RESEARCH PAPER

Phenotypic Detection of Crude Cytotoxic Necrotizing Factor Type 1 (CNF1) of Hemolytic and Non-Hemolytic Uropathogenic E. coli Isolates Using Different Tissue Culture Cell lines

Estabraq Saad Rehan[1]*, Nada. H. A. Al-Mudallal[2]

[1,2] *Department of Microbiology, College of Medicine. Al-Iraqia University, Baghdad, Iraq*

ABSTRACT

Background: Cytotoxic necrotizing factor type 1 (CNF1), a cytoplasmic protein presents in certain diarrheagenic and uropathogenic strains of *Escherichia coli* (UPEC), is a member of a family of bacterial toxins that target the Rho family of small GTP-binding proteins in mammalian cells contributing to inflammation and tissue damage, particularly in ascending urinary tract infections.

Aim: Comparatively evaluate the Phenotypic expression of crude cytotoxic necrotizing factor type 1 (CNF1) produced by hemolytic and non-hemolytic uropathogenic *E.coli* isolates using diverse tissue culture cell lines.

Methodology: Eight well identified uropathogenic *E.coli* which isolated from prostate and bladder cancers Iraqi patients and all harboring the *cnf1* gene. These isolates were tested for their hemolytic ability by culturing on blood agar media. Cytotoxic necrotizing factor 1 preparations were made for chosen hemolytic and non-hemolytic isolates by cell lysis through harvesting the bacterial cells and sonicate the suspension at 10 µm peak amplitude for 5 min in an ice bath. The obtained cell lysate centrifuged at 6000 × g, 4 °C for 15 min. then the supernatant was collected and sterilized by Passing through a 0.22 µm filter to remove any remaining bacterial cells. The toxin concentration was determined using Bradford method. The cytotoxic effects were determined using MTT assay on three tumor cell lines including HeLa cells (human cervical carcinoma), Du145 cells (prostate cancer), and a normal cell line, HDF (human dermal fibroblast) at various dilution ratios, and cell viability was measured spectrophotometrically (OD Absorbance was quantified at 570 nm utilizing an ELISA reader values) after 24 hours of exposure. cells were stained with Giemsa solution after fixation. The stained cells were examined under a light microscope (40× objectives), and representative images were captured for analysis.

Results: Three UPEC isolates were hemolytic and five were non-hemolytic from total eight. Only two isolates were used to determine phenotypic expression of CNF1 (one hemolytic and the other non-hemolytic). The protein concentrations of crude CNF1 from both isolates after extraction were 0.4 and 0.25 µg/ml respectively. Results of cytotoxic activity of CNF1 on the three tumor cell lines, on HDF cells, hemolytic *E. coli* caused a marked decline in viability, showing the lowest O.D value at 1:2 dilution (0.18), indicating strong cytotoxicity while non-hemolytic isolate showed less effect, with the highest OD at 1:8 (0.5). Similarly, HeLa cells exposed to hemolytic *E. coli* exhibited a decrease in OD (~0.28–0.3 at 1:4 and 1:8), while non-hemolytic isolates showed minimal cytotoxicity (OD ~0.48 at 1:8). In DU-145 cells, hemolytic *E.coli* isolates demonstrated the greatest cytotoxic effect at 1:2 dilution with OD (0.34), whereas non-hemolytic *E.coli* isolates showed higher OD (0.49 at 1:8), indicating lower toxicity.

Conclusion: The data indicate a significant effect of crude cell lysate of both hemolytic and non-hemolytic UPEC on HDF cell lines , OD values generally increase with hemolytic UPEC, reaching the lowest values at 1:2(0.18), reflecting higher cytotoxicity, In contrast, non-hemolytic UPEC exhibited a high increase in OD at higher dilutions, with the highest at 1:8 (0.5) suggesting lower cytotoxic effects.The morphological changes of hemolytic UPEC ranging between sever damage cosist with apoptotic /necrotic featutre in dilution ration 1:1 to very mild or non near to control in dilution ration 1:8. The effect of non-hemolytic UPEC morphological changes ranging between stress with prominent apoptotic/ necrotic feature in dilution rate 1:8**.**

Keywords:N\A.

How to cite this article: Rehan ES, Al-Mudallal NHA, Phenotypic Detection of Crude Cytotoxic Necrotizing Factor Type 1 (CNF1) of Hemolytic and Non-Hemolytic Uropathogenic E. coli Isolates Using Different Tissue Culture Cell lines..Int J Drug Deliv Technol. 2026;16 (1s): 397-411; DOI: 10.25258/ijddt.16. 397-411

Source of support:. None

Conflict of interest: None

INTRODUCTION

Uropathogenic *Escherichia coli* (UPEC)-induced urinary tract infections (UTIs) usually evoke cystitis, pyelonephritis, and prostatitis (1,2). In addition, UPEC was also reported to accelerate the prostate cancer progression in the genetically engineered Hi-Myc mouse prostate cancer model, and increase the risk of bladder cancer through promoting CDKN2A methylation (3,4). These pathogenic strains carrying several virulence factors that allow them to colonize, invade and cause damage to the host (5,6). Group of cyclomodulins is genotoxins and/or cell cycle modulating toxins that contribute to tumorgenesis through modulate cellular differentiation, apoptosis and proliferation. These cytotoxins, include Cytolethal distending toxin CTD, cytotoxic necrotizing factors CNFs, Colibactin and Cycle inhibiting factor (CIF) (7). The main

**Author for Correspondence: estabraq.s.rehan@aliraqia.edu.iq*

function of cyclomodulins is still not completely clear and understood. Reports and studies revealed that cellular modulating effects suggest that they are attributed to the bacterial colonization, persistence and the development of chronic infection (8).

The strains of *E. coli* that produce CNFs are belong to the pathotype necrotoxigenic *E. coli* (NTEC), which are associated with the infections of intestinal and extraintestinal in humans and animals. Most of CNFs include chromosomally encoded gene *cnf1*, and plasmid-encoded gene *cnf2* (3,9). The size of CNF1 is a 115 KDa protein toxin which activates the deamination of Rho GTPase proteins, leading to cytoskeletal alterations and some effects in the cell cycle with subsequent macropinocytosis, inducing specific effect (CPE) known as megalocytosis, induce multinucleation and enlargement. According to (10) CNF1 is an AB-type toxin produced by pathogenic strains of *E. coli* that are responsible for urinary tract infections and newborn meningitis. (11). A notable correlation was identified between CNF1, haemolytic activity, and the products of the pap/sfa genes (12). CNF1 permanently activates small Rho-family GTPases by the deamidation of glutamine 63 in RhoA and glutamine 61 in Rac1 and Cdc42.The constitutive activation of these small GTPases by CNF1 induces *in vitro* alterations such as actin cytoskeletal rearrangement, multinucleation, cell spreading, lamellipodia and filopodia production, and death of urothelial cells. Reduced phagocytic capacity of polymorphonuclear leukocytes (PMNs) and activation of nuclear factor-κB (NF-κB) (13). UPEC strains have the ability to produce hemolysin, this toxin plays an important role in the forming of pore in the cell membrane. Hemolysin is produced as either a free toxin or an outer membrane vesicle (14). Studies showed that (50% (from bacteria that cause UPEC have the ability to produce the enzyme hemolysin (15). One of the most important ways to detect the ability of bacteria to produce this enzyme is to hydrolyze blood agar medium, and this ability is related to the pathogenesis of E. coli strains, especially in acute infection (16). Hemolysin is a significant virulence factor for pyelonephritis and urosepsis isolates In vitro, the E. coli hemolysin is clearly cytotoxic to primary cultured renal cells (17–19) showed that the hemolysin contributed to renal parenchymal damage in a murine model of urinary-tract infection (UTI), but it did not influence colonization levels in the kidney (19). Previous studies demonstrated that the cytopathic effect is a distinctive phenotypic feature for the CNFs and had been differentiated from the β-hemolytic activity of uropathogenic E. coli (20).

In Iraq, there are many bladders and prostate cancers cases were reported. Some initial reports suggested that these cases are due to war radiation and weapons (21,22) without investigation of the role of bacterial virulence factors like CNF1 and hemolysin in the cytotoxicity and genotoxicity on human cells through simulating some physiological processes such as differentiation, apoptosis, proliferation, megalocytosis, and cells enlargement. The aim of this study Comparative evaluation of the Phenotypic expression of crude cytotoxic necrotizing factor type 1 (CNF1) produced by hemolytic and non-hemolytic uropathogenic *E.coli* isolates using diverse tissue culture cell lines.

