IJPSR (2018), Volume 9, Issue 3 (Research Article)

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 30 May, 2017; received in revised form, 13 August, 2017; accepted, 29 August, 2017; published 01 March, 2018

ROLE OF AUTO FLUORESCENCE IN APOPTOSIS ANALYSIS OF MCF-7 AND MDA-MB231 HUMAN BREAST CARCINOMAS TREATED WITH THYMOQUINONE

Amin Soltani [1], Ghadir Tavakoli Hafshejani [2] and Mahdi Ghatrehsamani[*3]

Plant Research Center [1], Department of Immunology [2], Faculty of Medicine, Cellular and Molecular Research Center [3], Shahrekord University of Medical Sciences, Shahrekord, Iran.

Keywords:

Autoflourescence, Thymoquinone, MDA-MB231, MCF-7

Correspondence to Author:
Mahdi Ghatrehsamani

Cellular and Molecular Research Center, Shahrekord University of Medical Sciences, Shahrekord, Iran.

E-mail: ghatreh.m@SKUMS.ac.ir

ABSTRACT: Thymoquinone (TQ) is the major active component of black seed essential oil which possess encouraging effects against cancer cells. The antitumor activities of TQ were verified in breast carcinoma *in-vitro* and *in-vivo*. Most of the times, the cellular responses to apoptosis, after TQ treatment, are evaluate by flow cytometry using annexin V-propidium iodide (PI) staining method. Although flow cytometry has become an important tool to study characteristics of individual cells but induction of auto fluorescence (AF) can decreases the technique sensitivity. In order to investigate the effect of TQ on AF induction, we assessed the fluorescence after TQ treatment. We present evidences suggesting that the change in AF is depend on cell type and TQ concentration. We show here, MDA-MB231 breast cancer cell line treated with different concentration of TQ exhibits elevated level of auto fluorescence (AF) when analyzed by flow cytometry. Consistent with this view, MCF-7 cells did not show any AF upon TQ treatment, in contrast to MDA-MB231. Finally, we presents data demonstrating that changes in AF can affect the flow cytometry results and confound the findings obtained with common dyes using for apoptosis investigation. Conclusively the effect of TQ treatment on AF induction should be considered when we want survey apoptosis effect on breast cancer cells.

INTRODUCTION: Thymoquinone (TQ) is one of the most used herb in folk medicine in the Mediterranean area and West Asia. It have been mainly considered for its anticancer activity. TQ's first isolated from a natural product of the *Nigella sativa* Linn. black seed. *Nigella sativa* is well-known for its healing potential [1]. Scientific research supports the traditional medicine utilization of the TQ as an anti-inflammatory, antimicrobial and anticancer agent both *in-vitro* and *in-vivo* [1].

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.9(3).996-01

Article can be accessed online on: www.ijpsr.com

DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.9(3).996-01

There is powerful evidences proposed that TQ induces apoptosis in many cancer cell lines by different pathways [2 – 4]. Various techniques designed to distinguish, quantitate and describe apoptosis, but flow cytometry remains the approach of choice to investigate the apoptotic cells [5]. With flow cytometry, it is feasible to measure several parameters simultaneously and details about physical and biological properties of single cells in a population are attained [6, 7]. It is an assigned technique for study cell biology [7]. Flow cytometry has the great advantage to analysis cell populations at the single-cell level. It connects direct and quick analysis to determine numbers, cell size distribution, extra biochemical and physiological phenotype of individual cells which, revealing the heterogeneity present in a population [8]. Furthermore, in combination with appropriate

fluorescent dye, flow cytometry affords a powerful and sensitive tool to describe physical and biochemical features of individual cells [9]. Annexin V-FITC/propidium iodide (PI) flow cytometric assay has been thoroughly used for the analysis of apoptosis induction effect of TQ, in various experimental models. In these studies samples were analyzed for green fluorescence (FITC) and for red fluorescence (PI) by flow cytometry [10 – 12].

Flow cytometric analysis of mammalian cells is usually complicated by high proportions of auto flourescence (AF). The AF range of most cell types is typically very vast; the emission ranges from 500 - 700 nm, with a peak emission at 550 nm [13]. Thus the background caused by AF is a typical problem with fluorescein-conjugated probes [14]. These possibility discrepancies point to the need for careful protocol development and choice of proper controls to extract reliable and biologically relevant information from flow cytometry assay [9].

