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The roles of TNFSF10 in apoptosis of Prostate Cancer-(PC3)-Cell Line that treated by Pyocyanin

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Summary. Fifty isolates of *pseudomonas aeruginosa* were got out of wounds and burn specimens. isolated of the colonies and diagnosed it depending on the morphology and biochemical test that include catalase and oxidase tests and then used of VITEK 2-Compact to conform it. Pyocyanin (PNC) at concentrations (12.5,25,50,100,200 and 400) was significantly increased the percentage of cytotoxic effect on Prostate Cancer-(PC3)-Cell Line compared to control. The results of recent study reveal that the PNC, induces apoptosis of Prostate Cancer-(PC3)-Cell Line and Human Tumor Necrosis Factor Ligand Superfamily Member 10 (TNFSF10) human kit was demonstrated it.

Keywords: Pyocyanin, *P.aeruginosa*, Prostate Cancer-(PC3)-Cell Line, TNFSF10.

Introduction

Cancer is irregular growth for some cells that represent the uncontrolled proliferate and cause metastasis for attack many tissues.

Cancer formation from another organs, tissues and structure. Progress as an irregular products of genetics or/and epigenetics.

prostate cancer (PC3) at western countries consider public situation of men which start as irregular growth of men sexual organs [1,2].

PNC, is a pigment produced by Pseudomonas aeruginosa and consider as an extracellular secondary metabolite, its shows redox activity and in mammalian has cytotoxic action, It's has been a new and a powerful different to cancer treatment [3].

Apoptosis is regulated conserved cell death program, acting as a key role in normal physiological action, for example, (embryogenesis and mature tissue homeostasis), also consider as a famous cause of its role as a suppressor mechanism of tumor. Apoptosis is a cell's death by regular physiological mechanism and responding to several stimulation like damage or infection, which following by radiotherapy program or cytotoxic treatments [4].

The TNF superfamily (TNFSF) of cytokine-like molecules is currently composed of 19

ligands, while the TNF receptor (TNFR) superfamily (TNFRSF) containing proteins that bind to these ligands consists of 29 related receptors. Interactions among these TNFSF ligands and TNFRSF receptors mediate signaling that controls the survival, proliferation, differentiation, and effector functions of both immune and non-immune cells. While members of the TNFSF/TNFRSF system (collectively TNFSF/TNFRSF) generally exhibit proinflammatory properties that are partially due to their activation of NF-κB signaling pathways, TNFSF/TNFRSF activities can also trigger apoptosis and other forms of cell death. Thus both beneficial and harmful effects have been described for various TNFSF/TNFRSF members, and several of these effects have been linked to hereditary and acquired human diseases. With respect to cancer treatment, studies in the later 1980s showed that only a few cancer cells actually died when treated with TNF, and that TNF treatment of patients led to a lethal inflammatory shock syndrome [5].

Materials and methods

Isolation:

50 specimens collected from the Al-Diwaniya Teaching Hospital. These samples included: burn and wounds. Burns and wound samples were collected using sterile cotton swabs. All samples moved to the laboratory and cultured into cetrimide agar incubated at 37℃ for 24hr. Isolates purified many times until pure isolated were got, diagnosed under microscopic and biochemical test [6].

Identification via VITEK 2-Compact:

Procedure:

According to manufacturer's instruction. (Biomerieux)

1-Added 3 ml. normal saline in a tube of test and inoculate by isolated colony.

2-Tube of test joined with Dens examine apparatus for colony standardization to Mc Farland solution. ($1.5*10^8$ cell/ml).

3- putted inside cassette the Standard vaccines and count of specimen in computer software by code.

4- VITEK-2 cards reading from the code have been putted in card so cards connected with ID sample.

5-puted of Cassette inside filler unit when cards filled, cassette transported out to reader / incubator.

6-All of the steps handled by device which check incubation temperature visual read of card, then constantly watching then transfer computer test data for analyses. While test cycle complete automatically system removed card inside waste container [7].

Extraction and purification of PNC:

According to[8].

1. The cetrimide agar was cut to 1.5-cm2 and added to a sterile bottle.
2. Add 20ml. from chloroform
3. Shake the mixture strongly, the chloroform was colored to blue
4. Add 0.2m of HCL and shake, the solution will color to red pigment.
5. The solution was removed and add of 0.2M NaOH and the blue color was removed.
6. Add 20ml of chloroform and shake strongly to get blue pigment.

