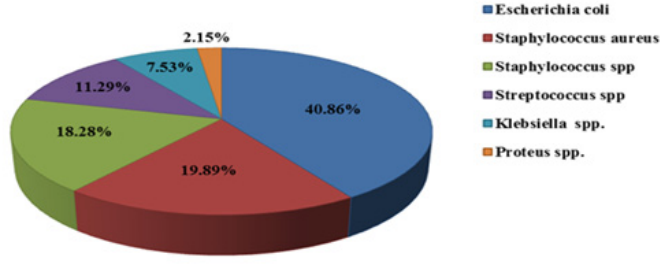


**Figure (1)** Percentage of Bacterial isolates from sample

**Detection of cnf1 gene by Polymerase Chain Reaction (PCR)**

Cytotoxic necrotizing facter-1 gene obtained from *E. coli* was used as PCR marker for detection of CNF-1 in *E. coli* which are isolated in the this factor was detected in whole DNA genome extract. cytotoxic necrosis factor 1 genes (cnf1) within 76 E. coli clinical isolates by PCR with specific primers. Each DNA extracted sample was subjected to PCR reaction with primer cnf1 at size (543) showed the presence of cnf1 genes in four E. coli clinical studied isolates This selected primer appeared to be highly specific for E. coli, and identification of E. coli at the species level was confirmed by PCR detection of cnf1 genes. showed in figure (2)

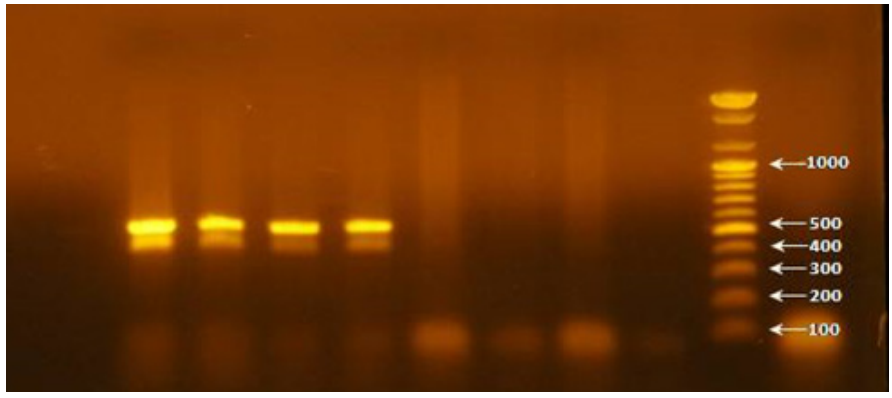


**figure 2:** Detection of cnf1 gene in whole DNA of bacterial isolates by PCR on agarose gel (1.5%) at 5 V/cm for 1 hour, stained with ethidium bromide and visualized on a UV transiluminator documentation system The size of amplified gene at 543pb

**Purification of CNF1**

**Ammonium sulfate precipitation**

The CNF1 was the concentrate by ammonium sulfate in the 60% saturation and gave good activity CNF1(15)

**Ion exchange chromatography**

The ion exchange chromatography technique by DEAE-Sephadex column was used as a second step to purify CNF1 of E. coli. 25mM Tris-HCl at pH 7.4 solution used as equilibration buffer, there was one protein peak appeared in the washing step, while two main protein peaks appeared in the elution by a gradient concentrations of sodium chloride . Protein peaks were assayed to detect CNF1activity.The results indicated in the figure (3) showed two peaks appeared in the eluted fractions, the first peak was the purified CNF1 from E. coli (No.23) (34 -37 fractions), have the CNF1 activity, the fractions were analyzed by SDS-PAGE, HEP2 multinucleation assays and western blotting.

The used DEAE-Cellulose in the purification of CNF1is very important because have High resolving power, High protein binding capacity, simple preparation and activation ( 16).

