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# Evaluation of the apoptotic effect of the triazole analog TAN on human colorectal Cancer cell line HCT 116 by flow cytometry

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ABSTRACT

The flow cytometry capability for fast and individualized analysis of a big number of cells would seem to make it typically selected for the cell death assessment. It offers the capability to revise large numbers cells separately rather than a mixed population, and investigates concurrently many proteins expression like cell specific markers and apoptotic indicators. Annexin V assay is one of the most widely used approach to detect apoptosis as it is easy-to-use, fast , needs no fixation and allows a large number of cells to be analyzed at any one time. Annexin assay is ideal for solitary cell suspensions and so flow cytometry. In this study we try to evaluate the apoptotic effect of the investigational drug TAN on human cancer cell line HCT116 in comparison with the triazoles: itraconazole (ITC), difenoconazole (DIF), epoxyconazole (EPO), and the standard anticancer agent's doxorubicin (DOX) and 5-fluorouracil (5FU) by flow cytometry. To assess the apoptotic effect of the triazole analog TAN on human colorectal cancer cell line HCT116using annexin V dye via flow cytometry assay.

The results of flow cytometry reveal a significant differences between treated and untreated HCT116 cells in all drugs based on mean ±Std. in all drugs that are used in this study , TAN showed a significant apoptotic effect after DOX ,DIF and EPO with P value = . 0005, the following conclusions as the TAN has significant and promising anticancerrole through induction of apoptosis in HCT116 cells and flow cytometry is a reliable technique to measure the apoptotic percentages in cells.

**Keywords:** apopotic effect, Colorectal Cancer, Flow Cyclometry

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## INTRODUCTION

Colorectal cancer (CRC) is the most popular kind of gastrointestinal cancer which can be resulted from genetic alterations that evolve during a lifetime, the CRC begins as a benign adenomatous polyp in the colon and rectum and prevalence initially into the colon wall and possibly metastasis into the lymphatic nodes and the other organs [**1-2**]. CRC is the 3rd most commonly occurred cancer in men after lung and prostate cancer and the 2nd in women after breast cancer worldwide, with over 1.2 million new cases and 608,700 deaths estimated to have been occurred in 2008 [**3**]. According to GLOBOCAN 2018, CRC incidence rate is about 10.2% of all new cases and mortality rate is about 9.2% of total cancer death cases. In the USA, total CRC incidence and mortality rates raised from 2000 to 2014 incidence and death rates are 30% and 40% higher in males than in females, respectively because females have a longer life expectancy [**4**]. Relative studies in the Iraqi Cancer Registry from 1965-1994 appeared an elevated the incidence of CRC from 25% to 50% with incidence rate 2.6% [**5**] and number of cases with CRC in 2011 are about 1086 per 100,000 with increase in the incidence rate about 3.26% [**6**]. The 5-year survival prediction is greatly depending on the cancer stage. While displaying over 90 percent survival for patients with stage I CRC, it barely reaches 10 percent for patients with stage IV CRC [**7**]. Apoptosis detection in response to cancer therapy represents one of successful diagnostic techniques to evaluate the anticancer drug effectiveness [**8**]. The two models of cell death, apoptosis and necrosis, differ basically in their morphological, biochemical and biological relevance [**9**]. Depending on the type of the cell and the activator, a cell may die by either of these 2 distinguished mechanisms. Necrosis is a nonspecific way of cell death, it is recognized by the cell and the mitochondria swelling, which leads to separation of the cell membrane and Lyses [**10**], release of the cytoplasmic content results in an inflammatory response. Apoptosis seems to be an active mechanism that has a role in the development of tissues and organs [**11**], immune responses regulation [**12-13**], or differentiated cells natural death at the end of their life time. Precise characterization and quantification of the mechanism of cell death ongoing in a specific state is a mandatory requirement to understand the biological process that occurs. Apoptosis can be distinguished by morphological characteristics, like condensation and margination of chromatin, shrinkage of cell, blebbing of membrane, and the apoptotic body production [**14-15**]. Apoptosis has also been identified by enzymatic inter nucleosomal DNA destruction biochemically [**16-17**], but, evaluation of apoptosis of morphologically and biochemically lack precise quantity and sensitivity assessment. While characterization of cell death is exclusively depending on light or electron microscopy [**18**], approaches used for quantification are involving microscopy, assays of colony-formation, exclusion of vital dye tests and