Nonetheless, with TQ treatment the potential of this technology has not been exerted completely. The main problems are AF and the selection of the most suitable fluorescent dyes that had been originally developed for mammalian cells.

In the present study, it was attempted to overcome some of these problem. It became apparent that TQ increase AF under standard conditions in flow cytometric analysis. These background signals need to be taken into account when cell lines are evaluated with fluorescent probes.

MATERIAL AND METHODS: TQ was purchased from Sigma - Aldrich (Germany) and was kept at - 20 ^{o}C as 100 mM stock solutions in DMSO. Appropriate test concentrations were obtained by dilution with cell culture medium immediately before use.

Cell Lines and Culture Conditions: MCF-7 and MDA-MB231 human breast cancer cell lines were purchased from Pasture Institute of Iran. MCF-7 and MDA-MB231 breast cancer cells were maintained in RPMI 1640 (Sigma-Aldrich; USA) supplemented with 10% fetal bovine serum (FBS; Canada), 100 U/ml of penicillin and 100 µg/ml of streptomycin (Invitrogen, Canada) and 2 mM L-glutamine (Sigma-Aldrich). Cells were cultured in 75 cm^2 culture flasks at 37 ^{o}C in a humidified atmosphere of 5 % CO_2. Cells were harvested when they reached about 80 - 85 % confluence using a 0.5 % trypsin - EDTA solution (Sigma-Aldrich) diluted in phosphate buffered solution (PBS) (Sigma - Aldrich).

MTT Assay: Cells were seeded in clear 96-well plates (Sarstedt, Canada) at a density of 10,000 cells/well (MCF-7, MDA-MB-231). After 24 hours, the culture medium was replaced by fresh medium containing different concentrations of TQ or DMSO (solvent). Concentrations ranged from 10 µM to 90 µM. In some of the wells, only culture medium was added.

These wells were used as negative controls and were referred to as untreated control cells. Cell number was evaluated using the MTT cell proliferation assay. The absorbance was measured at 570 nm on a microplate spectrophotometer reader (Benchmark, Bio-Rad, USA). Each compound was evaluated in at least 3 independent experiments with eight replicates in each experiment.

Apoptosis Assay: Mode of cell death induced by the TQ was evaluated by using Annexin V-FITC/PI Apoptosis Detection Kit (BD Biosciences). Annexin V-FITC was used as a marker of phosphatidyl serine exposure and PI as a marker for dead cells. This combination allows differentiation among early apoptotic cells (annexinV-positive, PI-negative), late apoptotic cells (annexin V-positive, PI-positive), necrotic cells (annexin V-negative, PI-positive) and viable cells (annexin V-negative, PI-negative).

Cells were seeded and treated with the indicated compounds or solvent for the cell apoptosis. After 48 h of treatment, the untreated and treated cells were harvested and washed with cold PBS, and aliquots of 5×10^5 cells were washed with PBS and resuspended in 500 µl of binding buffer 1X provided with the kit. A volume of 5 µl of annexin V-FITC and 5 µl of PI were added to 100 µl of cells suspension and cells were incubated at room temperature in the dark for 30 min. Approximately 20,000 cells were analyzed by flow cytometry using a flow cytometer (Partec, Milton Keynes, UK) with a 488 nm blue laser. The percentage of cells in each category was determined. The experiments were performed three times separately.

RESULTS:

Inhibition of Cell Growth: TQ was tested for its growth inhibition in cells of human MDA-MB231and MCF-7 breast carcinoma. The IC50 value after 48h exposure to the test compound was measured. In MDA-MB231 cells TQ was efficacious at IC_{50} (48 h) = 30 µM and 50 µM for MCF-7 cells.