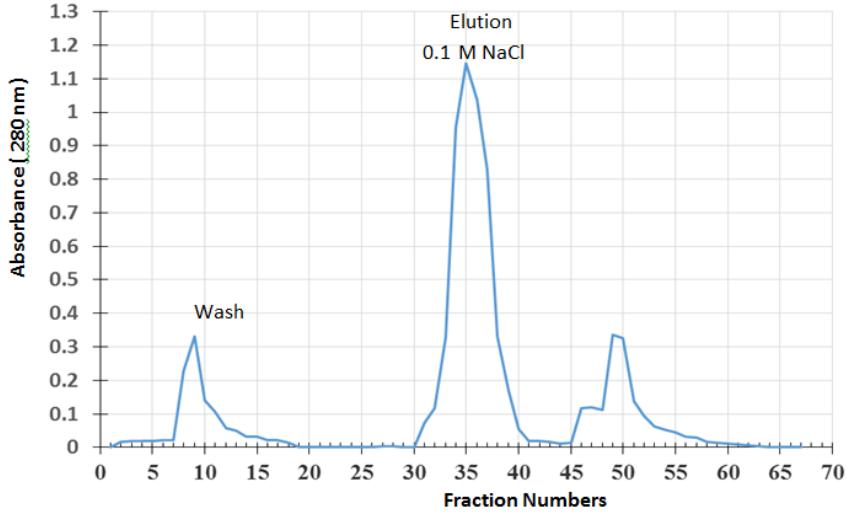


**Figure 3:** Ion-exchange chromatography for CNF1 from E. coli (No.23) using DEAE-Sephadex (2.7x 9cm) column equilibrated with 25mM Tris-HCl buffer at pH 7.4 The CNF1 eluted with gradient of 0.1-0.5M NaCl in the same buffer. With flow rate 30ml/hr. Collected fraction 5ml for each tube.

**Gel filtration chromatography**

After purification by ion exchange, fractions representing CNF1 activity were collected and concentrated for applying to Sephacryl S-100 previously equilibrated 500mM NaCl in 25 mM Tris-HCl at pH7.8, Sephacryl S-100 ,which allow larger ability of separation with high degree of purification. Results in figure (4) showed only one peaks appeared in the eluted fractions. The first peak purified CNF1 from E. coli (No.23) (9 -10 fractions), have the CNF1 activity, were analyzed by SDS-PAGE, HEP2 multinucleation assays and western blotting .

The used Sephacryl S-100 in the gel filtration chromatography is very important because have ability wide range of protein, non-effect charge and used to determination molecular weight of proteinsAll results in the ammonium sulfate precipitation, Ion exchange chromatography and

Gel filtration chromatography agreement with (17).

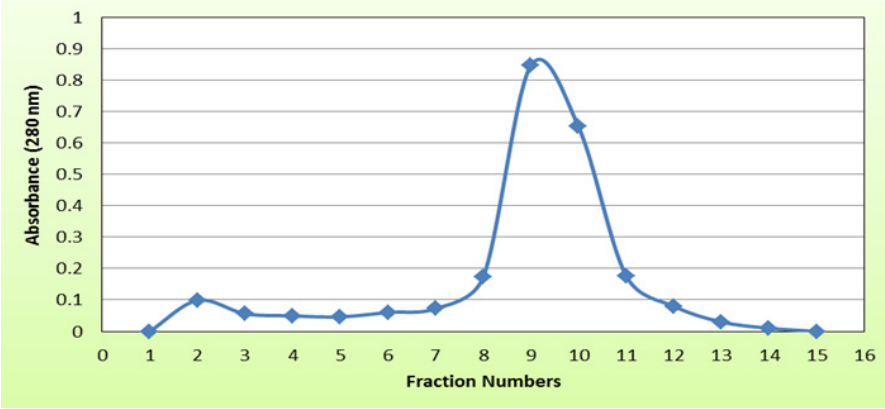


**Figure 4:** Gel filtration chromatography for CNF1 from E. coli (No.23) using Sephacryl S-100 (1.6 x60cm). Column washed with 500mM NaCl in 25 mM Tris-HCl at pH7.8, with flow rate 30ml/hr. Collected fraction 5ml for each tube.