flow cytometry [**19**]. The flow cytometry capability for fast and individualized analysis of a big number of cells would seem to make it typically selected for the cell death study. Many approaches of flow cytometry are available which basically rely on DNA alterations, light scatter properties, or surface membrane to evaluate cell death [**20**]. So, apoptotic cells can be identified by their diminished staning with DNA specific fluorochromes like propidium iodide (PI), acridine orange, DAPI, or Hoechst dyes, due to DNA lyses and its successive leaking from the cell. In adverse to apoptotic cells, necrotic cells usually do not demonstrate a decrease in DNA stains ability at once. So, the distinguishing between normal live or necrotic cells is not possible when it is based on just one DNA content analysis parameter. Morphologic alterations in cells passing through apoptosis influence their light scattering criteria. The forward light scatter reduction can be seen in thymocytes stimulated to pass through apoptosis, paralleled by a rise in side scatter. Necrotic cell death leads to a rise of the forward and side scatter signals. In apoptosis the cellular plasma membrane integrity and most of the membrane functions are intact [**21**]. Thus, apoptotic cells expel "viability assay" stains such as PI or trypan blue. This is in opposite to necrotic cells, in that one of the earliest alterations is the loss of function and integrity of the membrane. By their capability to extrude PI, apoptotic cells can be wrongly classified by flow cytometry as viable cells. To assess apoptosis in a mixed population of cell, characterization of cell phenotype is mandatory. An assay of cell viability based on cell staining with annexin V used to distinguish early apoptosis from late apoptosis, built on determining the of phosphatidyl serine translocation from the internal to the external layer of the plasma membrane of early apoptotic cells, stained by annexin V.

## MATERIAL AND METHODS

### HCT-116 colorectal carcinoma cell line

HCT 116 (ATCC® CCL-247™) USA

### Drug solutions for flow cytometry assay

### TAN solution

This solution was prepared in a final concentration of 100 µg / ml for application of flow cytometry assay and micro titration of this compound was done as the experiment required.

**Itraconazole (ITC), Difenoconazole (DIF) and Epoxyconazole (EPO) solutions:** These solutions were prepared as that mentioned for TAN.

### Doxorubicin (DOX)

The molecular weight for this drug is 580.0 and chemical formula: $C_{27}$ $H_{29}NO_{11}$, HCl. This solution was prepared in a final concentration of 1000 µg/ml for application of cytotoxicity assay, and for use micro titration of this compound was done as the experiment required.

### Fluorouracil (5-FU) solution

This drug was purchased as contains 500mg/10 ml, from which the solution was prepared in a final concentration of 1000 µg/ml for application of cytotoxicity assay, and for use micro titration of this compound was done as the experiment required.

### Materials and Reagents of flow cytometry assay

- Annexin V FLUOS staining kit (Annexin V- FITC binding kit Thermo fisher, USA).
- The kit contains ready-to-use Annexin-V-FLUOS solution, and incubation buffer.

### Procedure

1. Harvest cells ($1 \times 10^6$ cells) in a T25 culture flask (in triplicate for experiments) and 3 T25 culture flask for control (unstained, Annexin only).
2. Collect the supernatant (floating apoptotic cells) after 48 h of incubation, and trypsinized the adhesive cells (~$2 \times 10^6$ cells) from every T25 flask (combine corresponding floating cells and trypsinized cells and this will result in 6 tubes).
3. The collected cells are washed twice with PBS and centrifuged (670 × *g*, for 5 min, at RT).
4. Each pellet (~$2 \times 10^6$ cells) is re-suspended in PBS (400 µl).

For experimental cells (Triplicate) - (400 µl of cells + 100 µl of incubation buffer with 2 µl of Annexin [1 mg/ml] .

