Resistance Studies, *in vitro* Model, of Myeloid Leukemia Cell Lines HL-60 Against Thymoquinone and Doxorubicin in the Presence of Type I Collagena

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Abstract: *Purpose*: The prognostic of Acute leukemia is cell drug resistance dependent, which is principal cause of death. The bone marrow microenvironment is directly implicated as source of chemio resistance. Several researchers have studied *in vivo* and vitro the effect of the bioactive molecules such as the Thymoquinone (TQ) on cancers chemo resistant. The aim of this study is to compare the activities of Thymoquinone to Doxorubicin on presence and on absence of collagen type I, which is the major component of cell extra matrix (CEM).

Methods: Cell line HL60 resistance against Doxorubicin and Thymoquinone was tested on presence and on absence Type I collagen at concentration 25, 50 and 100 µg /cm^2 TQ and Dox cytototoxicities was evaluated with counting using KOVA Glasstic Slide and phase contrast microscopy. HL-60 cells were seeded at 10 cells/well for 24h in the presence or not of collagen and treated or not with 200nM of Dox or 10 µM of TQ. After incubation, apoptosis was determined using Annex V and Dead Cell Assay kit (Millipore) and Caspase 3/7 Assay kit (Millipore).

Results: cell line HL60 proliferation is more resistance against Doxorubicin in presence Type I collagen than Thymoquinone

Conclusion: Collagen induce cell HL60 resistance against Doxorubicin, But not against Thymoquinone. Combination Thymoquinone, bioactive molecule, to Doxorubicin can decrease the drug resistance and improve leukemia prognostic.

Keywords: Thymoquinone, doxorubicin, collagen, cytotoxicity, annexine V, caspase3/7.

1. INTRODUCTION

Despite the improvement of cure rates of leukemia and the innovation of novel therapeutic approaches, chemo resistance remain the major challenge for the clinicians and cause of death. Recently, evidence has accumulated suggesting that the microenvironment is implicated in therapy failure and can interfere with treatment by giving leukemic cells a list of tumor promoting factors that may intensify cancer malignancy (Rodrigo Jacamo and Michael Andreef, 2015).

Two forms of Environment mediated-drug resistance (EM-DR) have been identified: The first one is mediated by the soluble factors (SFM-DR, Soluble Factor-Mediated Drug Resistance such as cytokines, chemokines and the growth factors (Ting Wu and Yun Dai, 2016).

Y. Chen and al have shown that resistance to cytarabine was mediated by the interaction of CXCR4 receptor to SDF1 chemokine leading to the activation of MYC and BCL-XL in acute myeloid leukemia (AML) cells (Y. Chen and al., 2013). On the other hand, the interaction of integrins with the extracellular matrix components (fibronectin, type I collagen, vitronectin…) was the second form of EMDR named (CAM-DR, Cell Adhesion-Mediated Drug Resistance) (Ting Wu and Yun Dai, 2016). Recently, many studies have shown that type I collagen plays a crucial role in the protection of leukemic cells against chemotherapy inducing apoptosis. In fact Mohamed Amine and all have shown that collagen, among the β1 integrin receptor, can protect the leukemia cells by reduction intracellular Doxorubicin level and apoptosis event [Mohamed Amine El Azreq and al., 2012].

For the last decades of the twentieth century, several researchers have studied *in vivo* and vitro the effect of the bioactive molecules such as the Thymoquinone (TQ) on cancers chemo resistance. TQ is active compound of *N. Sativa*. Several workers have shown its anti-tumor activity, for example it has been found to possess anti neoplastic effects in-vitro against Ehrlich ascites carcinoma, Dalton's lymphoma ascites, and sarcoma (S-180) cell lines. Another study has identified it as an inducer of cell cycle arrest and

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apoptosis in human colon cancer HCT-116 cells *via* p53-dependent mechanism [Shashi Rajput and al., 2013; Ismail Norsharina and al., 2011; Ghosheb OA *et al*., 1999].