Treatment with TQ Enhanced Auto Fluorescence when Measured by Flow Cytometry: MDA-MB231 treated for 48 h with TQ showed an increased fluorescence compared to untreated cells measured by flow cytometry. To investigate the AF of populations seen after TQ treatment, populations gates (**Fig. 1A**) were set in the FSC-SSC dot plot, and the fluorescence intensity distribution for population was analyzed (**Fig. 1B**). With TQ treatment a bimodal distribution was observed after 48 h in MDA-MB231. While no annexin V-FITC/ PI was used, treated population with 50 µM concentration of TQ showed a higher fluorescence of FITC and PI in compare to the untreated control group (**Fig. 1C**). As seen in **Fig. 1D**, the treated population which stained with annexin V-FITC/PI showed a fluorescence intensity distribution higher than in untreated population.

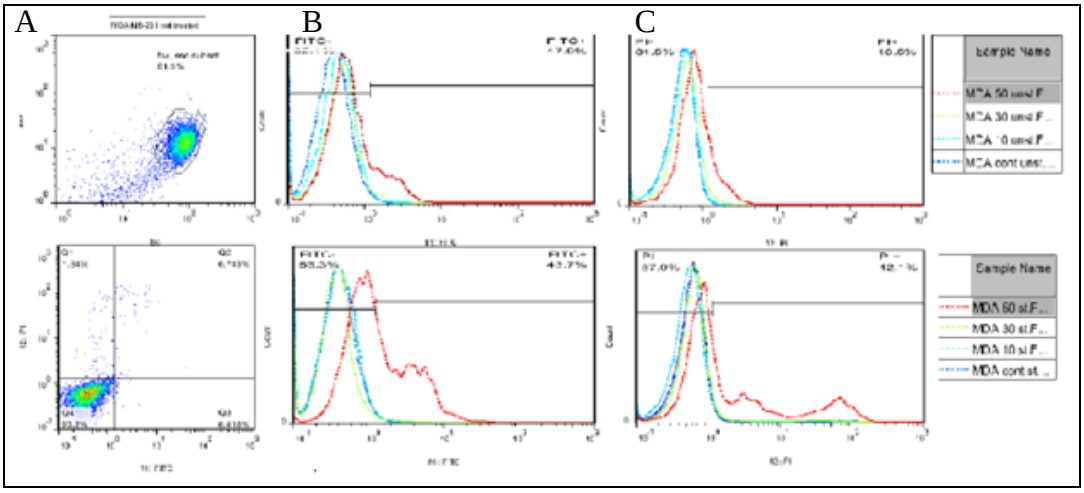

FIG. 1: FLOWCYTOMETRY ANALYSIS OF MDA-MB231 CELLS (A) The population were defined in FSC and SSC of the whole population. (B) Dot plots with of the untreated control. (C) The distribution of auto fluorescence intensity of treated groups are plotted in a histogram and The AF of control and treated cells exposed for 48 h to TQ (10 µM, 30 µM, 50 µM) is compared. (D) MDA-MB231 cells were treated with TQ for 48 h and were stained with annexin V and PI, and analyzed by FACS. The AF of MDA-MB231 increased after 48 h of treatment with TQ, IC_{50} values were 30 µM

MCF-7 Cells Fail to Increase AF upon TQ Treatment: To investigate the AF of TQ on other cell line which, have different biological and molecular characteristics, MCF-7 cell line was used. MCF-7 cell were examined after treatment with TQ and compared to the untreated cells under the same conditions. Surprisingly TQ treated MCF-7 cells show no AF at the same dose or even higher dose of which was used in MDA-MB231 cells (**Fig. 2**). Thus, these data further support the hypothesis that increased AF depend on the drug kinds and cell lines.

Increasing Concentrations of TQ Associated with Elevated Levels of AF in MDA-MB231: To further investigate the other factor that has crucial role in elevating AF, cultures treated with TQ were examined at different concentration. The concentration dependency of the AF was analyzed by exposing MDA-MB231 and MCF-7 to different concentrations of TQ for 48 h (**Fig. 1c** and **2**). TQ were added to MDA-MB231 and MCF-7 cell lines at three different concentrations (IC 50, less than and more than IC_{50}) corresponding to 10, 30 and 50 µM for MDA-MB-231, 30, 50 and 70 µM for MCF-7 cells. In MDA-MB231 the auto fluorescence levels decreased at low concentrations corresponding to 10 µM and increase at high concentration but the AF did not change in various dose schedules in the treatment of MCF-7 cell line. Thus, the results show that the levels in AF observed are both dependent on the concentration treatment drug and cell line.