MATERIAL AND METHODS

Bacterial isolates

Eight clinical isolates of *E.coli* previously confirmed harboring *cnf1 gene* by moleculer methode (PCR) technique were icluded in this study.The isolates were taken from prostate and bladder cancer Iraqi patients (23).

Determination of the hemolytic activity of *E. coli* isolates

The *E. coli* isolates were assessed for haemolytic activity on blood agar enriched with 5% sheep blood. The blood agar plates were made by aseptically incorporating 5% sheep blood into freshly sterilised and cooled nutrient agar media at a temperature of 45–50 °C. The E. coli test isolates were then streaked onto blood agar plates and incubated overnight at 37 °C (24). After incubation, the haemolytic activity of the streaked isolates was examined, and those producing a clear zone of haemolysis (beta hemolysis) were classified as haemolytic (Hly+).

Bacterial growth and preparation of the crude cell lysate containing cytotoxic necrotizing factor-1 (CNF1)

Inoculate *E. coli* isolates into tryptone soya broth (TSB). Incubate at 37 °C for 20 h under shaking (200 rpm) to allow sufficient growth. Dilute overnight cultures 100-fold in RPMI medium supplemented with 10% FBS. Incubate until the culture reaches an $OD_{600} \approx 0.08–0.1$ (~2 h). Secondary Culture by transfer the prepared inoculum into 20 ml aliquots of TSB contained in sterile 50 ml conical flasks.

Incubate again at 37 °C, 200 rpm for 20 h, the cell lysis through harvest the bacterial cells and sonicate the suspension at 10 μm peak amplitude for 5 min in an ice bath. The obtained cell lysate then Centrifuged at 6000 × g, 4 °C for 15 mi. Collect the supernatant and sterilize it by Pass the supernatant through a 0.22 μm filter to remove any remaining bacterial cells. Finally the filtrate is the crude CNF-1 lysate (25).

Determining of Protein Concentration

The Bradford technique was used to determine the protein concentration(Bradford, 1976), A stock solution of bovine serum albumin was prepared by dissolving 0.1g in 100ml of D.W.

Certain concentrations of Bovine serum albumin were prepared (10 mg/ml,20 mg/ml, 100 mg/ml). Serial dilutions of bovine serum albumin ranging from 10-100 mg/mL were prepared by diluting the stock solution with distilled water, Bradford reagent (4.9 ml) was added to each tube, and the tubes were left to incubate in the dark for 10 minutes. The absorbance was then measured at 595 nm using a spectrophotometer, and the OD of each concentration. A volume of 4.9 ml of Bradford reagent was added to plastic test tubes and 0.1 ml of the Protein solution was added to the reagent and incubated at 30C for 10 minutes, the optical density was measured at 595nm the protein concentration was estimated depending on the standard curve of Bovine Serum Albumin. The protein concentration was calculated according to this equation:

Protein concentration (mg/ml) = O.D (595nm)/ (slope* 1000)

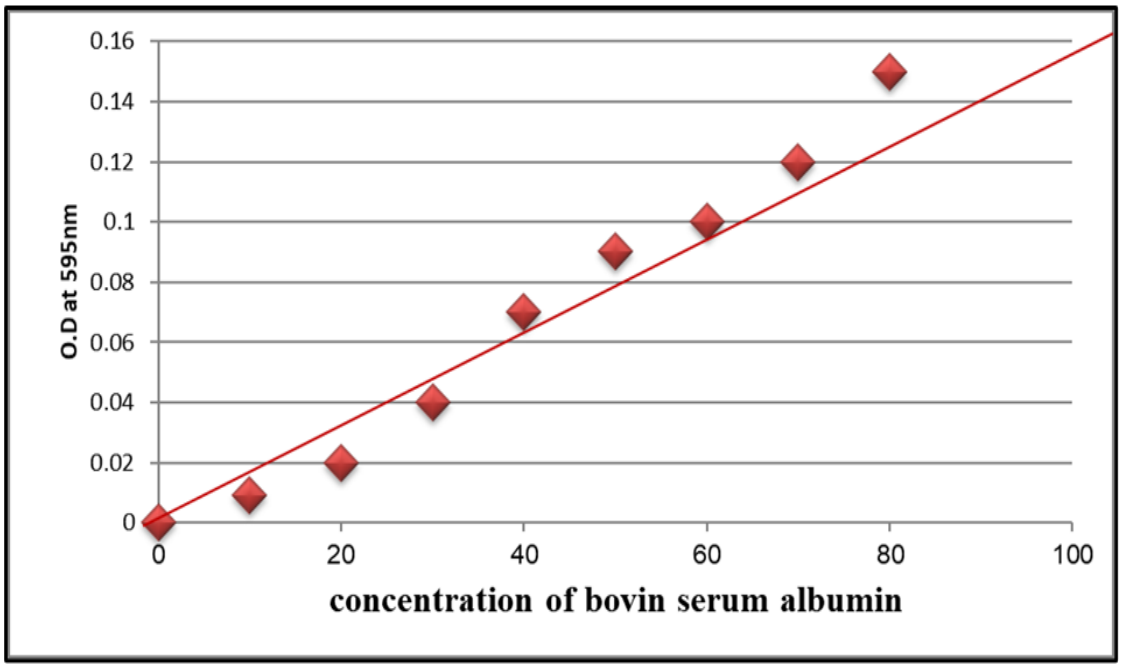

Figure1: BSA standerd curve performed via Bradford approch

Cell lines

This study employed tumor cell lines including HeLa cells (human cervical carcinoma), Du145 cells (prostate cancer), and a normal cell line, HDF (human dermal fibroblast) were obtained from the Iranian Biological Resource Center. All lines were pre-authenticated by the center using standard assays, including STR profiling and mycoplasma testing (https://ibrc.ir/fa). The samples were acquired from the Chemistry Analysis Centre (CAC) in Baghdad, Iraq, and were initially stored in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) (Capricorn, Germany), 100 IU/mL of streptomycin, and 100 µg/mL of penicillin (Ajenta Pharm, India). The cells were passaged with Trypsin-EDTA, reseeded at 50% confluence, and exhibited a doubling in number within one week, subsequently incubated at 37 °C. (Alqaisi Mouruj A Al aubydi Assis Lecturer, 2023

Sample Preparation

Cell Seeding/ Treatment and MTT Assay

Cells were seeded at a density of 5-6 $\times 10^3$ cells per well in 96-well plates. Plates were incubated at 37°C with 5% CO_2 for 24 hours to promote cell adhesion. After incubation, remove the medium and replace it with 100 µL of cell lysate-medium at the stated doses. Plates were incubated for another 24 hours under identical conditions. Following 24 hours of treatment, the culture media was withdrawn(*Mtt-Assay-v1-10xzzkvo*, n.d.).

Cytotoxicity Assay

The MTT cell viability experiment utilizing MTT reagent (Merck, Germany) was conducted on 96-well plates to assess cytotoxicity. Cell lines were inoculated at a density of 5-6 $\times 10^3$ cells per well in 96-well plates. Plates were incubated at 37°C with 5% CO_2 for 24 hours to facilitate cell adhesion. Subsequent to incubation, the medium was discarded and substituted with 100 µL of cell lysate-medium at the specified concentration (0.4 µg/ml and 0.24 µg/ml) for both hemolytic and non-hemolytic respectively. Plates were incubated for a further 24 hours under identical conditions. Following 24 hours of treatment, the culture medium was discarded. Each well was administered 20 µL of MTT solution (0.5 mg/mL in PBS). Plates were incubated for 3 to 4 hours at 37°C. The MTT solution was thereafter eliminated, and 100 µL of DMSO (Dimethyl Sulphoxide, Merck, Germany) was introduced to each well to solubilise the formazan crystals. Absorbance was quantified at 570 nm utilising an ELISA reader (BioRad, USA). The cytotoxicity percentage, and cell viability representing the rate of suppression of cell growth, was determined as (26).