**Effect of CNF1 on different cancer cell lines using MTT assay**

The 3- (dimethyl thiazol-2-yl)-2,5- biphenyl- tetra zolium bromide (MTT) assay is end point assay which detect cell viability at certain-time point. MTT assay was achieved to determine the (IC50) values of partial purified CNF1 on seven types of cell lines which were, AMN3, RD, Hela, liver hepatocellular cancer cells (Hep G2), and Prostate cancer cells (PC-3) for purified CNF1.( No.23).Data analysis carried out in g/L and log values of g/L is being plotted in graph pad prism using log (Inhibitor) versus normalized response Curve. Best values were chosen for the most significant IC 50 values. Cell viability at each time-point was determined by MTT colorimetric assays. the IC 50 value of partial purified CNF1-treated Rd cells after 24,48,72 hours respectivly of incubation at 37ºC was 333.3, 203.2, 119.3 µg /ml while results showed that the IC50 of partial purified CNF1-treated Hela cells after 24, 48,72 hours respectivly of incubation at 37º C was 568, 348.9, 70.94 µg /ml. showed that the IC50 of purified CNF1-treated Amn3 cells after 24,48,72 hours respectivly of incubation at 37ºC was 352.1,112, 74.5 µg /ml .The IC50 of partial purified CNF1(No.23)-treated prostate cancer (PC-3) cells was 170 µg /ml after 48 hr. As shown in figure (5), also the IC50 of partial purified CNF1-treated HePG2 cells after 48 hours was 104 µg /ml in shown same figure and from all these results we found that the HePG2 cells were more sensitive to word CNF1 than other cell lines used in this study. Cell viability was determined by MTT colorimetric assays after 24,48,72 hr of exposure. In general the effect of CNF1 was shown in high concentration in all cell lines.

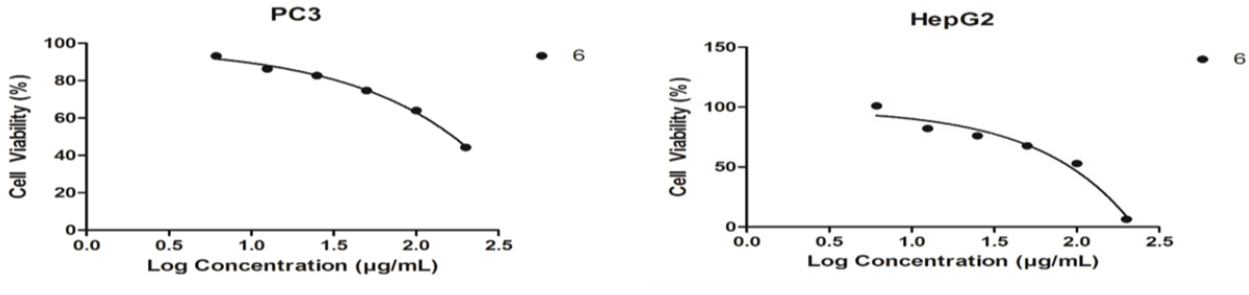


**Figure 5:** Cytotoxicity effect of partial purified CNF1 on PC3,HepG2 cells after4 8, hours of incubation at 37ºC.

**Cytotoxic effect of CNF1 on HePG2 cells**

High content screen analysis(HCS) of partial purified cnf1 on viability, membrane permeability, mitochondrial membrane permeability and Cytochrome C realizing using HePG2 cells.The results of cell viability reveal that there is a significant reduction in cell count HepG2 cells treated with high concentration of purified CNF1. The changes in cell viability are directly correlated to the toxic effect of the purified cnf1tested. We found that HePG2 cell loss was need high concentrations of purified CNF1 which affect the cellular survival of HepG2 cell . The results of nuclear intensity Figure (6) showed that HepG2 nucler intensity decrease significantly when treated with 6.25, 12.5, 25 and 100 µg/ml of purified CNF1 and comparative with positive control, also the Figure (7) showed that the intensity of HepG2 cell permeability decrease significantly when treated with same concentration of purified CNF1 comparative positive control, these results also revealed that the effect of purified CNF1 was dose dependent. It has been reported that changes in cell membrane permeability are often associated with a toxic or apoptotic responses, and the loss of cell membrane integrity is a common phenotypic feature of marked cytotoxicity (18).The results from figure (8) revealed that 6.25, 12.5, 25 and 100 µg/ml of purified CNF1 caused increase in mitocondrinl membrane potential intensity comparative control, and the effect of purified CNF1 was dose dependent. Purified CNF1 caused a significant decreasing in cytochrome C releasing intensity comparative positive control shown figure (9). The cytotoxic outcomes of HepG2 cell- CNF1 interaction were evaluated by high content screening analysis after 24 hour of exposure. The evaluation of the HCS images acquired from figure (10) showed that the Hoechst dye enables monitoring of cell loss, nuclear morphology changes and DNA content which are proportional to the total Hoechst intensity per nucleus. Multiparametric analysis of compound toxicity at the level of individual cells using cellular imaging-Basel approaches such as high- content screening (HCS) have played key roles in the detection of toxicity and classification of compounds based on observed patterns of reversible and irreversible cellular injury (19).