The aim of this study was to compare the effect of type I collagen at different concentration on the resistance of leukemic cells to Doxorubicin principal molecule used in the treatment of acute myeloblastic leukemia, and to TQ.

2. MATERIALS AND METHODS

2.1. Preparation of Collagen

Acid-extracted, non-pepsinized Type I collagen from rat tail tendons was prepared as described by Garnotel (Garnotel and al., 2000). Lyophilized collagen was dissolved in a sterile 18 mM acetic acid solution at a concentration of 3 mg/ml.

2.2. Cell Culture

For 2 D cell culture, collagen was dissolved in a sterile 18 mM acetic acid solution at a concentration of 35µg/ml and seeded in 24-well plates (250µl /well). The wells were dried overnight at room temperature then washed two times with 1mL of DPBS (GIBCO®). HL-60 cells were seeded at 10^5 cells/well and were pre-activated or not for 4 h with Collagen, after which they were treated or not for 16 h with Doxorubicin and TQ.

2.3. Cytotoxicity Assay

HL-60 cells were treated or not with a concentration range of TQ for 24 h in the presence or not of collagen. TQ cytotoxicity was compared with that of Doxorubicin (Dox). TQ and Dox cytotoxicity's was evaluated with counting using KOVA Glasstic Slide and phase contrast microscopy. The results were analyzed with Graph Pad Prism 6software.

2.4. Apoptosis

HL-60 cells were seeded at 10 cells/well for 24h in the presence or not of collagen and treated or not with 200 nM of Dox or 10 µM of TQ. After incubation, apoptosis was determined using Annex V and Dead Cell Assay kit (Millipore) and Caspase 3/7 Assay kit (Millipore).

2.5. Annexin V and Dead Cell Assay

For 100 µl of cell suspension (2.10^4 to 10^5 cells), we add 100 µL of the Muse™ Annex V and Dead Cell Reagent. The suspension was vortexed and incubated for 20 minutes at room temperature. Apoptosis was analyzed on a Muse™ Cell Analyzer (Millipore).