% Cell viability= (Absorbance of treated cell/Absorbance of non-treated cell) *100

% Cytotoxicity= 100-cell viability

Gimmsa Staining

Cells were seeded at a density of 4-6 $\times 10^4$ cells/well in 24-well plates. The Plates were incubated at 37 °C with 5% CO_2 for 24 hours to allow for cell attachment. After incubation, the medium was removed and replaced with 1ml of cell lysate-medium at the indicated concentrations. Plates were incubated for an additional 24 hours under the same conditions. The culture medium was removed, and the cells were gently washed once with PBS to remove residual medium. The cells were then fixed with 500 µl cold methanol (100%) for 10 minutes at room temperature,

*Author for Correspondence: estabraq.s.rehan@aliraqia.edu.iq

followed by air-drying. Fixed cells were stained with 500 µl Giemsa solution and incubated for 15 minutes at room temperature, protected from light. The wells were then rinsed thoroughly with distilled water to remove excess stain and allowed to air-dry completely. The stained cells were examined under a light microscope (40× objectives), and representative images were captured for analysis (27).

Statistical analysis: Data are expressed as mean, SD, and other descriptive statistics. Fisher's exact and percentage were used in the current study to calculate the p-value for some parameters. Values of $p>0.05$ were considered statistically non-significant, while $p\leq0.05$ were considered significantly different. The statistical analysis was carried out by SPSS Inc., Chicago (28).

Data analysis: Each experiment was performed in triplicate, and data were expressed as mean ± standard deviation (SD). The degree of cytotoxicity was compared among isolates and cell lines to determine the variation in CNF1 phenotypic expression.

Ethical approval: This study was approved by ethical approval committee of college of medicine Al-Iraqia Unevirsity No.FM.SA.157 in 28/4/2025

RESULTS

Protein Concentration

The protein concentrations of crude CNF1 from both isolates (hemolytic and non-hemolytic) after extraction were 0.4 and 0.25 µg/ml respectively

Hemlytic Activities Testing of E. Coli Isolates

A total of 8 isolate only 3 isolate (37.5%) showed clear zone of hemolysis on blood agar containing 5% sheep blood (β hemolytic phenotype) These isolates were considered hemolytic (Hly+) figure 2. The other 5 (62.5%)isolate show non-hemolytic activity wer consider hemolytic (Hly-) figure3.

Figure 2: hemolytic E.coli
Figure 3: non-hemolysis E.coli

Cytotoxic effect of Hemolytic and Non-Hemolytic *E. coli* on HeLa Cells

Cytotoxicity Analysis: The untreated control (UT) showed the highest optical density (OD ~0.5) for both hemolytic and non-hemolytic E. coli, indicating no cytotoxic effect. For diluted samples (1:1 to 1:8), OD values generally decreased with hemolytic E. coli, reaching the lowest values at 1:4 and 1:8 dilutions (~0.28–0.3), reflecting higher cytotoxicity shown in Table 1 In contrast, non-hemolytic E. coli exhibited a slight increase in OD at higher dilutions, with the highest at 1:8 (~0.48), suggesting lower cytotoxic effects. Overall, hemolytic E. coli demonstrated greater cytotoxicity toward HeLa cells at intermediate dilutions show in (Table 2), whereas non-hemolytic E. coli maintained higher cell viability across all dilutions as shown in (Table 3)

Cytotoxic effect of Hemolytic and Non-Hemolytic *E. coli* on HDF cell

Cytotoxicity Analysis: The untreated control (UT) showed the highest optical density (OD ~0.5) for both hemolytic and non-hemolytic E. coli, indicating no cytotoxic effect. For diluted samples (1:1 to 1:8), OD values generally increase with hemolytic *E. coli*, reaching the lowest values at 1:2(0.18), reflecting higher cytotoxicity shown in Table 1. In contrast, non-hemolytic E. coli exhibited a high increase in OD at higher dilutions, with the highest at 1:8 (0.5) suggesting lower cytotoxic effects. Overall, hemolytic

E. coli demonstrated greater cytotoxicity toward HDF cells at intermediate dilutionsm show in (Table 2), whereas non-hemolytic E. coli maintained higher cell viability across all dilutions show in (Table 3)

Table 1: cytotoxicity and optical density(OD) of hemolytic UPEC on different cell lines

Tested cell lines	Tested concentration at 24 hr. Mean ±SD					P valu1	P valu2
	C	1.1	1.2	1.4	1.8		
HEM/ HDF	0.538±0.01	0.204±0.01	0.189±0.01	0.203±0.01	0.217±0.01	0.001	0.72
HEM /HELA	0.523±0.04	0.274±0.01	0.273±0.02	0.266±0.05	0.291±0.04	0.001	0.9
HEM/ DU145	0.500±0.02	0.341±0.04	0.424±0.01	0.481±0.02	0.491±0.04	0.001	0.81
P value 1 tested groups vs control/ p value 2= between tested groups only /P < 0.05 is considered statistically significant							

C ytotoxic effect of Hemolytic and Non-Hemolytic *E. coli* on DU145 cell

Cytotoxicity Analysis: the untreated control (UT) showed highest optical density (OD ~0.5) for both hemolytic and non-hemolytic E. coli, indicating no cytotoxic effect. For diluted samples (1:1 to 1:8), OD values generally increase with hemolytic E. coli, reaching the lowest values at 1:2 (0.34), reflecting higher cytotoxicity show in Table 1. On other hand non-hemolytic E. coli exhibited a high increase in OD at higher dilutions, with the highest at 1:8 (0.49), suggesting lower cytotoxic effects show in (Table 2). Overall, hemolytic E. coli demonstrated greater cytotoxicity toward DU145 cells at highest dilutions, whereas non-hemolytic E. coli maintained higher cell viability across all dilutions show in (Table 3).

Table 2: cytotoxicity and optical density (OD) of non-hemolytic UPEC on different cell lines

Viability Tested cell lines	Tested dilution					X^2P value (Tested dilution)	
	C	1.1	1.2	1.4	1.8		
HEM/ HDF	100	38	35	38	40	$X^{2=}$ 0.009	0.9
HEM /HELA	100	52	52	51	56	$X^{2=}$ 0.11	0.7
HEM/ DU145	100	68	85	96	98	$X^{2=}$ 0.8	0.35
Non-HEM/ HDF	100	60	76	83	95	$X^{2=}$ 5.7	0.01
Non- HEM/ HELA	100	48	64	78	91	$X^{2=}$ 0.2	0.58
Non-HEM/ DU145	100	80	88	89	99	$X^{2=}$ 0.002	0.98
X^2P value (Tested dilution)	Non-c	$X^{2=}$ 3.4 0.17	$X^{2=}$ 7.1 0.02	$X^{2=}$ 13.9 0.001	$X^{2=}$ 13.9 0.0009		
P < 0.05 is considered statistically significant							

Tested cell lines	Tested concentration at 24 hr. Mean ±SD	P valu1	P valu2

	C	1.1	1.2	1.4	1.8		
Non-HEM/ HDF	**0.538±0.01**	**0.323±0.01**	**0.409±0.02**	**0.444±0.01**	**0.510±0.01**	**0.001**	**0.01**
Non- HEM/ HELA	0.523±0.04	0.249±0.03	0.335±0.04	0.409±0.04	0.477±0.02	**0.001**	0.17
Non-HEM/ DU145	0.500±0.01	0.401±0.01	0.440±0.03	0.443±0.02	0.494±0.02	**0.001**	0.93
P value 1 tested groups vs control/ p value 2= between tested groups only /P < 0.05 is considered statistically significant							

Table 3: cytotoxic effect of hemolytic and non-hemolytic UPEC on viability of different cell lines