**For control cells:**

**Control1**: (unstained) - (without any stain (400 µl of cells + 100 µl of incubation buffer)

**Control2**: (Annexin V only) - (400 µl of cells + 100 µl of incubation buffer with 2 µl of Annexin (1 mg/ml))

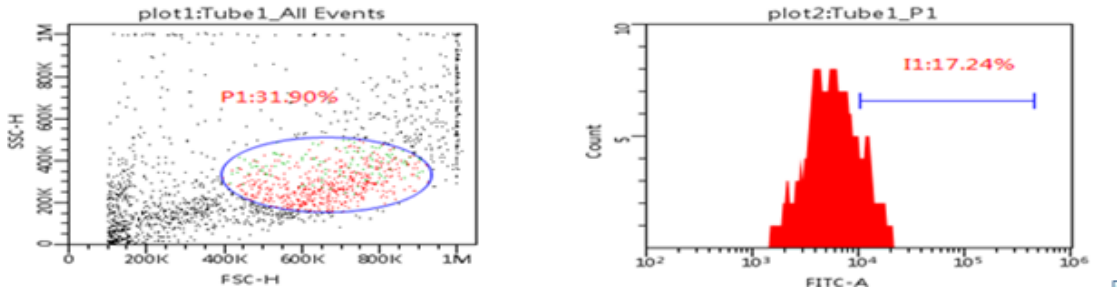
5. The cells are analyzed by a flow cytometry without washing.

Cells which were Annexin V negative are regarded healthy, cells, while Annexin V positive cells are regarded apoptotic [**22**].

## RESULTS

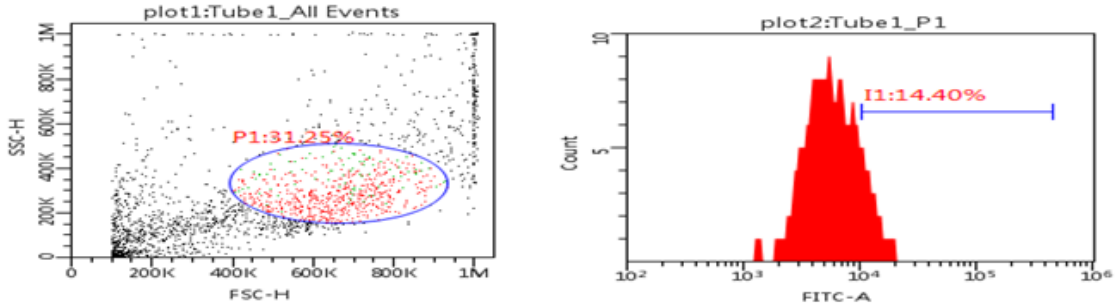
### Annexin V Apoptosis Detection in HCT116 Cells using Flow cytometry assay

Untreated and treated with the IC50 concentration of standard and test drugs( that obtained from our research: Assessment of the antineoplastic role of the triazole analog TAN in blocking hedgehog signaling pathway smoothened receptors on the human colorectal cancer cell line HCT116, {IC50 which was for TAN: 63.94µg/ ml , for ITC: 21.85µg/ ml, for DIF: 6.316.75µg/ ml, for EPO: 281.843µg/ ml,5FU: 82.77µg/ ml and DOX: 345.176µg/ ml}), HCT $_{116}$ cells were stained using Annexin V ITC apoptosis detection kit. The results were expressed as plots by software as the forward and lateral scattered in the following figure that reflects the apoptotic percentages for each tested sample,. Data from different drugs treated samples was acquired in a work list using the Annexin V FITC assay for IC50 concentration for each drug (figures1 - 7).




