



CYTOTOXIC ACTIVITY OF COMPOUNDED ANTHRACYCLINE AGAINST RHABDOMYOSARCOMA CANCER CELL LINE

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Abstract

High selectivity of Cytotoxic Activity of Compounded Anthracycline against Rhabdomyosarcoma Cancer Cell Line that effect of DMT between cancer cell and normal cell line increase the prospects that this compound could serve as leads for novel anticancer drugs and hence may be potential candidates for use in human cancer therapy, however, further investigations will be required in this regard to validate this hypothesis.

Key words : Cytotoxic activity, anthracycline, human cancer therapy, anticancer drugs.

Introduction

Rhabdomyosarcoma (RMS) is a malignant tumor of mesenchymal origin; it is the most common pediatric soft tissue sarcoma, accounting for approximately 5% of childhood cancers (Alvarez-Allende and Dasgupta, 2016), RMS belongs to the group of small round blue cell tumors displaying different degrees of striated muscle differentiation; this type can be found virtually at any site in the body, including sites where striated muscle is not found normally .the peak incidence is seen early in childhood with a median age of about 5 years at diagnosis, (Hajdin, 2008; Kashi *et al.*, 2015).

In Iraq, the range is nearly 3% of childhood cancer cases in less than 14 years of age in 2010 (Hadad *et al.*, 2011). Al-Niimi (2006) demonstrated that the incidence of RD in north of Iraq is about 0.8%, whereas in Mosul it is about 2.4% (Al-Kzayer, 2010) and in Basrah RD constitutes about 5.94% of cancer (Mahdi *et al.*, 2007).

doxorubicin (DOX) is a potent anthracycline antibiotic; it is a widely used drug to treat a variety of human malignancies, but its cardiotoxicity has long been recognized as a complicating factor; in spite of its known efficacy in RMS therapy, because of cardiotoxicity, its

use is not justified in low-risk patients who have an excellent chance of cure with vincristine, actinomycin with or without cyclophosphamide, and primary tumor treatment (Arndt, 2012; Ejam, 2016). Another drug used in this study is Methotrexate (MTX), which is another effective and extensively used chemotherapeutic agent to treat range of malignancies, but its therapeutic use is limited because of dose dependent hepatotoxicity influence and it can induced acute liver injury (Al-Fatlawi and Al-Shammari, 2017).

The part of doxorubicin in treatment of rhabdomyosarcoma (RMS) has been questionable for a long time. In spite of its known movement in RMS, in light of its danger of cardiotoxicity, its utilization is not legitimized in generally safe patients who have a magnificent shot of cure with vincristine, actinomycin with or without cyclophosphamide, and essential tumor treatment.

Materials and Methods

Chemicals and reagents

Methotrexate, doxorubicin hydrochloride and penicillin/streptomycin purchased from Sigma Aldrich (Gillingham, UK). DMEM and RPMI 1640 media (GIBCO, USA), supplemented with 10% heat inactivated

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foetal bovine serum (Capricorn Scientific, Germany). MTT (3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium bromide) purchased from Bioworld (USA). All other reagents were of analytical grade and used as received.

Synthesis of target compound

The target compound was prepared by Fischer-Speier esterification (Otera and Nishikido, 2009) by mixed 0.5 g of doxorubicin (2 mmol) with methotrexate (25 mL) then 0.412 g of N,N-dicyclohexylcarbodiimide (DCC) (2 mmol) were added with continuous stirring on a magnetic stirrer. The reaction mixture is stirred at 70°C for 6 hours.

Chemical analysis of drug

HPLC analysis

The analysis depends on union method and parameter (Kadhim, 2014) in detection of drugs DOX, MTX and MDT Agilent T1 of column C18 Type of column C18 Agilent (4 μ m, 3.9 \times 150 mm), Injection volume 20 μ l, Detector UV-visible.

Fourier transform infrared spectrophotometer (FTIR)

DOX, MTX and the synthesized compound (DMT) are milled with potassium bromide (KBr) to form a fine powder, this powder was compressed into a thin pallet and analyzed directly; the FTIR spectrophotometer (Shimadzu, FT-IR-8400S, Japan) was used to determine the functional group present in the drugs, each sample was run at infrared region between 400 and 4000 nm to analysis the functional groups (Hussein *et al.*, 2016).

Dose response curve

Dose response curve was performed to observe the cytotoxic activities of designed drug DMT, in addition to DOX, MTX and (DOX+ MTX) mixture, in cancer RD cell line and normal REF cell line by MTT assay as following several doses of drugs were prepared in concentrations (0.5, 2.5, 5, 10, 20, 30, 40, 50) μ g/mL in (DMSO + media) or just media.