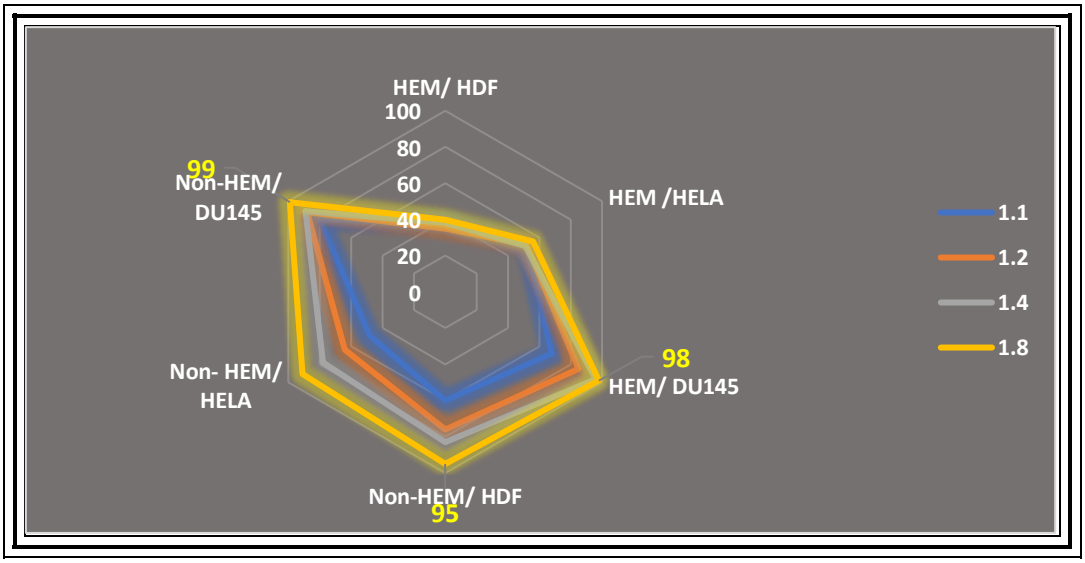

Figure 4: Radar plot of viability tested call lines performance at various concentration of crude cell lysate

Phenotypic detection of Hemolytic and Non-Hemolytic E. coli on different cell lines

HDF cell line: the effect of hemolytic UPEC changes ranging between sever damage cosist with apoptotic /necrotic featutre in dilution ration 1:1 to very mild or non-morphological change near to control in dilution ration 1:8 (figure 5). The effect of non-hemolytic UPEC morphological changes ranging between stress with prominent apoptotic/ necrotic feature in dilution rate 1:8 (figure 6)

HeLa cell line: the effect of hemolytic UPEC in dilution 1:1 exhibite significant stress and clear signe of cell injury, while in dilution ratio 1:8 the stress is increased and more appearent signes of cell death. The effect on non-hemolytic UPEC on 1:1 dilution ratio very mild stress but overall morphology similar to control (figure 7) but in 1:8 dilution ratio appear minimal cytotoxicity effect, stress response mild and less sever than 1:4 (figure 8)

3-DU145 cell line: the effect of hemolytic UPEC in dilution 1:1 exhibite clear signe of stress and cytotoxicity of high lysate concentration but in dilution ratio 1:8 we notice stress but not sever as 1:1, appear intermedite (figure 9)

The effect on non -hemolytic UPEC on DU-145 in 1:1 dilution ratio show extensive cell death (apoptotic and necrotic feature) major reduction in cell density, while in dilution ratio 1:8 show mild cytotoxicity effect mostly early morphological changes comparable to control (figure 10).

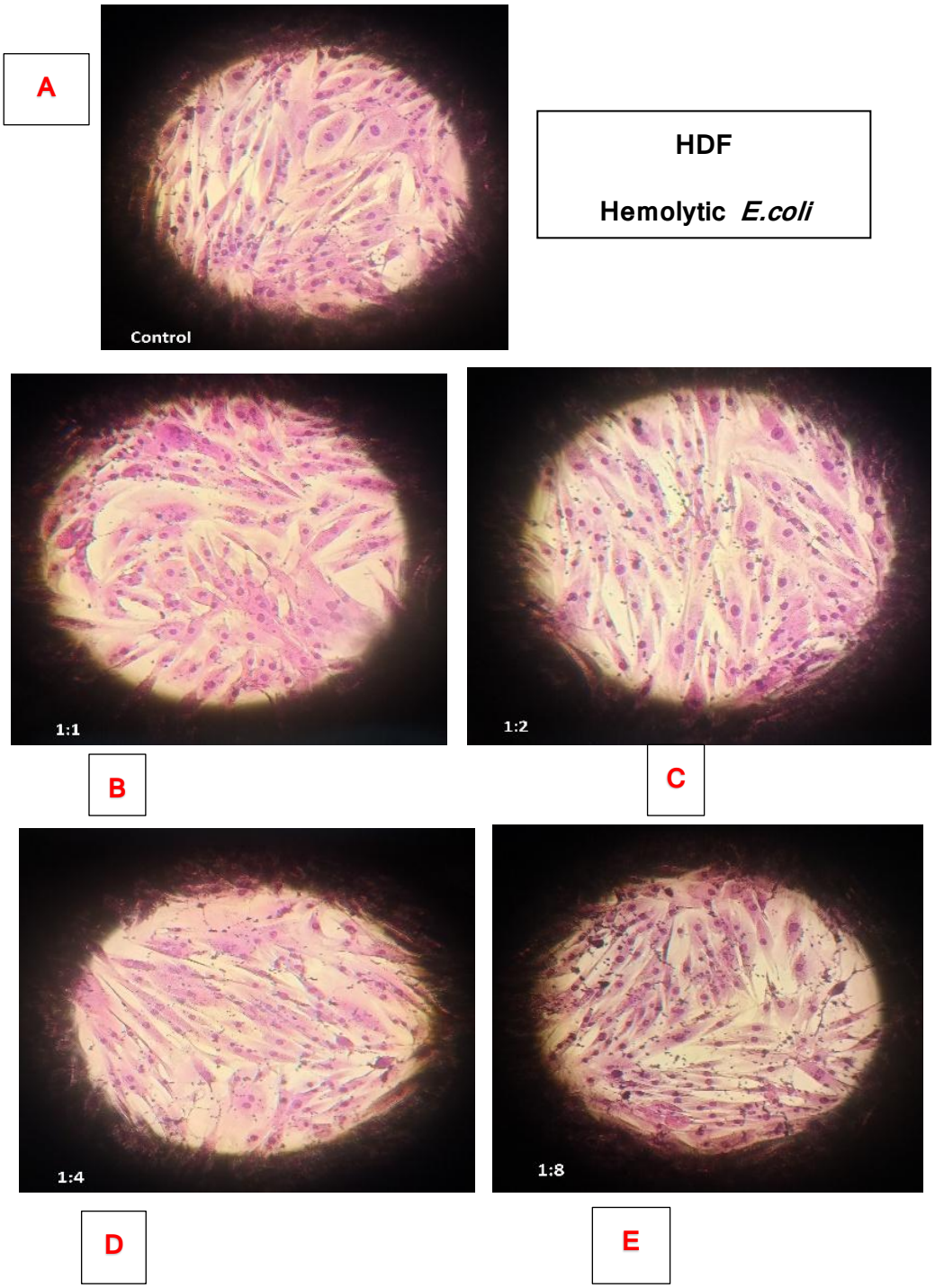

Figure 5: Morphological changes in HDF cells exhibited by crude cell lysate of hemolytic UPEC isolates. A: untreated cells, B-E: treated HDF cells by different dilution ratio from 1:1 to 1:8 after 24h exposure exhibit Condensed and less extended of cytoplasm to intact minimal sign of condense Nucleus: Condensed (pyknotic), to Normal appearance, clear and distinct. Damage: Severe – consistent with apoptosis/necrosis and strong cytotoxic effect to Very mild to none – morphology nearly identical to control.