7. Removed of chloroform solution and filtered it with special filter paper.
8. Incubate in an oven at 40℃ over night.
9. Get of blue crystal and weigh it.
10. Added of the chloroform again to solubilize the crystals.
11. Injection of solution in HPLC (High-performance liquid chromatography) to get pure PNC.

Cytotoxicity assays:

MTT assay:

Procedure:

- According to [9]. At the finish of the during exposure time, the medium was removed from the walls and then the cells were cleaned with PBS. A blanking controlling was carried to assess unspecific formazaning conversion.
- A volume of 1.9 ml of MTT solution (9 mg/ ml) were added to 1 ml medium to obtain last concentration of 3 mg/mL. Then, Added of 30 µl of the resulting solution in each well.
- The plated were incubated for 9 hours at 37°C until intracellular red formazan crystals were visible under invert microscope.
- The supernatant was removing and 10 µl DMSO were added in each wall to dissolve the resultant formazaning crystals.
- The plated were incubated at rooming temperature for 90 minutes until the cells have lyse and red crystals have dissolved.
- Absorbance was measured by a microplate analyzing at 570nm. The absorbance read of the blank must be taken from all samples. Absorbance read from testing samples must then be dividing by the controlling and multiplied by 100 to give percentage celling viability or proliferation. Absorbance valuable greater than the controlling lung indicate cell proliferation, while little values suggest cell dead or inhibiting of proliferation.

Crystal violet Assay

1-crystal violet (CV) used in order to determine visual density in microtiter plate wells after the cytotoxicity was complete, crystal violet_(CV), a triarylmethane stain, it's attached to cell nucleus especially in DNA and consider as proportionate with cell biomass. Dead cells will be separated from plate and then removed out of the plate during culture period but viable adherent will stay attached to the plate.

2-Disposed the maintenance medium and the test substance and washed the wells by PBS (100 µL) using an automatic pipette.

3-fixated cell culture with 10% of formalin stored in room temperature for 20 min.

4-removed fixative solution and 100 µL of 0.1% crystal violet put in all wells.

The effect of PNC on Prostate Cancer-(PC3)-Cell Line:

Three replicates walls in seven columns of 96_wall plate were cultured with Prostate Cancer-(PC3)-Cell Line in a concentration of $9*10^5$. Three replicates walls in column No.1 were considered as a control group and each one of the remaining six columns 9 walls replicates were exposed to 200 µL of each of the serial dilutions of PNC. Then the plate was covered with a self-glass lid and incubated once for 48 hrs. After the end of the exposure, the walls washed with 200µl of PBS. calculate of toxin action was by MTT assay for PC-3 growth.

Sandwich ElISA

Assay Procedure:

1. Prepare all reagents, standard solutions and samples as instructed. Bring all reagents to room temperature before use. The assay is performed at room temperature.

2. Determine the number of strips required for the assay. Insert the strips in the frames for use. The unused strips should be stored at 2-8°C.

3. Add 50ul standard to standard well. Note: Don't add antibody to standard well because the standard solution contains biotinylated antibody.

4. Add 40ul sample to sample wells and then add 10ul Human TNFSF10 antibody to sample wells, then add 50ul streptavidin-HRP to sample wells and standard wells (Not blank control well). Mix well. Cover the plate with a sealer. Incubate 60 minutes at 37°C.

5. Remove the sealer and wash the plate 5 times with wash buffer. Soak wells with 300ul wash buffer for 30 seconds to 1 minute for each wash. For automated washing, aspirate or decant each well and wash 5 times with wash buffer. Blot the plate onto paper towels or other absorbent material.

6. Add 50ul substrate solution A to each well and then add 50ul substrate solution B to each well. Incubate plate covered with a new sealer for 10 minutes at 37°C in the dark.

7. Add 50ul Stop Solution to each well, the blue color will change into yellow immediately.

8. Determine the optical density (OD value) of each well immediately using a microplate reader set to 450 nm within 10 minutes after adding the stop solution.

Results and Discussion

Isolation and Diagnosis of *P.aeruginosa*

50 clinical specimens, from burned patients and from wounds of patients. Specimen inoculated on cetrimide media. The diagnosis was completed with the automated VITEK-2 compact.