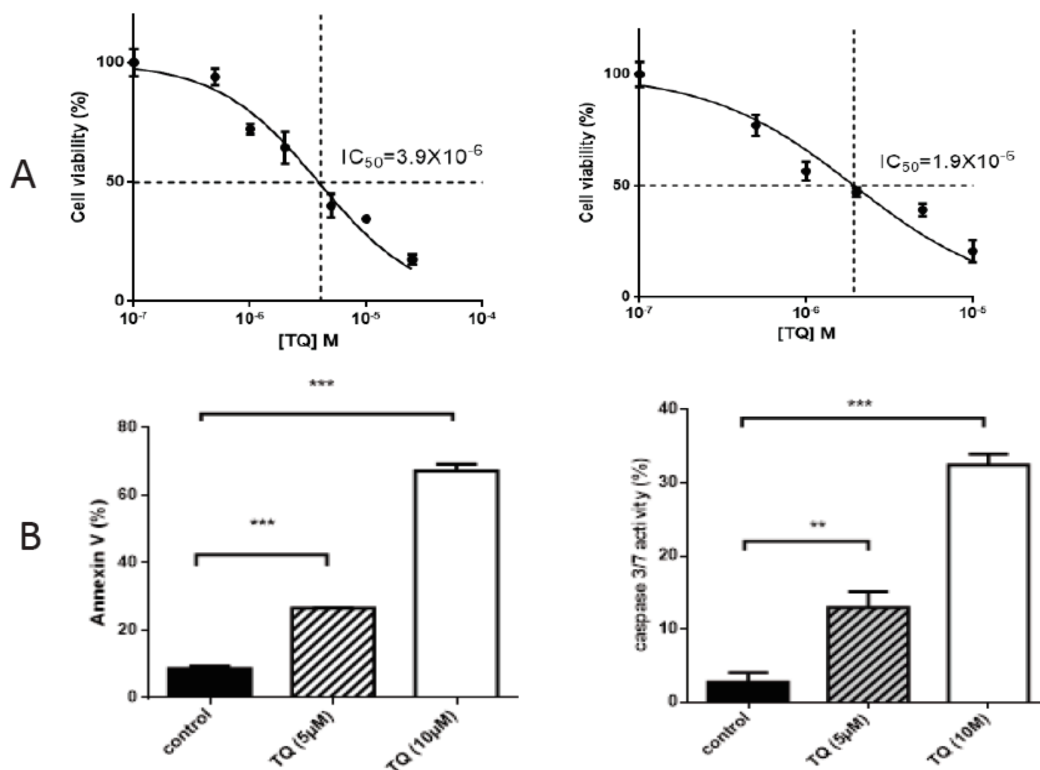
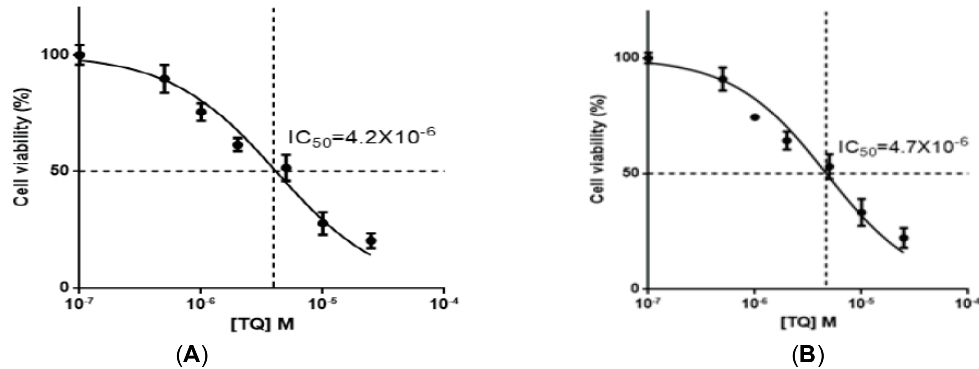


Figure 1: Effect of TQ in HL-60 cytotoxicity and apoptosis. (**A**) Determination of TQ IC_{50} in HL-60 cells. (**B**) The effect of TQ on phosphatidyl-serine externalization and caspase 3/7 activity following treatment of cells with TQ.



(**A**) (**B**)

Figure 2: Effect of Type I collagenon TQ cytotoxicity in HL-60 cells. (**A**) Determination of TQ IC_{50} in HL-60 cells on plastic support. (**B**) Determination of TQ IC_{50} in HL-60 cells in the presence of Type I collagen (coating 2D).

2.6. Caspase 3/7 Analysis

Cells were treated as described above. Then, samples were stained using the Muse™ Caspase 3/7 Assay kit (Millipore), according to manufacturer's instructions. Data were acquired on the Muse™ Cell Analyzer (Millipore).

2.7. Statistical Analysis

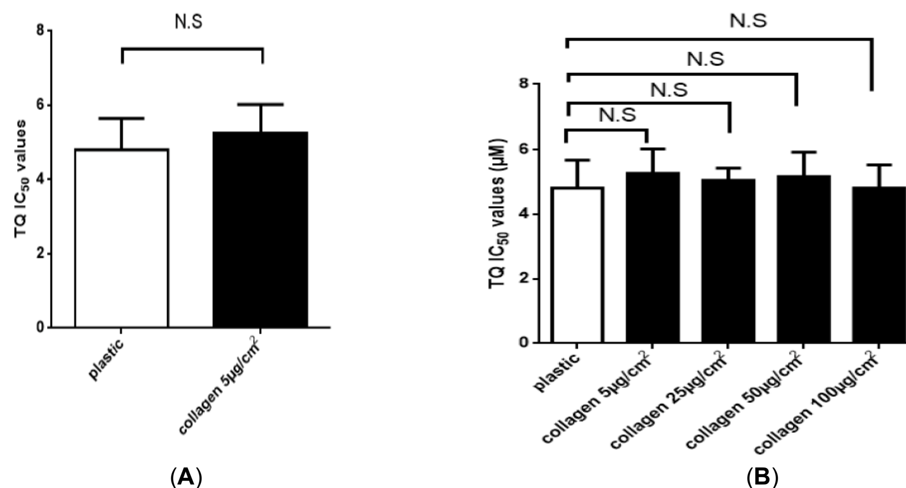
Results were analyzed and illustrated with GraphPad Prism (version 5; GraphPad Software). Statistical analyses were performed using one and two-way ANOVA. *p* value of 0.05 was considered statistically significant.