TAN TAN

**Figure (1): The assay lab research report that was automatically generated after acquisition and analysis. FSC-A vs. SSC-A plots were applied for gating cells & to detect any change in the scatter properties of the cells. Apoptotic percentages of TAN treated human cancer cell line hct116 detected by flow cytometry.**




ITC ITC

**Figure (2): Shows the apoptotic percentages of ITC treated human cancer cell line hct116 detected by flow cytometry.**

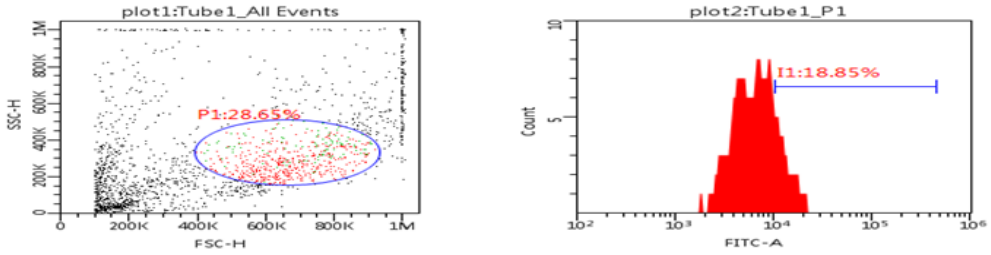
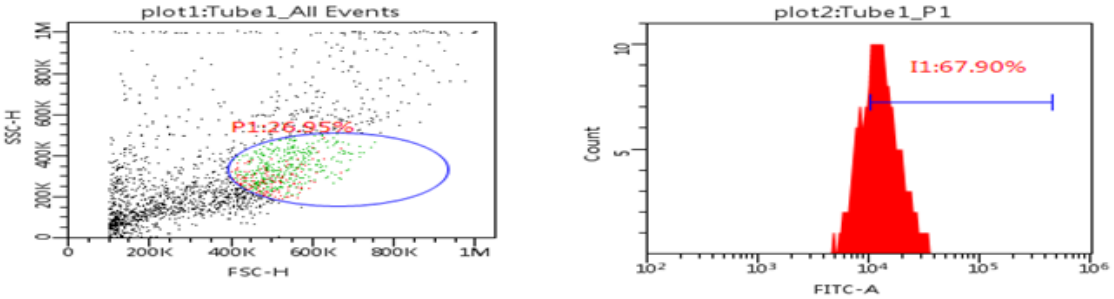
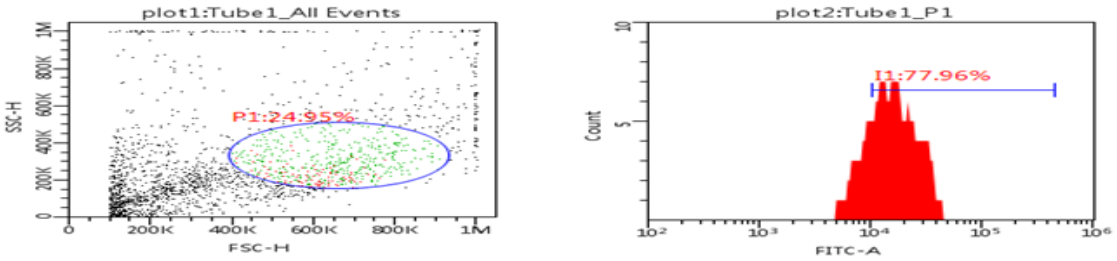