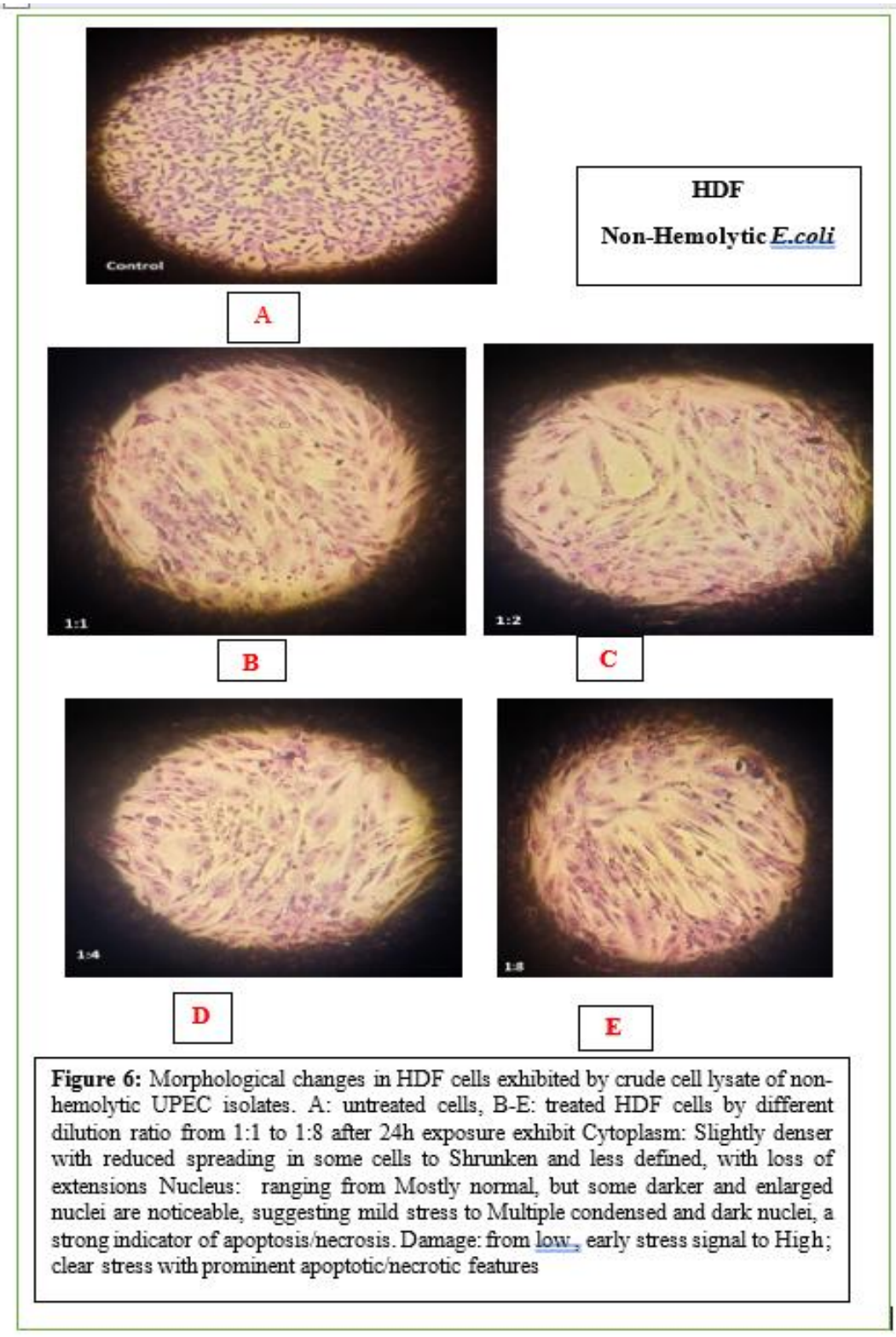

Figure 6: Morphological changes in HDF cells exhibited by crude cell lysate of non-hemolytic UPEC isolates. A: untreated cells, B-E: treated HDF cells by different dilution ratio from 1:1 to 1:8 after 24h exposure exhibit Cytoplasm: Slightly denser with reduced spreading in some cells to Shrunken and less defined, with loss of extensions Nucleus: ranging from Mostly normal, but some darker and enlarged nuclei are noticeable, suggesting mild stress to Multiple condensed and dark nuclei, a strong indicator of apoptosis/necrosis. Damage: from low., early stress signal to High; clear stress with prominent apoptotic/necrotic features

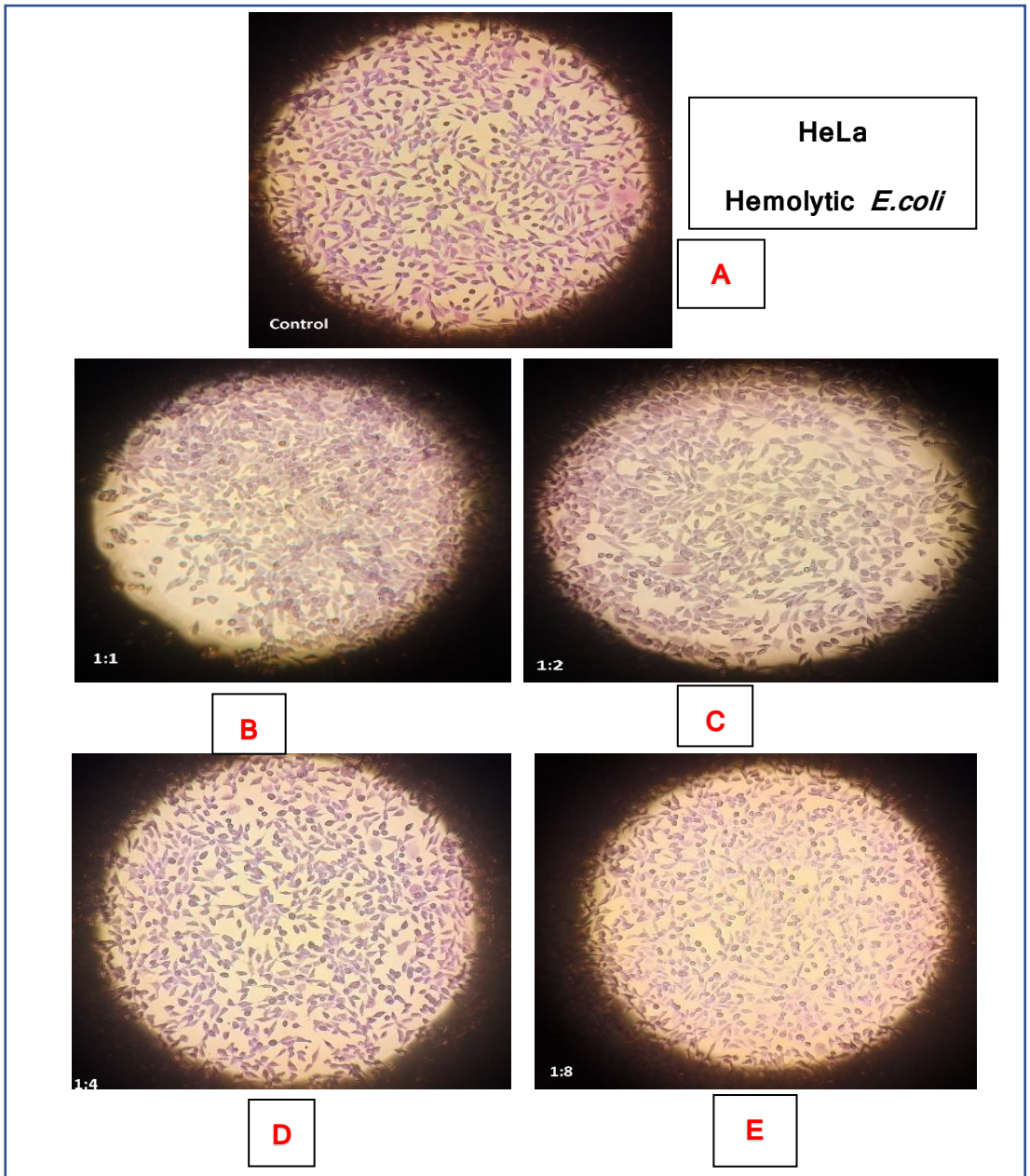

Figure7: Morphological changes in HeLa cells exhibited by crude cell lysate of hemolytic UPEC isolates. A: untreated cells, B-E: treated HeLa cells after 24h exposure to different dilution ratio exhibit Condensed and less extended of cytoplasm, early apoptosis, Significant stress and clear signs of cell injury. to Shrunken and contracted in multiple cells. Of cytoplasm, there is an indicating apoptosis/necrosis the damage Increased stress compared to 1:4; more apparent signs of cell death