3. RESULTS

The data indicate that treatment of cells with different concentrations of TQ resulted in significant inhibition of cell viability as compared to controls (Figure **1**). TQ inhibited the growth of HL-60 in a dose and time-dependent manner. The IC_{50} values after 24 h and 48 h of TQ incubation were 3.9 μM and 1.9 μM respectively. Overall, the IC_{50} value of TQ was found to be <50 mM, suggesting that TQ could exert strong anti-proliferative effect on HL-60 cells at low doses.

To determine if the decrease in proliferation and viability were a result of TQ induced apoptosis in HL-60 cell line, cells were stained with Annexin V, a Phospholipid-binding protein that detects translocation of phosphatidyl-serine to the outer cytoplasmic membrane, an event which takes place during the early stages of apoptosis. Activation of apoptosis was also represented by activity of caspase 3/7. As shown in (Figure **1**), we observed a dose-dependent increase in of the percentage of apoptotic cells following treatment with 5 and 10 μM of TQ for 24h.

The cytotoxicity was evaluated in the absence and the presence of collagen I (5μg/cm^2). As shown in (Figure **2**), no statistically significant differences were observed with or without the collagen. The IC_{50} values were respectively 4.7.10^{-6} and 4.2.10^{-6}μM.



(**A**) (**B**)

Figure 3: Collagen density and cytotoxicity of TQ. (**A**) Comparison of TQ IC_{50} values on plastic and in the presence of 5μg/cm^2 of collagen type I. (**B**) Comparison of TQ IC_{50} values on plastic and in the presence of different density of collagen type I.