In Briefly, 100 μ l cell suspension was added to the flat-bottomed micro-culture plate wells, separated plate for each cell line in triplicate and treated them with 100 μ l DOX, MTX and DMT extract, incubated for 24 h, 10 μ l of 5 mg/ml MTT in PBS solution was added to the wells and the cells were incubated for another 4 hr. at 37°C, dead cells and extra MTT were removed, (200 μ l) DMSO : isoprpanol (1:1) was added immediately to each well and shaken for 5 min. (The DMSO solution became purple). The optical density of each sample was recorded at wavelength of 490 in an ELISA reader. The inhibition

% of cells after the specified time were detected and the concentration that inhibit 50% of cell viability (IC_{50}) was fitted and calculated inhibition percentage was calculated using formula :

$$\text{Inhibition percentage (GI \%)} = [(A - A_1) \div A] \times 100.$$

Where, A = absorbance of untreated samples, A1 = absorbance of the treated test/standard.

The Interaction effect (IAI) of DOX, and MTX on RD cell line at IC_{50} values were compare in DMT as a compounded form and in (DOX + MTX) mixture as combined form by calculated as follows :

$$IAI = \frac{D1}{D_{y,1}} + \frac{D2}{D_{y,2}}$$

$$\left\{ \begin{array}{l} \leq 1, \text{ synergistic.} \\ = 1, \text{ additive} \quad \dots (\text{Tong } et al., 2015). \\ > 1, \text{ antagonistic.} \end{array} \right.$$

The selectivity index (SI) was determined by ratio between IC_{50} of each drug in normal REF cell line and IC_{50} of the same drug in cancerous cell line RD. SI value indicates selectivity of the drug to the cell lines tested. Drugs with an SI greater than 2 are considered to have a high selectivity towards cancerous cells (Badisa *et al.*, 2009).

Statistical analysis (Mean, SE and one way Anova) were performed using IBM SPSS statistics 21.0. In all tests, P value of significant was less than 0.05 halve inhibitory concentration was fitted by blotting of inhibition percentage versus log of concentration of any compound used.

Results

The result of Fisher esterification reaction between DOX and MTX in the presence of an acid catalysts N, N'-Dicyclohexylcarbodiimide (DCC) was formation powder compound named (DMT) the HPLC for DOX, MTX and DMT by (C18) column using a mobile-phase B in mobile-phase A with low organic mobile-phase concentration (10%) showed that retention time for DOX, MTX and DMT as (1.916, 0.806, 6.644) respectively and their areas were (425.51, 1261.72, 2237.48) respectively (fig. 1) and (table 1). The absorbance band of FTIR spectrum for DMT is shown in fig. 2. 1726 cm^{-1} was attributed to C = O stretching from esters.

Fig. 3 showed a significant decrease ($P \leq 0.05$) of growth after exposure to DMSO (in concentration of