P.aeruginosa is diagnosed by the odor into vitro. The smell as (grape-like, tortilla-like), all the isolates have been cultured on cetrimide media.

Finally, on Cetrimide Agar was used as a *P.aeruginosa* makes yellow-green to blue-

green colonies and fluoresces under UV light, as shown in Figure (1).

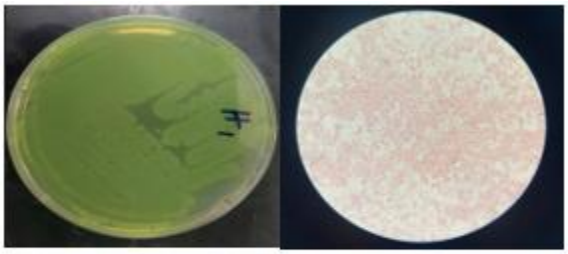


Figure (1) *P. aeruginosa* colony on Cetrimide agar and under light microscope

The results agree with [10]. which confirmed that *P.aeruginosa* is resistant to cetrimide media with concentration of up to 0.3g/L, and production of pigment is not inhibited with this range.

Extraction and purification of PNC:

PNC that produces from *P.aeruginosa* held for variable period time, the amount of PNC depending on the time. The results displayed that PNC extraction making the extreme volume from the plates held for 96hrs, followed by at 72hrs, 168hrs, 240 hrs. and finally, 48 hrs.

extraction of PNC was by technique described by [11] with some alterations. These alterations include using of cetrimide Agar instead of King's A Agar, incubation time is changed to get maximize pigment, dry chloroform was added to extract acid/base to get the last form of PNC.

In this study the method used that consisted of 2 steps:

1- The 1st step depended on properties of redox of PNC and changed the color when PNC moved from basic to acidic.
if the color of PNC is red that mean PNC was soluble in aqueous solvents. whereas the color is blue that's mean PNC was soluble in organic solvents. (Figure 2).

2- the 2nd step was the Standard PNC, which gives us a rotation time after inject it in HPLC to get pure PNC.

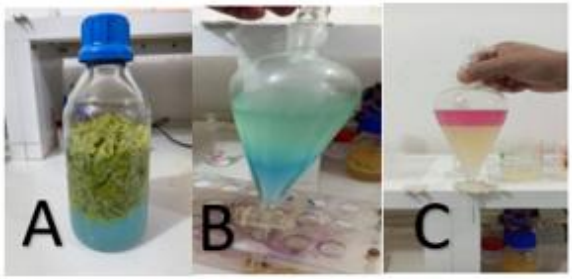


Figure (2) PNC extraction stage- (A)-Cutting cetrimide agar and putting in chloroform, (B)-Alkaline medium (NAOH), (C)- Acidic medium (HCL)

Measuring the Impact of PNC on the Viability of Prostate Cancer-(PC3)-Cell Line after 48 Hours-of Incubation by MTT Assay:

The comparison with the control group, PNC concentrations,(12.5, 25,50, 100, 200 and 400 µg/ml) cause significant reduction ($P<0.05$) in PC-3 cell line. Figure (3) shows that there was a significant effect on PC3 cell line with increasing of the concentration of PNC, particularly (200, 400 µg/ml).

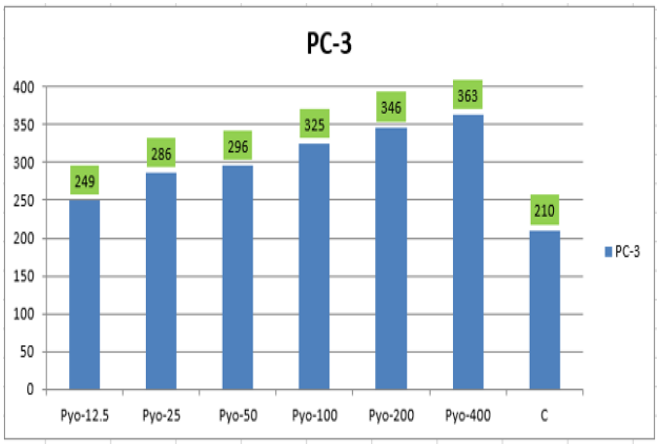


Figure (3): Impact of PNC on Prostate Cancer-(PC3)-Cell Line viability after 48 hours-of incubation