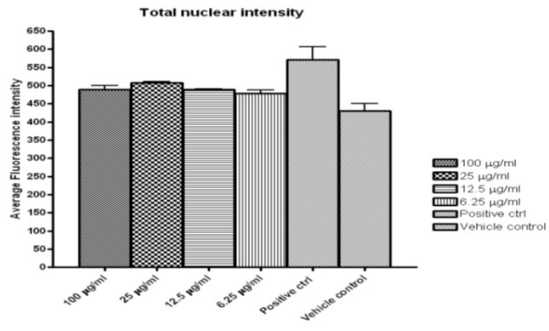


**Figure (6):** Effect CNF1 on total nuclear intensity via HCS

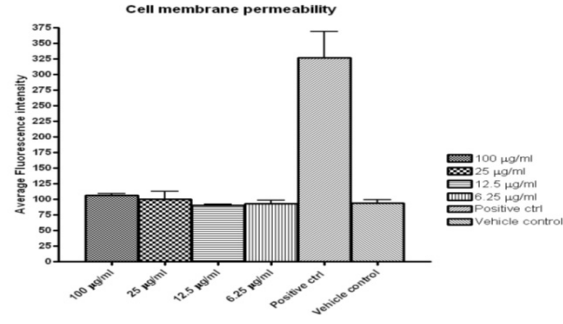


**Figure (7):** Effect CNF1 on cell memberane permeability via HCS

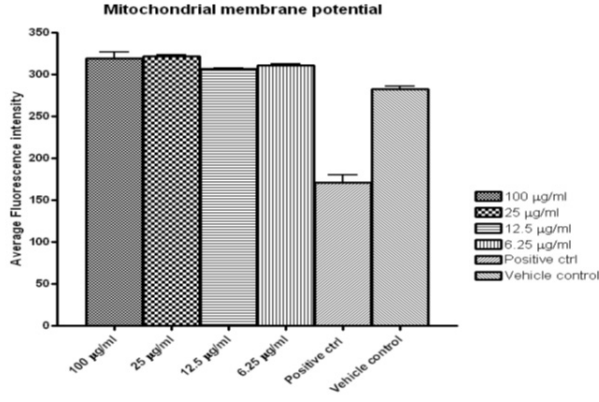


**Figure (8):** Effect CNF1 on cell mitochondrial membrane potential via HCS

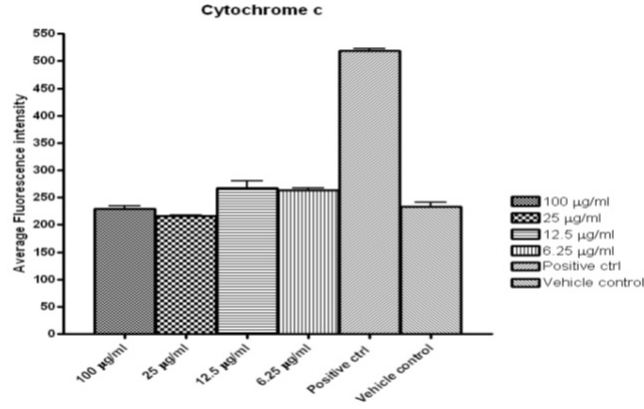


**Figure (9):** Effect CNF1 on Cytochrome C via HCS

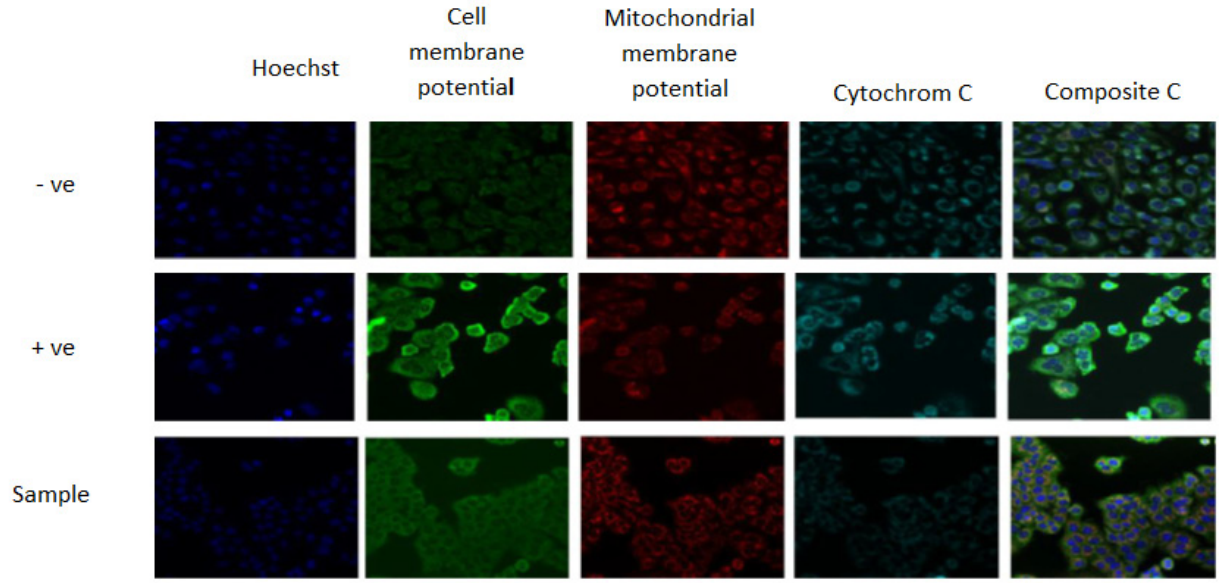


**Figure 10** Multiparameter cytotoxicity (HCS) analysis of purified CNF1 HepG2 cell line after 24 houre of incubation at 37ºC. Cells were stained with Hoechst dye which enables monitoring of cell loss, nuclear morphology changes and DNA content, which is proportional to the total Hoechst intensity per nucleus. also stained with permeability dye, which enables monitoring of membrane permeability, and cells were stained with MMP dye for mitochondrial membrane potential changes, and with goat anti mouse secondary antibody conjugated with DyLight TM for Cytochrome C releasing

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# عزل وتنقيه CNF1 (Cytotoxic necrosis factor 1) المنتج من بكتيريا الاشريشيه القولونيه ودراسة دورة المضاد لعمليه الموت المبرمج خارج الجسم الحي

مثنى حامد حسن[1]، مي طالب فليح[1]، ناهي يوسف ياسين[2]