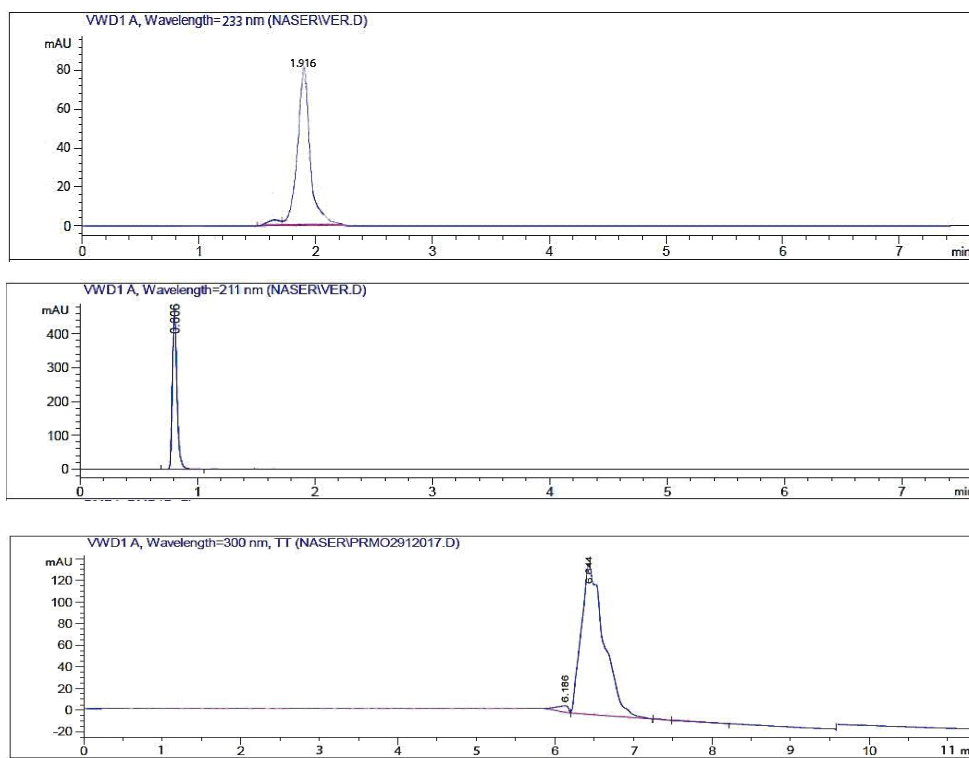


Fig. 1 : HPLC chromatogram of A: DOX, B: MTX and C: DMT solutions.

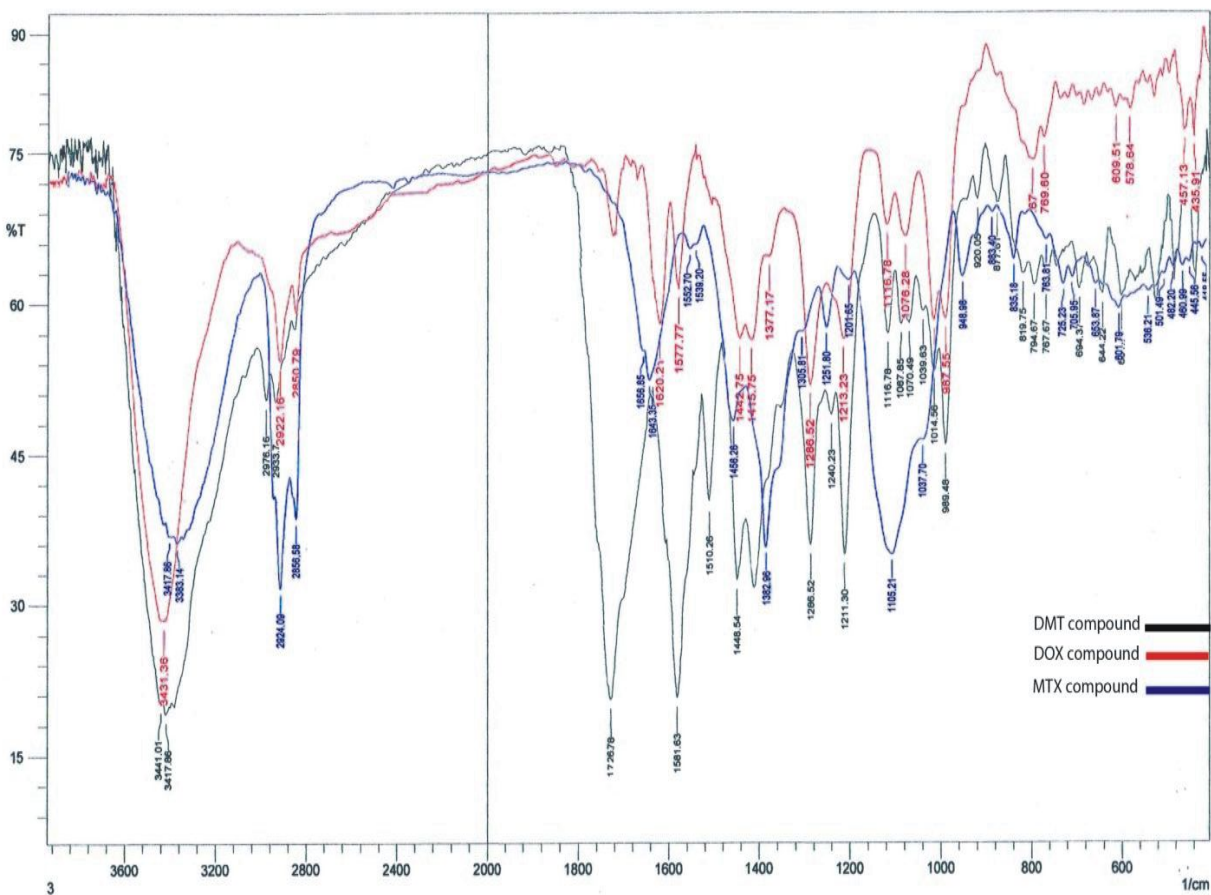


Fig. 2 : FTIR spectra of DOX —, MTX — and DMT —.