**Figure (3): Shows the apoptotic percentages of ITC treated human cancer cell line hct116 detected by flow cytometry.**



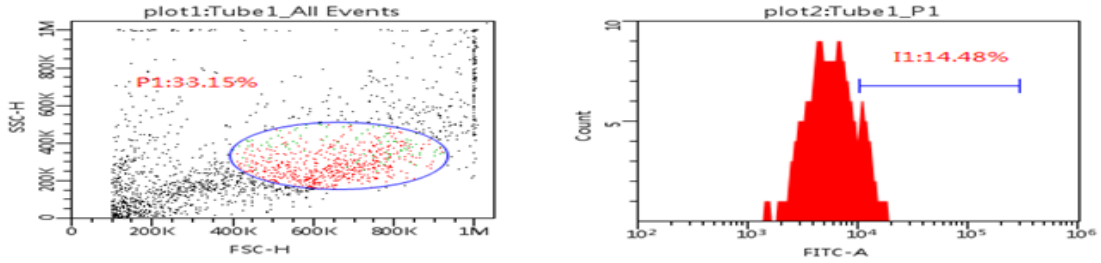

DIFDIF

**Figure (4): Shows the apoptotic percentages of DIF treated human cancer cell line hct116 detected by flow cytometry.**



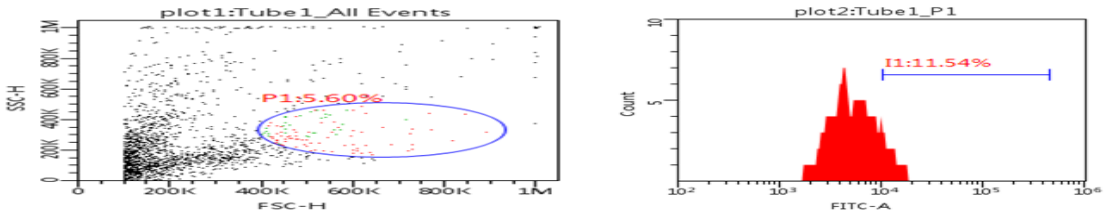

DOX DOX

**Figure (5): Shows the apoptotic percentages of DOX treated human cancer cell line hct116 detected by flow cytometry.**




5FU 5FU

**Figure (6): shows the apoptotic percentages of 5FU treated human cancer cell line hct116 detected by flow cytometry.**




control control

**Figure (7): Shows the apoptotic percentages of the untreated human cancer cell line hct116 detected by flow cytometry.**

After 24 hr. induced apoptosis DOX showed the higher apoptotic percentage in HCT116 cells. DIF had the 2nd place then EPO, TAN, ITC and 5-FU had the least effect on HCT116 cells, figure (8)

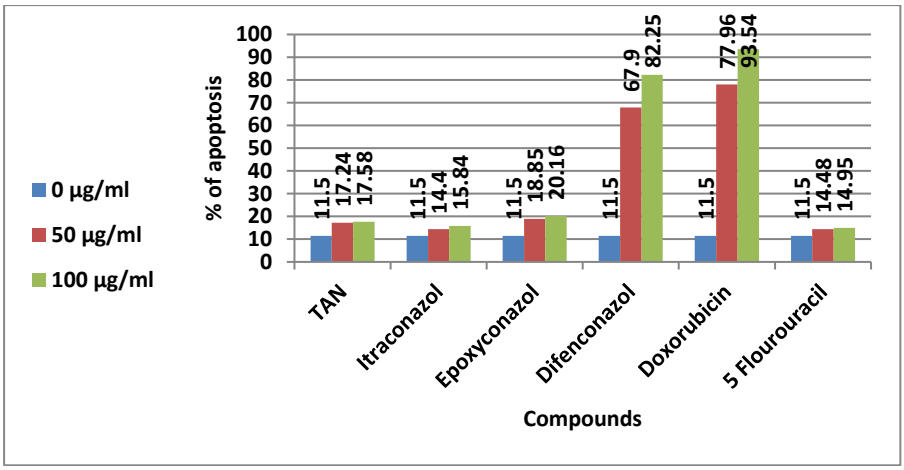



**Figure (8): Scheme shows the Apoptotic percentages of untreated and treated with different tested treatments groups for each drug, on HCT116 cell line detected by flow cytometry**

The results of flow cytometry reveal significant differences between treated and untreated HCT116 cells in all drugs based on mean ± Std. Deviation but with a variation in the level of significance. The higher significant difference was seen with DOX and DIF as P value equals to 0.000 in comparison with control group. EPO showed the 2nd high difference as its P value was 0.001, TAN was the 3rd with P value equals to 0.005 then 5-FU (P value: 0.014) and at last ITC (P value: 0.026) as shown in table (1).