Induction of Apoptosis by TQ: In order to further determine whether apoptosis responses to TQ differ in breast cancer cell lines, we measured the percentage of early apoptotic cells, late apoptotic and necrotic cells in the sub-G population by flow cytometry.

Fig. 3 shows the results of the annexin V-FITC binding and PI staining on treated and untreated MDA-MB231 and MCF-7, following exposure for 48 h to TQ at three different doses and the percent of cells in the sub-G population is shown.

The treated cells exhibited the higher percentage than the untreated control cells at all doses. As shown in **Fig. 3**, TQ was able to induce apoptosis in a dose-dependent manner in MDA-MB231 and MCF-7 breast cancer cell lines.

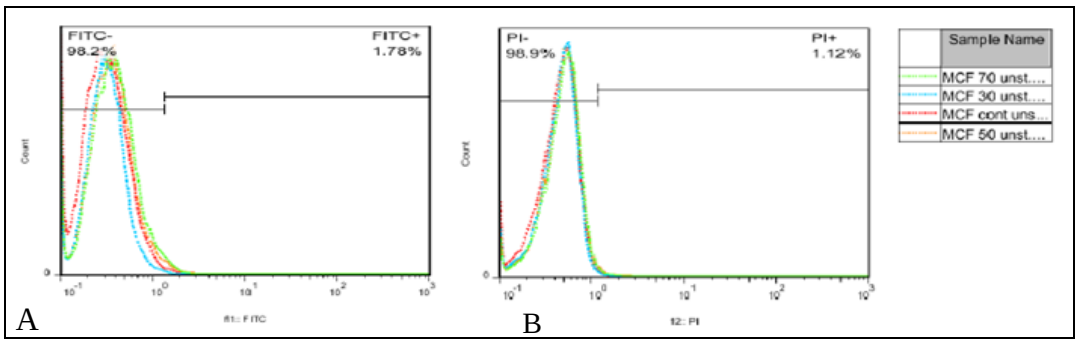

FIG. 2: FLOWCYTOMETRY ANALYSIS OF MCF-7 CELLS (A and B) The distribution of the fluorescence intensity of each cell groups was plotted in a histogram. The overlay of histograms from distinct treated samples is shown. Cell counts are plotted versus fluorescence intensity. MCF-7 cells was exposed to TQ (30 µM, 50 µM, 70 µM). The red line represents a no-drug control. Samples were taken 48 h after the addition of TQ