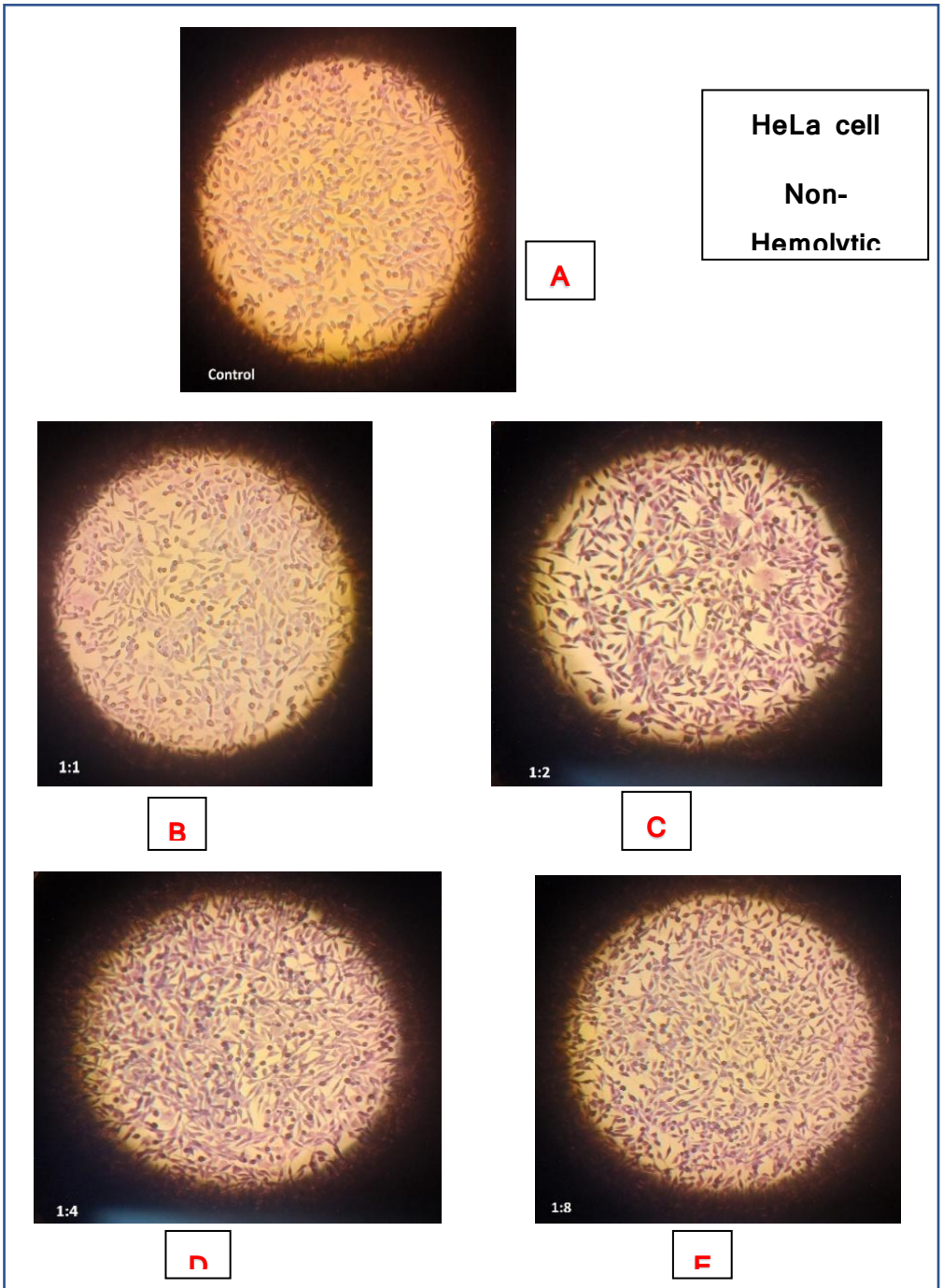

Figure 8: Morphological changes in HeLa cells exhibited by crude cell lysate of non-hemolytic UPEC isolates.A: untreated cells ,B-E: treated HeLa cells after 24h exposure to different dilution ratio exhibit different degree of cytoplasmic shrinkage minimal shrinkage and very mild stress to mild shrinkage in some cells with minimal cytotoxic effect , mild stress response less sever than 1:4

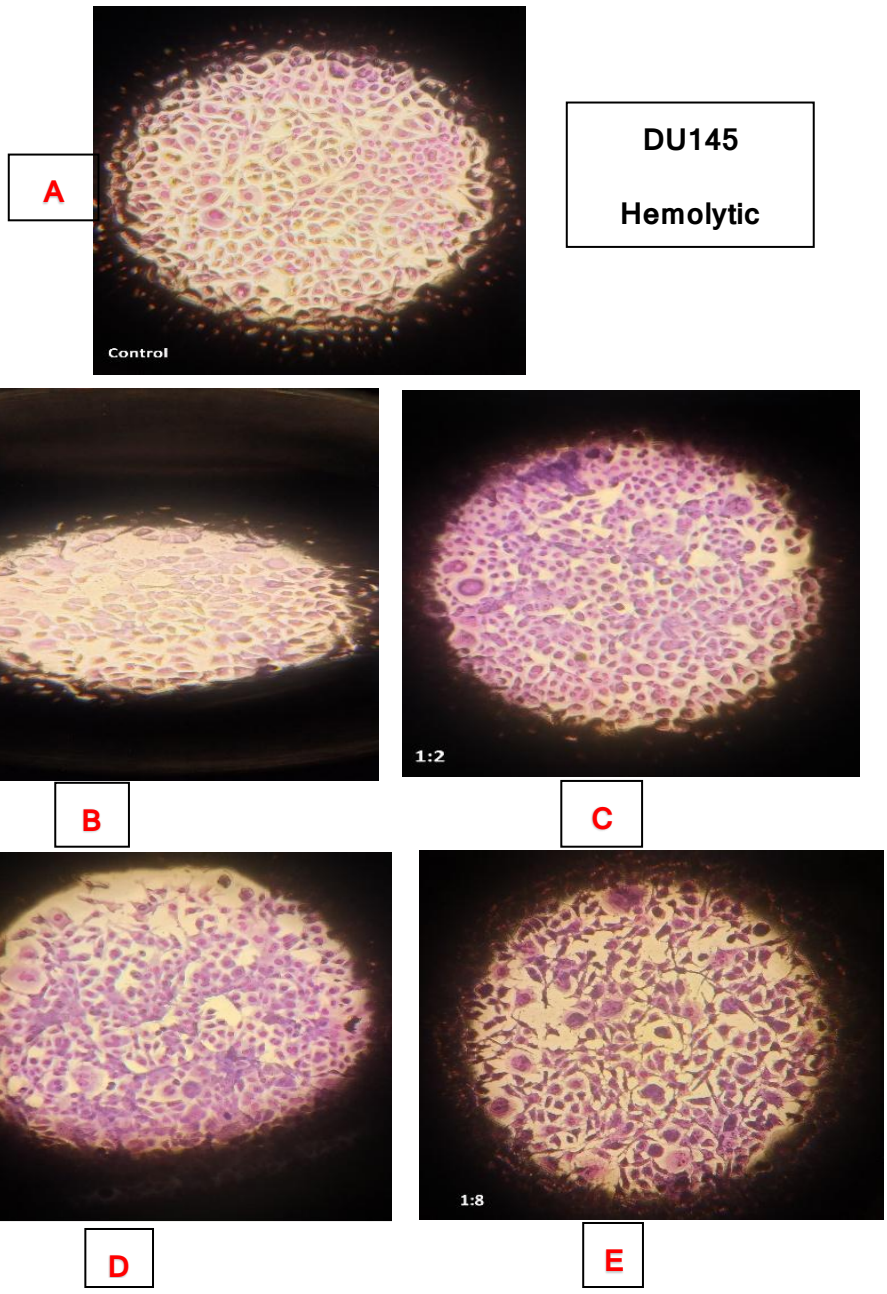

Figure 9: Morphological changes in DU145 cells exhibited by crude cell lysate of hemolytic UPEC isolates. A: untreated cells ,B-E: treated DU145 cells by different dilution ratio from 1:1 to 1:8 after 24h exposure exhibit of Cytoplasm: Denser staining, with signs of shrinkage in some cells to Some cytoplasmic disruption, with variable staining intensity.Nucleus: Several nuclei appear darker (pyknotic) or swollen, suggesting apoptosis/necrosis. Larger nuclei, with chromatin