Table 1 : Area and retention time of compounds.

No.	Drug	Retention time	Area.
1.	DOX	1.916	425.51
2.	MTX	0.806	1261.72
3.	DMT	6.644	2237.48

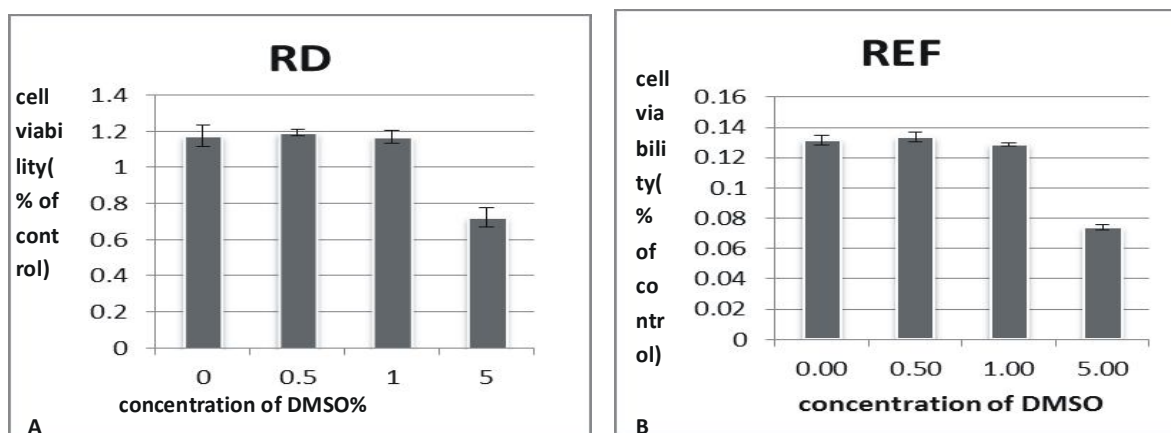
Table 2 : Cytotoxicity of DOX, MTX, DMT and (DOX+MTX) mixture.

Drug/Compound	RDIC ₅₀ ($\mu\text{g/mL}$)	REFIC ₅₀ ($\mu\text{g/mL}$)	SI	IAI
DOX	1.52	12.63	8.31	-
MTX	4.88	13.51	2.77	-
DMT	1.85	15.01	8.11	1.34
DOX & MTX	2.58	-	-	0.967

IC₅₀: Half-maximal inhibitory concentration, SI: Selectivity index, IAI: Interaction index.

Interestingly, the inhibition of proliferation of normal REF cells by the drugs DOX, MTX, DMT after 24 h of incubation were less than RD cells (IC₅₀ values).

The results in table 2 indicated that the combination of DOX and MTX exhibited synergistic effects (closely additive) in DMT and antagonism effects (middle antagonist) in (DOX+MTX) in RD cell line. The result showed that DOX, MTX and DMT has significant selectivity indices (SI > 2). However, the SI for the DOX and DMT, Results in (fig. 5), showed that the cells filled up the wounded area semi-completely in the control sample. On the contrary, in the RD treated samples, the area of the wounded region differed depending on the type of drugs. Interestingly, all drugs had a significant effect (P ≤ 0.05) on slowing wound healing as compared to control, and inhibited the cells from migrating towards the center, the RD cells were very sensitive to MTX,

**Fig. 3 :** Effects of vehicle (DMSO) on survival and viability of A: RD cells and B: REF cells in culture.

>1) for 24 h in RD and REF cells. A concentration of ≤ 0.1% DMSO did not show effect in cell viability after 24h.

The results of dose response curve indicated inhibitory activity with highly significant differences between control of RD (untreated) as compared with those treated with different concentrations of each compound (P ≤ 0.05). DOX was the most highly toxic compound to the RD cells as shown by nearly complete cell death at the 50 $\mu\text{g/mL}$ dose after 24 hr and its IC₅₀ about 1.52 $\mu\text{g/mL}$ (fig. 4), the MTX was not effective in producing RD complete cell death at higher concentration used at 50 $\mu\text{g/mL}$ dose over 24 hr. Results indicated that the incubation of RD cells with DMT showed a reduction in cell viability in a dose-dependent pattern by which the cell viability decreased by increasing the concentration of the compound. The lowest RD cell viability was recorded at concentration of 50 $\mu\text{g/mL}$ and its IC₅₀ value = 1.85 $\mu\text{g/mL}$.

within a 8.9%, then DOX within 16.86% and DMT within 27.09%, respectively.