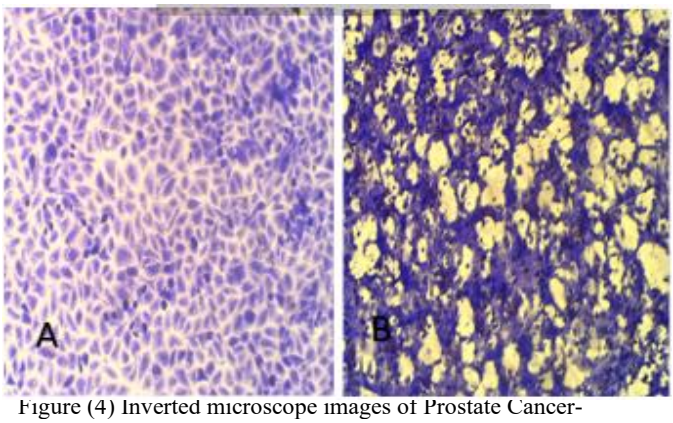


Figure (4) Inverted microscope images of Prostate Cancer-(PC3)-Cell Line as shown in (A)control cells-untreated with PNC and (B) treated cells with 200 µg/ml of PNC

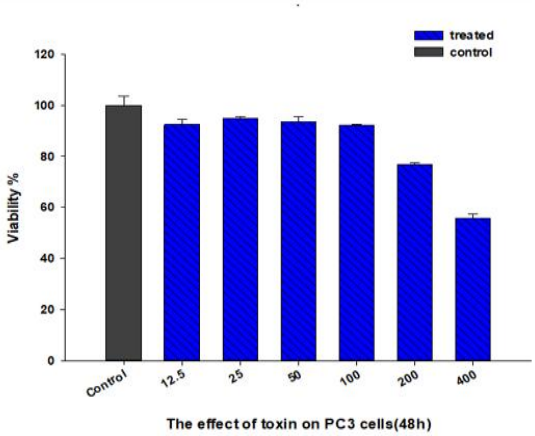
As shown in Figure (4), the effect of PNC on PC3 was significantly after adding 200 µg/ml of PNC to cell line and incubated for 48 hrs comparing untreated control cells.

The current study is consentient with a previous study which indicated that MTT test was employed to evaluate the cytotoxic action of purified PNC from PsC05 and PsE02 in vitro against 5 cancer cell lines (MCF-7, HePG-2, Hela, HCT-116, and PC-3) and 2 normal cells (WISH and WI-38). Doxorubicin worked as a positive control. IC50 values were used to express the concentration at which 50% of the cell monolayer was lost. [12].

A further study demonstrated the ELISA assay was utilized to compare the secretion levels of IL-2 in Human peripheral blood mononuclear cells (PBMCs), of prostate tumor patients, and normal control samples after and before treatment with PNC. All groups, after treatment with PNC, the level of IL-2 of PBMC was higher than that of pretreatment with PNC. [13].

Effect of TNFSF10 on Prostate Cancer-(PC3)-Cell Line

As shown in Figure (5), the effect of TNFSF10 on supernatant of PC3 which treated with Pyocyanin was significantly in all concentrations (12.5,25,50,100,200 and 400 µg/ml). TNFSF10 rise from low concentration to high concentration, Where the concentration increases, the effect increases also.



TNFSF10, also known as TRAIL or Apo-2 ligand [14],is a member of the TNF superfamily, and since its discovery it has been used as an antitumor protein because of its ability to induce apoptosis in a variety of human cancer cell lines while leaving normal cells unaffected [15]. Moreover, studies performed on TRAIL knockout mice show that TNFSF10 has an important role in normal tumor immunosurveillance as TNFSF10 knockout mice support tumor growth at a higher rate when compared with

normal mice and are more susceptible to tumor metastasis [16]. Cells undergoing TRAIL-induced death show many of the hallmarks of apoptosis, including DNA fragmentation, expression of pro-phagocytic signals on the cell membrane and cleavage of multiple intracellular proteins by caspases [17].

CONCLUSION

The study suggested that the ability of *P.aeruginosa* isolates are able to produce PNC on cetrimide agar, and the ability of PNC to Cytotoxic effect on PC3 at different concentrations and has little effect on normal cells. TNFSF10 increased with cells apoptosis that treated with pyocyanin.

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