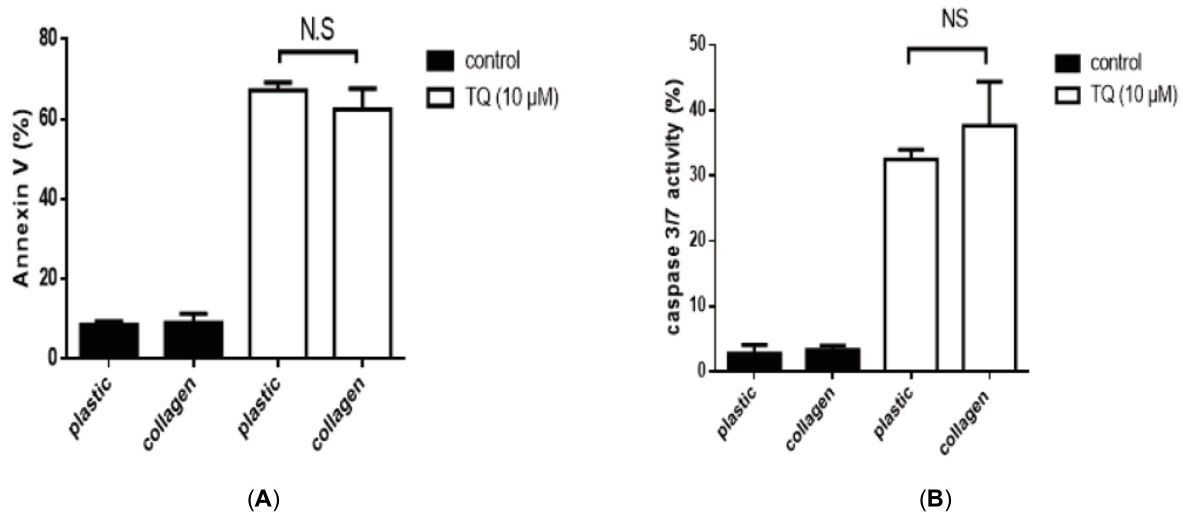


Figure 4: Annexin V and caspase 3/7 activity in HL-60 cells treated with TQ in the absence and presence of collagen type I. (**A**) Comparison of phosphatidyl-serine externalization on plastic support and on coating 2D. (**B**) Comparison caspase 3/7 activity on plastic support and on coating 2D.

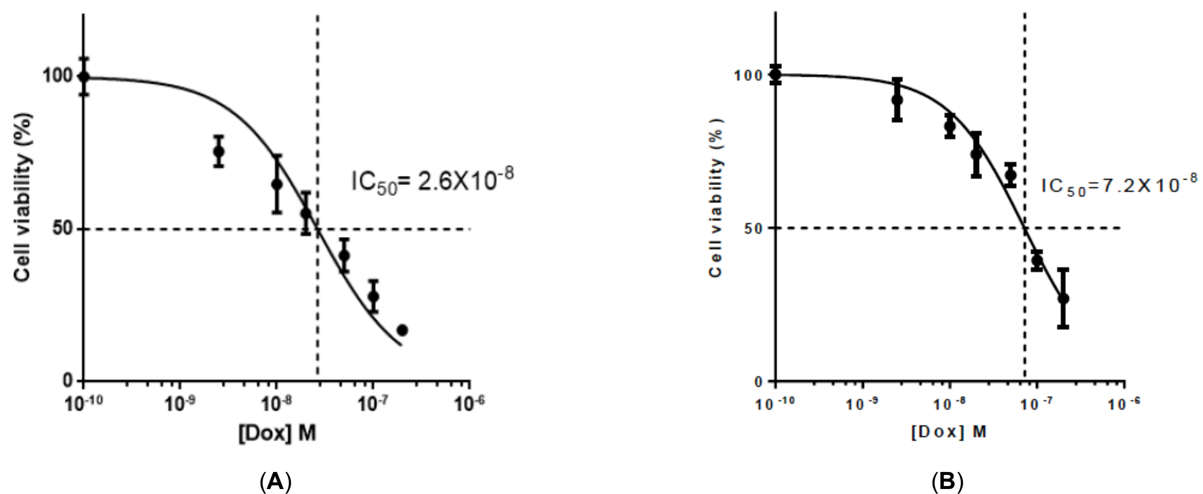


Figure 5: Effect of Type I collagenon Dox cytotoxicity in HL-60 cell line. (**A**) Determination of TQ IC_{50} on plastic support. (**B**) Determination of TQ IC_{50} in HL-60 cells in the presence of collagen type I.