**Table (1): Flow cytometry assay results represented by mean ± Std. Deviation and P Value in HCT116 cells**

| Drug | N | Mean ± Std. Deviation(HCT cells) | P valu |
|---|---|---|---|
| Control | 3 | 11.540±1.00 | |
| TAN | 3 | 17.580± 1.58 | .005 |
| ITC | 3 | 14.400±1.025 | .026 |
| EPO | 3 | 18.8500± .95 | .001 |
| DIF | 3 | 67.900±1.00 | .000 |
| 5-FU | 3 | 14.9400±.57 | .014 |
| DOX | 3 | 77.960±1.00 | .000 |

Comparison among groups revealed the following outcomes:
1. There is a significant difference between TAN and ITC treated HCT cells (P value: 0.043). TAN showed more apoptotic effect.
2. There is no significant difference between TAN and EPO treated HCT cells (P value: 0.299). EPO showed more apoptotic effect, although no significant.
3. There is a high significant difference between DIF and TAN treated HCT cells (P value: 0.000). DIF showed more apoptotic effect.
4. There is no significant difference between TAN and 5-FU treated HCT cells (P value: 0.071). TAN showed more apoptotic effect, although no significant.
5. There is a high significant difference between DOX and TAN treated HCT cells (P value: 0.000). DOX showed more apoptotic effect.
6. There is a significant difference between EPO and ITC treated HCT cells (P value: 0.005). EPO showed more apoptotic effect.
7. There is a high significant difference between DIF and ITC treated HCT cells (P value: 0.000). DIF showed more apoptotic effect.
8. There is no significant difference between ITC and 5-FU treated HCT cells (P value: 0.549). 5-FU showed more apoptotic effect, although no significant.
9. There is a high significant difference between DOX and ITC treated HCT cells (P value: 0.000). DOX showed more apoptotic effect.
10. There is a high significant difference between DIF and EPO treated HCT cells (P value: 0.000). DIF showed more apoptotic effect.
11. There is a significant difference between EPO and 5-FU treated HCT cells (P value: 0.008). EPO showed more apoptotic effect.
12. There is a high significant difference between DOX and EPO treated HCT cells (P value: 0.000). DOX showed more apoptotic effect.
13. There is a high significant difference between DOX and DIF treated HCT cells (P value: 0.000). DOX showed more apoptotic effect.
14. There is a high significant difference between DOX and 5-FU treated HCT cells (P value: 0.000). DOX showed more apoptotic effect.

## DISCUSSION AND CONCLUSIONS

Flow cytometry is standard and useful application for studying apoptosis. It offers the capability to revise large numbers cells separately rather than a mixed population, and investigates concurrently many proteins expression like cell specific markers and apoptotic indicators. Annexin V assay is one of the most widely used approach to detect apoptosis as it is easy-to-use, fast , needs no fixation and allows a large number of cells to be analyzed at any one time. Annexin assay is ideal for solitary cell suspensions and so flow cytometry. In our study and after 24 hr. induced apoptosis DOX showed the higher apoptotic percentage in HCT116 cells. DIF had the 2nd place then EPO, TAN, ITC and 5-FU had the least effect on HCT116 cells. The results of flow cytometry reveal significant differences between treated and untreated HCT116 cells in all drugs based on mean ± Std. Deviation but with a variation in the level of significance. The higher significant difference was seen with DOX and DIF as P value equals to 0.000 in comparison with control group. EPO showed the 2nd high difference as its P value was 0.001, TAN was the 3rd with P value equals to 0.005 then 5-FU (P value: 0.014) and at last ITC (P value: 0.026). The highly significant apoptotic effect of DOX suggesting that it induces an early and late apoptosis in addition to its ant proliferative role as documented by Silva V. et al, DIF and EPO apoptotic effect is evaluated for the first time through our study. The tested agent TAN showed a prominent apoptotic effect as compared with the parent drug ITC or with the positive control 5-FU.in a preclinical study on gastric cancer cells Hu Q et al confirmed the ant proliferative and the apoptotic effect of ITC especially when combined with 5-FU on the other hand Silva V. et al concluded that 5-FU did not induce increasing in the early and late apoptosis, suggesting that 5-FU has only anti-proliferative action.

1. The investigational drug TAN has significant and promising anticancer role through induction of apoptosis in HCT116 cells.
2. Flow cytometry is a reliable technique to measure the apoptotic process in the cells.

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