Discussion

The therapeutic activity of DOX was achieved through the processes of intercalating into DNA, inhibiting topoisomerase II and preventing DNA and RNA synthesis (Pommier *et al.*, 2010). Sensitivity of RD cells to MTX may be dependent on the time of exposure not on concentration and this agreement with the concept of Powis, (1985), which assumed that anti-folates display a time rather than a concentration-dependent antiproliferative effect.

Eventually these two drugs have toxic effects in the cells and this agreement with that mentioned by other researchers who found that DOX and MTX may exhibit an interesting profile of anticancer activity against cancerous cell line (Al-Kelaby *et al.*, 2016). The results indicated that the DMT, has superior anti-cancer

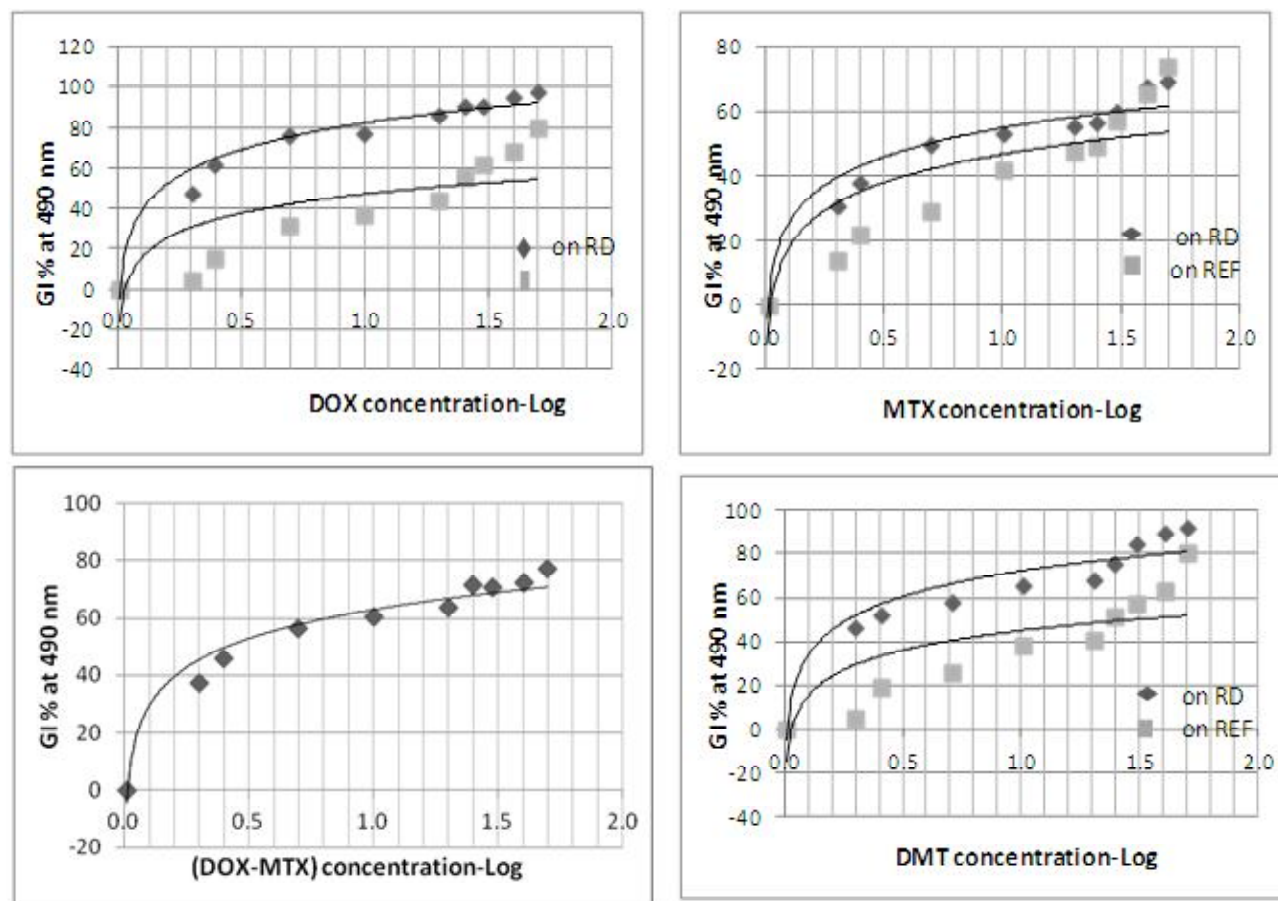


Fig. 4 : Dose-response curve of growth inhibition of; A: DOX; B:MTX; C:DMT; D:(DOX+MTX) mixture in RD and REF cell lines presented by plotting of drug concentration “log” versus GI% values.