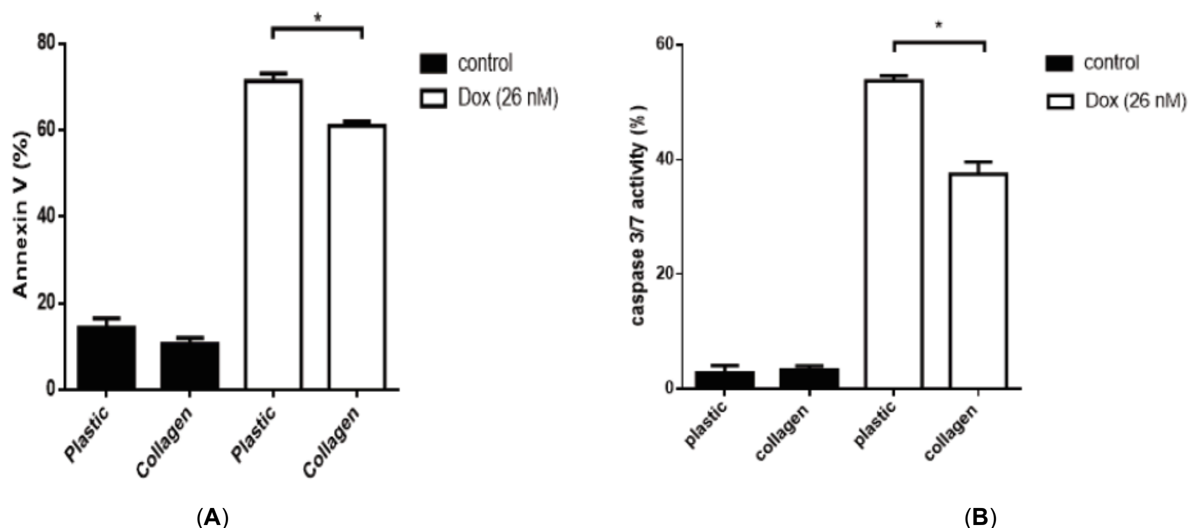
Then we were interested in the effect of collagen density on TQ cytotoxicity. For this, we treated cells with different density of collagen 25, 50 and 100 µg /cm^2. No differences were observed in the other treated groups compared to the plastic. (Figure **3**).

To confirm our results, apoptosis was detected by Annex in V/PI staining and caspase 3/7 activity. As illustrated in (Figure **4**), treatment of HL-60 cell line with 10 µM of TQ for 24h showed no differences on apoptosis rates in presence or absence of collagen I. These results demonstrated that the collagen I has no protected effect against the cytotoxic effect of TQ.

To evaluate the effect of collagen on Dox cytotoxicity, we defined the IC_{50}. HL-60 cells were treated with different concentrations of Dox (2, 10, 20, 50, 100 and 200 nM) in absence and presence of collagen I for 24h. Our data showed that at 100µg/cm^2 density, Type I collagen has a protected effect against the Dox. The IC_{50} values were $2.6.10^{-8}$ and $7.2.10^{-8}$respectively in absence and presence of collagen. These results reflected an increase by 2.76 of the IC_{50} in the presence of collagen I. (Figure **5**).

To define if the cytotoxicity of the Dox is associated to the apoptosis, we analyzed apoptosis by annex in V and caspase 3/7 activity.

Our data showed that in presence of collagen type I, the percentage of cells annexin V positive decrease.



(**A**) (**B**)

Figure 6: Annexin V and caspase 3/7 activity in HL-60 cells treated with Dox in the absence and presence of collagen type I. (**A**) Comparison of phosphatidyl-serine externalization on plastic support and on coating 2D. (**B**) Comparison caspase 3/7 activity on plastic support and on coating 2D.

Also our results demonstrate 3/7 activity decrease in the presence of collagen (Figure **6**).

These results showed that collagen is involved in apoptosis resistance.

4. DISCUSSION

The extracts of healing plants are important source of molecules pharmacologically bioactive and of their green syntheses. Nigella Sativa is an annual herbaceous, found in northern Africa and in southern Europe. The major of its active component is TQ (2-isopropyl-5-methyl-1,4-benzoquinone [Ghosheb OA and al., 1999]. Since 1960, many researchers have reported both *in vivo* and *in vitro* models, the benefits of the TQ's bioactivities as they are specially an anti-oxidative / anti-inflammatory and anti-proliferative in many types of cancer, such as colorectal cancer, breast cancer and leukemia. That, through different mechanisms, including anti-proliferation, apoptosis induction, cell cycle arrest, ROS generation and anti-metastasis / anti-angiogenesis. [Woo CC and al., 2011; Zubair H and al. 2013].