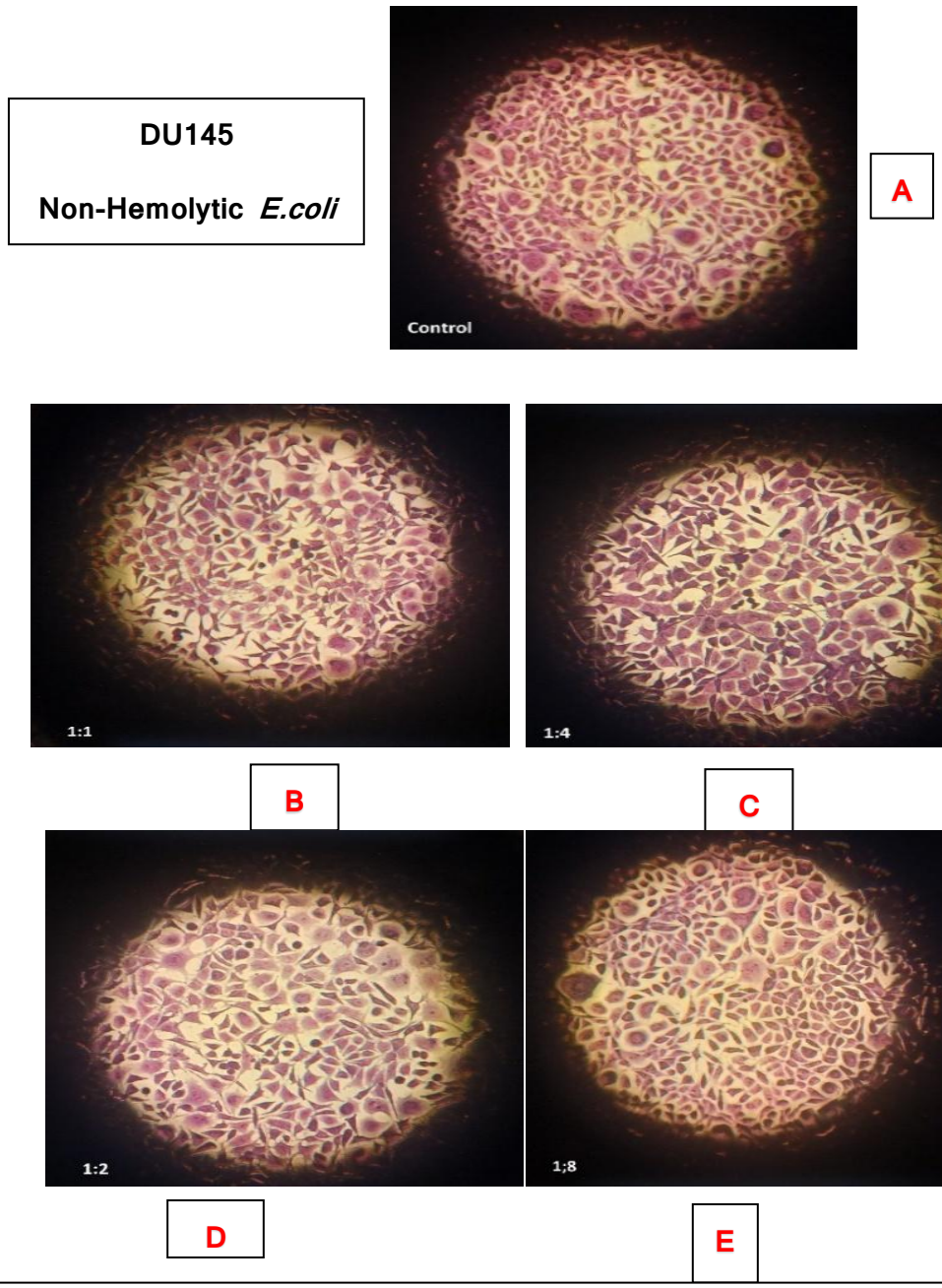

Figure 10: Morphological changes in DU145 cells exhibited by crude cell lysate of non- hemolytic UPEC isolates. A: untreated cells ,B-E: treated DU145 cells by different dilution ratio from 1:1 to 1:8 after 24h exposure exhibit denser Cytoplasm staining, with signs of shrinkage in some cells to Mild shrinkage and stress signs of cytoplasm; most cells still intact. Nucleus Dark, condensed (pyknotic nuclei), with irregular shapes; some

DISCUSSION

Experiments were based on detecting the hemolytic activity of the collected *E.coli* isolates followed by detection of characteristic of phenotypic detection and genotypic feature of toxin. Only 8 out of 24 isolate harbors *cnf1 gene*, 3 of them hemolytic the others non hemolytic.

The result of current study prevalence and pathological effect of cytotoxic necrotizing factor 1 (CNF 1) among both hemolytic and non-hemolytic UPEC recovered from prostate and bladder cancer patients in Iraq. A total of 3 β hemolytic isolates that harboring *cnf-1 gene* induced multinucleation and megalocytosis and affect on viability of treated cell lines . In healthy people who infected with recurrent UTI by *E.coli* , the toxin will kill the epithelial cell ,every time the bacterial toxin kills the cell the body regenerate and grew a new cells by the time and multiplication of new cell will lead to an error with DNA replication and repair system of DNA cannot work properly as the result of rapid speed replacement of damaged cells ,so this procedure will lead to many unproper high number of cells this will lead to progress in the occurrence of bladder and prostate cancer or any other type of cancer.

A study in Iraq among the 110 isolates obtained only 46 isolate (41.7%) were positive for hemolysin production on blood agar supplemented with 5% sheep blood. E. coli isolates harboring tested genes appeared cytotoxic effects against Rat Embryonic Fibroblast cells (REF) cells and this effect increased with the
increasing of multiplicity of infection(MOI) during the period of incubation (21).

Another study in Iraq The cytotoxic effect of different concentrations of CNF1 on cancer lines (HeLa, and PC3) at 72 h were examined .The effect was manifested multinucleated and enlargement in cell line (29).

A 2 academic research in Egypt The crude cell lysates of the haemolytic isolates were assessed on HeLa cell lines. Significantly, 13 of the 21 haemolytic isolates (61.9%) elicited morphological alterations, such as cellular expansion and mortality, within 24 to 48 hours of exposure. Seven of the thirteen cytotoxic isolates demonstrated multinucleation and cellular hypertrophy in HeLa cells (11). A separate investigation involving HeLa cells exposed to sonicate from cnf+ E. coli isolates revealed multinucleation and megalocytic cytotoxicity in a dose-dependent fashion. The visual evaluation of the HEp-2 cell line one hour after infection with 1 x 108 CFU/ml of the tested isolates showed no discernible impact on the HEp-2 cells. Two hours' post-infection, an investigation of 50 UPEC isolates demonstrated that 46 (92%) displayed cytotoxic effects, characterised by mild detachment and lysis of the HEp-2 cell line, as well as near-total detachment of the cell line. Three hours' post-infection, merely four isolates (8%) shown no impact on the cell line.

A 2 studies in USA in vitro cytotoxicity assays, infection with the cnf1+ isolates caused ~ 50% of HeLa cells to die, and the surviving cells exhibited an elongated morphology(Feng et al., 2017) .The observed cytotoxic effects cannot be ascribed to E. coli haemolytic activity, as E. coli haemolysin induces a cytocidal effect within 30 to 60 minutes and persists for 3 to 6 hours following treatment of cultured mammalian cells (30).

A study in Spain It was observed that E. coli strains responsible for urinary tract infections typically exhibit haemolysis and produce cytotoxic necrotising factor (CNF), demonstrating lethality in murine models. The production of haemolysis and synthesis of CNF were statistically correlated with strains whose sonic extracts were lethal when administered intraperitoneally to adult mice. crude as characterised by (25) and (20). Therefore, the presence of the Cnf 1 encoding gene in these isolates confirmed that Cnf 1 was being produced.

A study in Pakistan the UPEC Isolates demonstrated significant cytotoxic effects, with 20% testing positive for the cnf1 gene. Among the 27 UPEC isolates (46%) possessing the cnf1, hlyA, or both genes, the highest toxicity, characterised by 80-100% Vero cell death, was observed in isolates positive for the hlyA gene (n = 6). This was followed by isolates positive for both hlyA and cnf1 (n = 4), and those positive for cnf1 alone (n = 1). (31).

CONCLUSION

The cytotoxicity assessment revealed clear differences between hemolytic and non-hemolytic *E. coli* strains These findings are consistent with the known pathogenic mechanisms of hemolytic strains, which are capable of lysing host cells and impairing cellular function. The observed higher cytotoxic effect at intermediate dilutions might reflect an optimal balance between bacterial concentration and toxin activity, where very high concentrations may saturate the system or trigger defensive cellular responses. Conversely, the non-hemolytic strains, lacking potent membrane-damaging toxins, maintained cell viability across dilutions, aligning with their typically commensal or low-pathogenic behavior.