1 قسم علوم الحياة/ كليه العلوم/ جامعه بغداد
2 المركز العراقي لبحوث السرطان والوراثة الطبية/ الجامعة المستنصرية

**الخلاصه:**

خلال الفترة الزمنية الممتدة نيسان الى ايلول عام 2012 جمعت 186 عينه ادرار من خمس مستشفيات . تم الحصول على 76 عزلة Uropathogenic E. coli وشخصت تلك العزلات طبقا للمواصفات الزرعيه على وسط اكار الدم ووسط الايوسين المثيلين الازرق وباختبارات كيموحيويه وباستخدام جهاز الفايتك 2 وبنظام API 20 E. النتائج اظهرت بان اربعه عزلات من الاشريشيه القولونية قادرة على انتاج عامل CNF1((E4, E8, E23, E36 كانت (E23)اكثر كفاء من الاخرى على الزرع النسيجي ثم تم تنقيتها بطريقتين الاولى بطريقه كروموتوكرافيه المبادل الايوني باستخدام DEAE- Sephadex والثانية كرموتوكرافيه الترشيح الهلامي بواسطه Sephacryl S-100 . درس جين cnf1 بواسطه تقنيه PCR باستخدام بادئ الجين عند الحجم 543.
اختبرت تراكيز مختلفة من CNF1 في التاثيرعلى خلايا سرطانيه((RD, AMN3, , Hela, PC3 and HePG2 بعد مرور 72 ساعه وكانت التأثيرات تعدد الانويه وكبر حجم الخلايا وان اقل تركيز مثبط نصف الخلايا هو104µg/ml ضد HePG2 بعد مرور 72 ساعه ودرست فعالية الموت المبرمج لل CNF1 على الخلايا السرطانية بقياس التأثير على نسبه المايتوكوندريا كذلك درست مقاييس اخرى multiparameter cytotoxic assay وكانت النتائج قله تركيز السايتوكروم سي والمحتوى النووي الكلي والنفاذية الأغشية مقارنه مع عينه السيطرة الموجبة في حين كانت تراكيز عالية للمايتوكوندريا مقارنه بالسيطرة الموجبة وبتراكيز(12.5,6.25 ,100,25) .