In the present study, we evaluated the effect of TQ on the proliferation of HL60 in culture with and without collagen. Our data showed that TQ induces HL60 cytotoxicity dose and time-dependent. In fact, IC_{50} were 3.9 µM and 1.9 µM of TQ respectively after 24, 48hours of incubation (figure **1**). Overall, the IC_{50} value of TQ was found to be <50 mM, suggesting that TQ could exert strong anti-proliferative effect on HL-60 cells at low doses. [M.N. Norfazlina and al., 2013].

Several studies confirm the variability of the TQ cytotoxicity; it is time and dose dependent, but they controvert for the dose of IC_{50}. In fact, Latifah Saiful reported the IC_{50} is 19.48µM (24h) for HL60, which is 23 µM and 18 µM following respectively 24 and 72 hours of treatment for Mohamed Amine [Mohamed Amine El Azreq and al., 2012; Ismail Norsharina *et al*., 2011; M.N. Norfazlina *et al*., 2013]. IC_{50} is cancer type depend too, the treatment of the cell lines PA-1, A172 and H460 with TQ shown IC_{50} respectively 4.35µM ± 0.63, 18.41µM ± 1.70, 40.1µM ± 2.04 but cell line A549 had a larger IC50 256 µM D4 D5 D4and breast 20 µM Target apoptotic effects of TQ. The lower IC_{50} is an advent to use TQ without acute toxicity [M.N. Norfazlina and al., 2013; Banerjee S *et al*., 2009; Brown RK *et al*., 2014].

TQ exhibited its anti-proliferative activity in many types of cancer; human breast cancer and human ovarian adenocarcinoma, colon cancer cell line HCT-116 colon, small and none small-cell lung cancer and leukemic cell lines, without any toxicity to normal kidney cells or normal human pancreatic ductal epithelial cells [Shashi Rajput and al., 2013; B. N Ismail Norsharina and al., 2011; Banerjee S and al., 2005]. On the other hand, it's showing the beneficial effect to combine TQ at the Doxorubicin that reduce its chemoresistance and the cardiac toxicity [Banerjee S and al., 2009; Brown RK and al., 2014; Jafri H and al., 2010; Katharina and al., 2010].

The major problem for the clinician is the chemoresistance, which shows a risk relative of relapse in the most resistant group that was 9.8 times

higher than in the most sensitive group. [Dehghani H and al., 2015].

Many studies proved the microenvironment role in the mechanism of chemoresistance especially in the solid cancer, but only recently have researchers focused on it for blood cancers. The targeting of the cancer cells is to create an environment more suitable for its progress that by different way for example soluble factors and cell- cell contact [Emyr Bakkar and al., 2016].