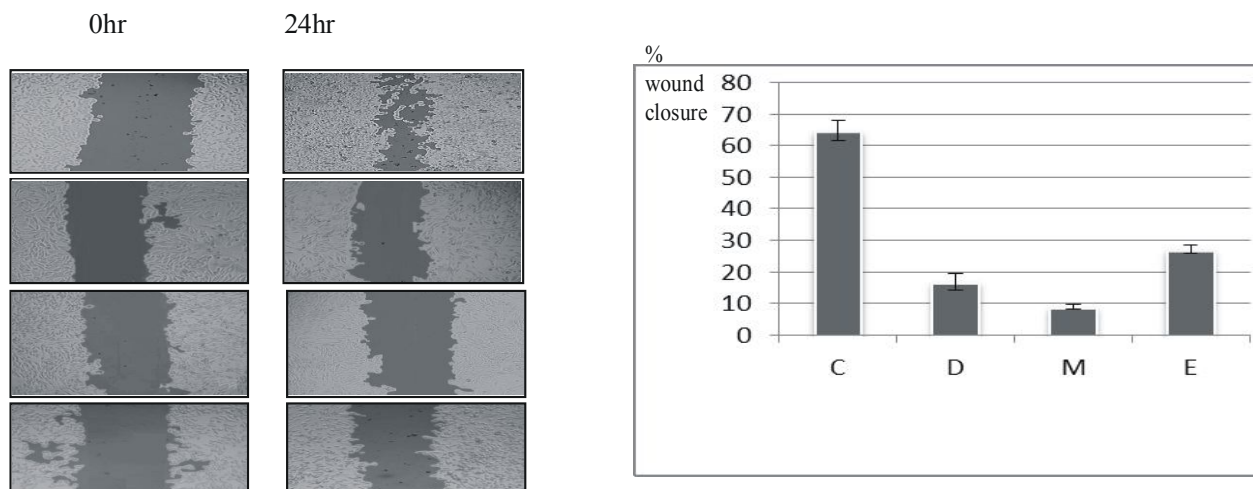


Fig. 5 : A, Photomicrograph of scratch at 0, 24 Scratch wound on RD cells: The wounded area in the control cells (untreated), and (0.25 µg/mL) of DOX, MTX, DMT. (400X). B, wound healing capacity of the RD cells after treatment of DOX, MTX, and DMT for 24 hours. C:control, , D: DOX, M: MTX, E:DMT.

properties when comparing it with MTX and DOX in RD cell line, therefore it can be said that it could be of greater value in the treatment of this cancer. In general, the effect of DMT may be related to its activity which come from the expression of esterase or may be from its

lysis inside the cell after the entrance of cell membrane or may be from its conversation to another substitution of DOX.

High selectivity in cytotoxic effect of DMT between cancer cell and normal cell line indicates that the drugs

are not cytotoxic to the normal cells as cancer cells. It is important for an anticancer agent to exhibit cytotoxicity but such activity should be specific for cancer cells only (Lai *et al.*, 2008). This result increased the prospects that this compound could serve as leads for novel anticancer drugs and hence may be potential candidates for use in human cancer therapy; however, further investigations will be required in this regard to validate this hypothesis. The results revealed a significant decrease in the migration of RD cells after treatment with MTX with high level, this finding was in agreement with the findings of Tomoda *et al.* (2006), who reported that the low-dose of methotrexate significantly inhibited the proliferation of endothelial cells *in vitro* in addition to reduce the number of lung metastatic nodules and the wet weight of the lungs. This observation is correlated with the general use of DOX in treating metastatic in some cancers like breast cancer (Beslija, 2003), but the present observation is not consistent with the findings of Pichot *et al.* (2009), who noted that DOX treatment alone only weakly affected the migration of MDA-MB-231 cells.

Conclusion

Results showed significant decrease in the migration of RD cells after treatment with DMT, these data suggest that an effective inhibited migration of RD cells through its antiangiogenic activity Nevertheless, modification of the concentration of DMT may further improve the actions.

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