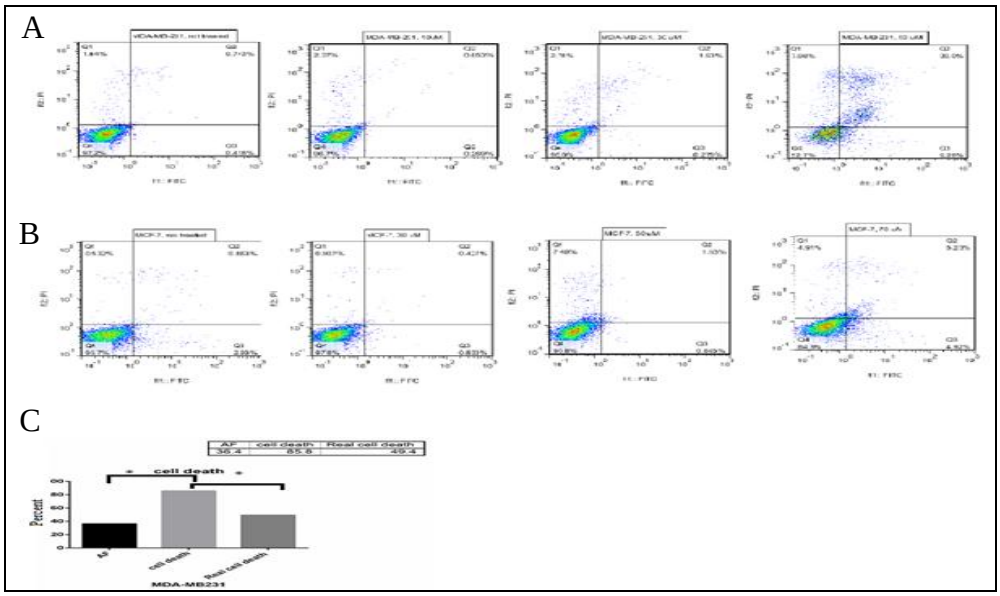

FIG. 3: THYMOQUINONE CONCENTRATION EFFECT ON AUTO FLUORESCENCE MDA-MB231 (A) and MCF-7 cells (B) (1×105) were incubated with indicated concentrations of TQ for 48 h and analyzed for annexin V-FITC/PI staining to determine apoptotic cells. (C) Calculation of real cell death at concentration of 50 µM by subtraction of total AF from total cell death

However, apoptosis induction was more pronounced in MDA-MB231cancer cells than in MCF-7 cell line (at the similar concentration). At the first glance, it seems TQ induce apoptosis in MDA-MB231 cell line more effectively than MCF-7 cell line. But, when we consider the effect of auto fluorescence and compare it with treated groups that stain with annexinV/PI, it seems the results are not true and highly variable in high concentration of TQ treatment (50 µM). Because auto fluorescence was much enhanced in high concentration of TQ, so to obtain the best results we decided to calculate total cell death by summing apoptosis with necrosis percent and subtract it from auto fluorescence percent as a real cell death (**Fig. 3**).

DISCUSSION: The results in this study shows that MDA-MB231 breast cell line treated with the black seed constituent TQ displays higher AF compared to untreated cells when measured by flow cytometry. Furthermore, the AF depends on the dose and cell line. We have found that various tumor cells have different sensitivity to TQ. In addition, TQ treatment caused AF augmentation MDA-MB231 breast cancer cell line after 48 h, without changing cell AF in MCF-7 breast cancer cell line. Also, dose increment of TQ can result in AF augment in MDA-MB231 breast cancer cell line. Quantitative estimation of (and amendment for) AF is an important prerequisite for the precise analysis of fluorescence data. The best method used to correct AF is subtracting the mean of the unlabeled population, from cells labeled with annexin V-FITC/PI.

TQ was found to apply its biological functions by modulating the physiological and biochemical procedures included in ROS formation. TQ acts as a powerful antioxidant and inhibits the production of superoxide radicals or augments the activities of the antioxidant enzymes. In tumors, however, TQ may induces ROS generation and decreases GSH levels in a dose-dependent manner [15]. Thus TQ has a dual role it may act as an antioxidant or a pro-oxidant. TQ reacts readily with amino or thiol groups of amino acids, and undergoes a series of oxido-reduction reactions resulting in the formation of semiquinone. The semiquinone can undergo redox cycling conducting to the oxidative stress and production of reactive oxygen species (ROS) [16]. ROS induced AF in cells has been suggested previously [17]. Although, the mechanisms of the formation of the oxidative stress- induced bright AF remain still unclear.

In this study, induction of AF was more pronounced in the breast cancer cell lines (MDA-MB-231), whereas the AF signal was weak in MCF-7, suggesting that TQ may act selectively by induction of ROS in these breast cancer cells. Similar effects of TQ and on apoptosis induction have also been reported in breast cancer cell lines. MDA-MB231 breast cancer cells showed the greatest sensitivity to TQ. In contrast, MCF-7 breast cancer cells were resistant to concentrations of TQ that were cytotoxic for the other breast cancer cell lines. There are papers reporting that TQ mediates ROS production as a mechanism to induce apoptosis and growth inhibition in various cancer cells including breast cancer [18 – 22]. It was also have shown that TQ significantly induced ROS production in MCF-7 cells as early as 30 minutes , and this induction was time-dependent up to 3 h after TQ treatment [11]. This suggests that hydroxyl radicals might be present upon TQ treatment transiently.