Overall, this study highlights the differential impact of hemolytic versus non-hemolytic *E. coli* on host cell viability and underscores the importance of bacterial virulence factors in cytotoxicity. These results could inform future investigations into host-pathogen interactions and the development of targeted antimicrobial strategies.

Acknowledgments (Times New Roman 12 Bold)

Acknowledgments Text (Times New Roman 12)

Conflict of Interest: (Times New Roman 12 Bold)

Conflicts of Interest: Authors must identify and declare any personal circumstances or interests that may be perceived as inappropriately influencing the representation or interpretation of reported research results. If there is no conflict of interest, please state "**The authors declare no conflict of interest**.".

REFERENCE

1. Bien J, Sokolova O, Bozko P. Role of uropathogenic Escherichia coli virulence factors in development of urinary tract infection and kidney damage. Int J Nephrol. 2012;2012(1):681473.

2. Mitsumori K, Terai A, Yamamoto S, Ishitoya S,

Yoshida O. Virulence Characteristics of Eschevichia Coli in Acute Bacterial Prostatitis. J Infect Dis. 1999;180(4):1378–81.

3. Tolg C, Sabha N, Cortese R, Panchal T, Ahsan A, Soliman A, et al. Uropathogenic E. coli infection provokes epigenetic downregulation of CDKN2A (p16INK4A) in uroepithelial cells. Lab Investig. 2011;91(6):825–36.

4. Simons BW, Durham NM, Bruno TC, Grosso JF, Schaeffer AJ, Ross AE, et al. A human prostatic bacterial isolate alters the prostatic microenvironment and accelerates prostate cancer progression. J Pathol. 2015;235(3):478–89.

5. Kaper JB, Nataro JP, Mobley HLT. Pathogenic escherichia coli. Nat Rev Microbiol. 2004;2(2):123–40.

6. Feng Y, Mannion A, Madden CM, Swennes AG, Townes C, Byrd C, et al. Cytotoxic Escherichia coli strains encoding colibactin and cytotoxic necrotizing factor (CNF) colonize laboratory macaques. Gut Pathog. 2017;9(1):71.

7. El-Aouar Filho RA, Nicolas A, De Paula Castro TL, Deplanche M, De Carvalho Azevedo VA, Goossens PL, et al. Heterogeneous family of cyclomodulins: smart weapons that allow bacteria to hijack the eukaryotic cell cycle and promote infections. Front Cell Infect Microbiol. 2017;7:208.

8. Lax AJ. New genotoxin shows diversity of bacterial attack mechanisms. Trends Mol Med. 2007;13(3):91–3.

9. Oswald E, De Rycke J, Guillot JF, Boivin R. Cytotoxic effect of multinucleation in HeLa cell cultures associated with the presence of Vir plasmid in Escherichia coli strains. FEMS Microbiol Lett. 1989;58(1):95–9.

10. Caprioli A, Falbo V, Roda LG, Ruggeri FM, Zona C. Partial purification and characterization of an Escherichia coli toxic factor that induces morphological cell alterations. Infect Immun. 1983;39(3):1300–6.

11. Morgan RN, Saleh SE, Farrag HA, Aboulwafa MM. Prevalence and pathologic effects of colibactin and cytotoxic necrotizing factor-1 (Cnf 1) in Escherichia coli: experimental and bioinformatics analyses. Gut Pathog. 2019;11(1):22.

12. Michaud JE, Kim KS, Harty W, Kasprenski M, Wang MH. Cytotoxic Necrotizing Factor-1 (CNF1) does not promote E. coli infection in a murine model of ascending pyelonephritis. BMC Microbiol. 2017;17(1):127.

13. Zinatizadeh MR, Schock B, Chalbatani GM, Zarandi PK, Jalali SA, Miri SR. The Nuclear Factor Kappa B (NF-kB) signaling in cancer development and immune diseases. Genes Dis. 2021;8(3):287–97.

14. McCoy CS, Mannion AJ, Feng Y, Madden CM, Artim SC, Au GG, et al. Cytotoxic Escherichia coli strains encoding colibactin, cytotoxic necrotizing factor, and cytolethal distending toxin colonize laboratory common marmosets (Callithrix jacchus). Sci Rep. 2021;11(1):2309.

15. Slavchev G, Pisareva E, Markova N. Virulence of uropathogenic Escherichia coli. 2013;

16. Sharma S, Bhat GK, Shenoy S. Virulence factors and drug resistance in Escherichia coli isolated from extraintestinal infections. Indian J Med Microbiol. 2007;25(4):369–73.

17. Keane WF, Welch R, Gekker G, Peterson PK. Mechanism of Escherichia coli alpha-hemolysin-induced injury to isolated renal tubular cells. Am J Pathol. 1987;126(2):350.

18. Mobley HL, Green DM, Trifillis AL, Johnson DE, Chippendale GR, Lockatell CV, et al. Pyelonephritogenic Escherichia coli and killing of cultured human renal proximal tubular epithelial cells: role of hemolysin in some strains. Infect Immun. 1990;58(5):1281–9.

19. O'Hanley áP, Lalonde G, Ji G. Alpha-hemolysin contributes to the pathogenicity of piliated digalactoside-binding Escherichia coli in the kidney: efficacy of an alpha-hemolysin vaccine in preventing renal injury in the BALB/c mouse model of pyelonephritis. Infect Immun. 1991;59(3):1153–61.

20. Landraud L, Gauthier M, Fosse T, Boquet P. Frequency of Escherichia coli strains producing the cytotoxic necrotizing factor (CNF1) in nosocomial urinary tract infections. Lett Appl Microbiol. 2000;30(3):213–6.

21. Alhadidi HAS, Al-Qaysi SAS, Al-Halbosiy MMF. Prevalence and cytotoxic effects of some colibactin and cnf genes among Escherichia coli isolated from urinary tract infections. Microbiol Biotechnol Lett. 2022;50(2):283–92.

22. Waly AHT, Barraj AH. Prostate Cancer in Iraq: A Comprehensive Literature Review of Epidemiology, Clinical Landscape, Challenges, and Public Health Strategies. Asian Pacific J Cancer Care. 2025;10(3):871–80.

23. Saad Rehan, E., & Hal-Mudallal N. Molecular Detection of Cytotoxic Necrotizing Factor Type 1 gene (CNF1) of Uropathogenic E.coli isolated from Bladder and Prostate Cancers Patients. 2025.

24. Dubois D, Delmas J, Cady A, Robin F, Sivignon A, Oswald E, et al. Cyclomodulins in urosepsis strains of Escherichia coli. J Clin Microbiol. 2010;48(6):2122–9.

25. Alonso P, Blanco J, Blanco M, Gonzalez EA. Frequent production of toxins by Escherichia coli strains isolated from human urinary tract infections: relation with haemagglutination. FEMS Microbiol Lett. 1987;48(3):391–6.

26. Al-Shammari AM, Alshami MA, Umran MA, Almukhtar AA, Yaseen NY, Raad K, et al. Establishment and characterization of a receptor-negative, hormone-nonresponsive breast cancer cell line from an Iraqi patient.

Breast Cancer Targets Ther. 2015;223–30.
27. Perry P, Wolff S. New Giemsa method for the differential staining of sister chromatids. Nature. 1974;251(5471):156–8.
28. Arbuckle J. IBM® SPSS® AmosTM 23 User's Guide. IBM. Recuperado de ftp://public. dhe. ibm. com/software/analytics/spss …; 2014.
29. Hassan MH. Isolation and Purification of CNF1 (Cytotoxic necrosis factor 1) produced from bacteria Escherichia coli and study its role against apoptosis in vitro. Iraqi J Cancer Med Genet. 2015;8(2).
30. Cavalieri SJ, Bohach GA, Snyder IS. Escherichia coli alpha-hemolysin: characteristics and probable role in pathogenicity. Microbiol Rev. 1984;48(4):326–43.
31. Bashir S, Haque A, Sarwar Y, Ali A, Anwar MI. Virulence profile of different phylogenetic groups of locally isolated community acquired uropathogenic E. coli from Faisalabad region of Pakistan. Ann Clin Microbiol Antimicrob. 2012;11(1):23