Dalila Nacihas shown the role of the collagen, major component of the extracellular matrix of the medullary micro-environment, in the chemoresistance of acute leukemia [Naci D and al, 2012. Collagen is the ligand of integrin, a major receptor which mediate cell-cell adhesion and cell adhesion to the surrounding extracellular matrix, can protect leukemia cells by increasing drug resistance [Ganapathi R and al, 1996]. Our results show the IC_{50} of HL-60 line incubated with TQ is 4.3 10^{6} and 4.7$10^{-6}$ respectively in the absence and the presence of collagen I (5μg/cm²). Also, our results show the collagen density 25, 50 and 100 μg /cm^2 have not effect on TQ cytotoxicity; but for the Doxorubicin, collagen density 100 μg/cm^2 decreased 2.76 fold its cytotoxicity. In fact, the viability cell pass from 2.6 10^{-8} in absence of collagen to 7.2$10^{-8}$ in presence 100 μg/cm2 collagen density (Figure **5**). The mechanism of leukemia cell resistance is not elucidated; different pathways can play a major role. The interaction between leukemia and micro-environment depending on the type of leukemia [Naci D and al, 2014]. ElAzraq and al report that collagen integrin decreased Doxorubicin efflux from inside by up regulation of ATP-binding cassette C1(ABCC1) expression and activity which correlated with reduction intracellular Doxorubicin level's in the human T-AL cell line (Jurkat). Collagen decrease Doxorubicin apoptosis by reduction of its intracellular content [Mohamed Amine El Azraq and al., 2012]. Collagen, fibronectin, laminin are a major component of extracellular matrix and integrin ligand can decrease drug apoptosis and induce the resistance of HL-60 cell line [Emyr Bakker and al., 2016]. Our study compares the collagen effect of Doxorubicin apoptosis to TQ apoptosis in HL-60 cell line, treated with 10 μM of TQ and with DOX for 24h on presence 100 μg/cm^2 of collagen (Figure **6**). Our results show that no differences on TQ apoptosis rates in presence or absence of collagen I (figure **5**) In contrast for the Dox collagen involved in apoptosis resistance. Type I collagenis a major matrix in the bone marrow micro-environment. However, its role is not elucidated in the hematological malignancies. Collagen binding integrin can protect cancer cell from drug induced apoptosis with different mechanisms type cancer and drug dependent [Ganapathi R and al., 1996]. In fact, leukemia cell adherent to the matrix by interaction to laminin, fibronectin and collagen are protected by inhibition of the apoptosis induced by Doxorubicin and a chemotherapy drug in therapy cancer. Collagen active the ERK localized in cell T cytoplasm by phosphorylation. The complex MAPK/ERK restored Mcl-1 and active p38 MAPK pathway. P38 can mediate B cell leukemia survival by anti-apoptotic function. [Dalila Naci and al. 2012, Naci D, Aoudjit F, 2014; Ganapathi R and al., 1996].

Matsunaga and al demonstrated that the adhesion leukemia cell to osteoblasts through integrin induced secretion Wnt antagonists which block the Wnt pathway and decreased the chemo-sensitivity of acute myeloid leukemia, in another hand involved the activation of a cell survival pathway controlled by GSK3β/NF-kB. [Matsunaga and al., 2003; De Toni F. and al., 2006].

The intracellular efflux of the TQ, which is a major compound of Nigella Sativa, is solvent dependent [Lalitha Priyank D and al. 2012; Salim LZ and al., 2013]. It's induced HL60 cell death through different pathways about 4% by cytotoxicity effect such as necrotic cell and 90% apoptotic effects [M.N. Norfazlina and al., 2013; Slim LZ and al., 2013].

In fact, our result shown the HL60 cell proliferation is time incubation dependent with TQ and DOX, the cell viability increased with collagen level in presence Dox but not change in presence TQ. We don't noted difference significantly of the caspase 3/ 7 level, marker of the apoptosis event, in presence and in absence of collagen for the TQ. TQ is able to increase intrinsic reactive oxygen species (ROS) generation. The generation of ROS is associated with mitochondrial proteins such as Bcl-2 and Bax. These proteins can play a major role in apoptosis. TQ act as pro-oxidant, it is able to breakage cellular DNA, and regulate apoptosis by different pathways. It active caspase-3, -6, and -7, they coordinate in the execution phase of apoptosis by cleaving multiple structural and repair proteins [Zubair H and al., 2013; Slim LZ and al., 2013].

TQ up-regulated phosphorylation of phosphatidylinositol 3-kinase/Akt (PI3K/Akt) and mitogen-activated protein kinases ([MAPKs] p38kinase, ERK-1/-2, and JNKinase [Naci D and al., 2014].

CONCLUSION

The findings of our research are quite convincing. It shown that TQ, bioactive molecular for *Nigella Sativa,* can decrease the HL60 resistance in presence of Collagen Type I. Our study supports the searches which recommend combining TQ at Doxorubicin and the use of equimolecular mixture drug increase fourfold Doxorubicin effect on Leukemia cells. Also the combination of TQ and synthetic drug or bioactive molecule can reduce the chemoresistance and limit drug toxicity.

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CONFLICT OF INTEREST

None.

DISCLOSURE STATEMENT

The authors have nothing to disclose.

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