As our analysis suggest that an increase in fluorescence had been observed after treatment with TQ and taken as experimental evidence for the presence of intracellular hydroxyl radicals. This suggests that hydroxyl radicals might be present TQ treatment. However, the main factor that enhance apoptosis upon TQ treatment may be due to AF and TQ was found to apply its biological functions by modulating the physiological and biochemical procedures included in ROS formation. TQ acts as a powerful antioxidant and inhibits the production of superoxide radicals, or augments the activities of the antioxidant enzymes. In tumors, however, TQ induces ROS generation and decreases GSH levels in a dose-dependent manner [20]. Thus TQ has a dual role it may act as an antioxidant or a pro-oxidant. TQ reacts readily with amino or thiol groups of amino acids, and undergoes a series of oxido-reduction reactions resulting in the formation of semiquinone.

The semiquinone can undergo redox cycling conducting to the oxidative stress and production of reactive oxygen species (ROS) [21]. ROS induced AF in cells has been suggested previously [22]. Although, the mechanisms of the formation of the oxidative stress- induced bright AF remain still unclear. But certainly, AF can interfere with specific fluorescent probes in flow cytometric analysis of TQ treatment at doses dependent manner.

CONCLUSION: Our study shows for the first time to our knowledge that, TQ could highly increase AF of MDA-MB231 but not MCF-7 cell line, which cannot be compensate by flow cytometry for both cells. Thus, to gain the best real results, we should decrease the percent of apoptosis from AF or using drug induced cell for unstained sample. Further investigation will be needed to understand the molecular reason for AF in more detail.

ACKNOWLEDGEMENT: This work was supported by the Shahrekord University of Medical Science (Grant numbers 2176).

CONFLICT OF INTEREST: The authors declared no potential conflicts of interest.

REFERENCES:

1. Tavakkoli A, Ahmadi A, Razavi BM and Hosseinzadeh H: Black Seed (*Nigella Sativa*) and its Constituent thymoquinone as an antidote or a protective agent against natural or chemical toxicities. The Iranian Journal of Pharmaceutical Research 2017; 16(0): 2-23.
2. Majdalawieh A and Fayyad M: Anti-cancer properties and mechanisms of action of thymoquinone, the major active ingredient of *Nigella sativa*. Critical Reviews in Food 2017.
3. Kotowski U, Heiduschka G, Kadletz L, Fahim T, Seemann R, Schmid R, Schneider S, Mitterbauer A and Thurnher D: Effect of thymoquinone on head and neck squamous cell carcinoma cells *in-vitro*: Synergism with radiation: Oncology Letters 2017; 14(1): 1147-1151.
4. Hsu H-H, Chen M-C, Day CH, Cecilia Hsuan, Lin YM, Li SY, Tu CC, Padma VV, Shih HN, Kuo WW and Huang CY: Thymoquinone suppresses migration of LoVo human colon cancer cells by reducing prostaglandin E2 induced COX-2 activation. World Journal of Gastroenterology 2017; 23(7): 1171-1179.
5. Kuystermans D, Avesh M and Al-Rubeai M: Online flow cytometry for monitoring apoptosis in mammalian cell cultures as an application for process analytical technology. Cytotechnology 2016; 68(3): 399-408.
6. Tirino V, Desiderio V, Paino F and Papaccio G: Cytometry and pathology. In: Humana Press, New York, NY 2016; 65-84.
7. Adan A, Alizada G, Kiraz Y, Baran Y and Nalbant A: Flow cytometry: basic principles and applications. Critical Reviews in Biotechnology 2017; 37(2): 163-176.
8. Clift MJD, Fytianos K, Vanhecke D, Hočevar S, Petri-Fink A and Rothen-Rutishauser B: A novel technique to determine the cell type specific response within an *in-vitro* co-culture model *via* multi-colour flow cytometry. Scientific Reports 2017; 7(1): 434.
9. Renggli S, Keck W, Jenal U and Ritz D: Role of autofluorescence in flow cytometric analysis of *Escherichia coli* treated with bactericidal antibiotics. Journal of Bacteriology 2013; 195(18): 4067-4073.
10. Motaghed M, Al-Hassan FM and Hamid SS: Cellular responses with thymoquinone treatment in human breast cancer cell line MCF-7. Pharmacognosy Research 2013; 5(3): 200-206.
11. Woo CC, Hsu A, Kumar AP, Sethi G and Tan KHB: Thymoquinone inhibits tumor growth and induces apoptosis in a breast cancer xenograft mouse model: the role of p38 MAPK and ROS. PLoS One 2013; 8(10): e75356.
12. Soltani A, Pourgheysari B, Shirzad H and Sourani Z: Antiproliferative and apoptosis-inducing activities of thymoquinone in lymphoblastic leukemia cell line. Indian Journal of Hematology and Blood Transfusion 2016: 1-9.
13. Liu P, Jing X, Yu F, Lv C, Chen L, Heimann A, Lehr HA, Bhakdi S, Meyer J and Kempski O: A near-infrared fluorescent probe for the selective detection of HNO in living cells and *in-vivo*. Analyst 2015; 140(13): 4576-4583.
14. Erben T, Ossig R, Naim HY and Schnekenburger J: What to do with high autofluorescence background in pancreatic tissues - an efficient Sudan black B quenching method for specific immunofluorescence labelling. Histopathology 2016; 69(3): 406-422.
15. Taha MME, Sheikh BY, Salim LZA, Mohan S, Khan A, Kamalidehghan B, Ahmadipour F and Abdelwahab SI: Thymoquinone induces apoptosis and increase ROS in ovarian cancer cell line. Cellular and Molecular Biology 2016; 62(6): 97-101.
16. Chae IG and Chun KS: Thymoquinone induces apoptosis through inhibition of JAK2/STAT3 signaling *via* production of ROS in human renal cancer Caki cells. Cancer Research 2016; 76.
17. Almshawit H and Macreadie I: Fungicidal effect of thymoquinone involves generation of oxidative stress in *Candida glabrata*. Microbiological Research 2017; 195: 81-88.
18. Ashour AE, Ahmed AF, Kumar A, Zoheir KMA, Aboul-Soud MA, Ahmad SF, Attia SM, Abd-Allah ARA, Cheryan VT and Rishi AK: Thymoquinone inhibits growth of human medulloblastoma cells by inducing oxidative stress and caspase-dependent apoptosis while suppressing NF-κB signaling and IL-8 expression. Molecular and Cellular Biochemistry 2016; 416(1-2): 141-155.
19. Martinovich GG, Martinovich IV, Vcherashniaya AV, Shadyro OI and Cherenkevich SN: Thymoquinone, a biologically active component of *Nigella sativa*, induces mitochondrial production of reactive oxygen species and programmed death of tumor cells. Biophysics 2016; 61(6): 963-970.
20. Dergarabetian EM, Ghattass KI, El-Sitt SB, Al-Mismar RM, El-Baba CO, Itani, WS, Melhem NM, El-Hajj HA, Bazarbachi AAH, Schneider-Stock R and Gali-Muhtasib HU: Thymoquinone induces apoptosis in malignant T-cells *via* generation of ROS. Frontier in Bioscience 2013; 5: 706-719.
    Schneider-Stock R, Fakhoury IH, Zaki AM, El-Baba CO and Gali-Muhtasib HU: Thymoquinone: fifty years of success in the battle against cancer models. Drug Discovery Today 2014; 19(1): 18-30.
21. Miura Y, Huettmann G, Orzekowsky-Schroeder R, Steven P, Szaszák M, Koop N and Brinkmann R: Two-photon microscopy and fluorescence lifetime imaging of retinal pigment epithelial cells under oxidative stress. Investigative Ophthalmology and Visual Science 2013; 54(5): 3366-3377.

How to cite this article:
Soltani A, Hafshejani GT and Ghatrehsamani M: role of auto fluorescence in apoptosis analysis of MCF-7 and MDA-MB231 human breast carcinomas treated with thymoquinone. Int J Pharm Sci Res 2018; 9(3): 996-01.doi: 10.13040/IJPSR.0975-8232.9(3).996-01.

All © 2013 are reserved by International Journal of Pharmaceutical Sciences and Research. This Journal licensed under a Creative Commons Attribution-NonCommercial-ShareAlike 3.0 Unported License.

This article can be downloaded to **ANDROID OS** based mobile. Scan QR Code using Code/Bar Scanner from your mobile. (Scanners are available on Google Playstore)