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Anti-Proliferative Activity of *Althaea Officinalis* Extracts on Iraqi Breast Cancer Cell Line AMJ13

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Abstract

Background Breast cancer has been the highest-ranked malignancy among the Iraqi population since 1986. *Althaea officinalis* is a perennial plant and native to Iraq and Asia, Europe and United States of America.

Objective To evaluate the cytotoxicity of *Althaea officinalis* extracts on AMJ13 breast cancer cell line.

Methods The cytotoxic activity of crude, flavonoids, and phytosterols extracts were tested by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Apoptosis assay was done by Acridine orange–Ethidium bromide dual staining method.

Results The cell line was exposed to serial concentrations of Doxorubicin and extracts from (3.125 to 100 µg/ml) in triplicate of each concentration for 72 hours exposure period. The Inhibitory concentration fifty (IC50) values for Doxorubicin, crude, flavonoids, and phytosterols were as follows: 19.52 µg/ml, 25.18 µg/ml, 39.54 µg/ml, and 47.88 µg/ml, respectively.

Conclusion *Althaea officinalis* extracts have a significant cytotoxic activity and have the ability to cause apoptosis on AMJ13 breast cancer cells. Crude extract have the highest significant cytotoxic activity

Keywords Breast cancer, AMJ13 cell line, *Althaea officinalis*, cytotoxicity assay, apoptosis assay

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List of abbreviations: AMJ13 = Human breast cancer cell line-Iraq, AO/EtBr = Acridine orange–Ethidium bromide, DMSO = Dimethyl Sulphoxide, Dox = Doxorubicin, IC50 = Inhibitory concentration fifty, MCF-7 = Human breast cancer cell line-USA, MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, PBS = Phosphate buffer saline, TNF-α = Tumor necrosis factor alpha, VEGFR = Vascular endothelial growth factor receptor

Introduction

Breast cancer happens when cells in the breast begin growing out of proportion and results in malignant tumor that spreads throughout the body [(1)]. It is highly prevalent in females and accounts for second highest number of deaths worldwide [(2)]. Breast cancer has become a major threat to female health in Iraq, where it is the leading cause of death after cardiovascular diseases among women and it has been the highest-ranked malignancy among the Iraqi population in general since 1986 [(3)]. There are three main options available for the treatment of patients with solid tumors: surgery, radiotherapy and chemotherapy. Each treatment can either be applied alone or in combination, depending on the disease [(4)]. Chemotherapy remains the most effective form of treatment once the tumor has spread [(5)]. Although chemotherapeutic drugs and radiation are more powerful maneuvers for treatment of malignancy, they are associated with serious

adverse effects, and tumor resistance against therapy [6,7]. Plants and plant derived products possess the potential to be excellent lead structures and to serve as a basis of promising therapeutic agents for cancer treatment as these are simple, safer eco-friendly, low-cost, fast, and less toxic as compared with conventional treatment methods [8]. Phytochemicals are selective in their functions and acts specifically on tumor cells without affecting normal cells [7]. *Althaea officinalis* (Khatmi) is a perennial plant and native to Iraq and Asia, Europe and United States of America [9]. It is widely used traditionally for the treatment of the irritation of oral, pharyngeal mucosa and associated dry cough, mild gastritis, skin burns and for insect bites [10]. It is also used in catarrh of the mouth and throat, gastrointestinal tract and urinary tract complains, as well as for inflammation, ulcers, abscesses, burns, constipation and diarrhea [11]. The objective of this study was to investigate the cytotoxic activity of *Althaea officinalis* extracts on AMJ13 breast cancer cell line.

Methods

Cytotoxicity assay

To determine the cytotoxic effect of *Althaea officinalis* crude, flavonoids, and phytosterols compared to Doxorubicin cytotoxicity. The MTT cell viability assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was done using 96-well plates. AMJ13 cells were seeded at 1×10^4 cells/well. After 24 hours or a confluent monolayer was achieved, cells were treated with tested compounds at different concentration. Cell viability was measured after 72 hours of treatment by removing the medium, adding 28 µL of 2 mg/mL solution of MTT and incubating the cells for 2.5 h at 37 °C. After removing the MTT solution, the crystals remaining in the wells were solubilized by the addition of 130 µL of DMSO (Dimethyl Sulphoxide) followed by 37 °C incubation for 15 min with shaking [12]. The absorbency was determined on a microplate reader at 492 nm (test wavelength); the assay was performed in triplicate. The inhibition rate of cell growth (the percentage of cytotoxicity) was calculated as the following equation:

Cytotoxicity = A-B/A *100

Apoptosis assay

The induced death of (AMJ13) cells was performed using Acridine Orange-Ethidium bromide (AO/EtBr) dual staining method [13]. Briefly, the cells in 96-well plates were treated with Doxorubicin, *Althaea officinalis* crude, flavonoids, and phytosterols and incubated for 16 h. The cells were detached and washed twice using PBS (Phosphate buffer saline), and transferred to a clear 96-well plate. Dual fluorescent dyes (10 µL) were added into the cells at equal volumes. Finally, the cells were visualized under fluorescence microscopy.

Results

Cytotoxic activity of Althaea Officinalis crude, flavonoids, and phytosterols fractions on AMJ13 cell line

The study was done on (AMJ13) cell line. The effect of different concentrations of crude, flavonoids, and phytosterols fractions of *Althaea officinalis* and Doxorubicin from (6.25 to 100 µg/mL) on AMJ13 tumor cell line revealed significant cytotoxic effects on all cell lines where all test substances inhibited cell growth at highest concentrations and reduced at the lower concentrations with the highest growth inhibitory effect presented with Doxorubicin followed by crude, flavonoid, and phytosterols fractions (Table 1). The highest cytotoxic activity of Doxorubicin, crude, flavonoids and phytosterols fractions were achieved at high concentration (100 µg/mL) and the least activity observed at lower concentration (6.25 µg/mL) after 72 h exposure period and the cytotoxic activity increases with concentration as concentration dependent (Figures 1, 2, 3 & 4). Histopathologically, the AMJ13 tumor cells in control group showing continues cell growth and monolayer formation, while the tumor cells treated by

tested agents showed cells detachment and presence of cytoplasmic vacuolation (Figure 5). The IC50 values for Doxorubicin, crude, flavonoids and phytosterols on AMJ13 cell line were calculated by the logarithmic equation. They were equal to 19.52 µg/mL, 25.18 µg/mL, 39.54 µg/mL, 47.88 µg/mL respectively.

Table 1. Cytotoxic activity of serial concentrations of *Althaea officinalis* crude, flavonoids and phytosterols fractions compared to Dox on AMJ13 cell line after 72hrs exposure period, (p<0.05)

Concentration (µg/mL)	Doxorubicin IR% (Mean ±SEM)	Crude IR% (Mean ±SEM)	Flavonoids IR% (Mean ±SEM)	Phytosterols IR% (Mean ±SEM)
0 (Control)	0±0.02	0.0±0.02	0.0±0.02	0±0.02
6.25	22.81±2.79	11.67±1.2	10.33±2.03	9.33±1.45
12.5	33.0±2.082	25.0±3.61	24.0±2.08	24.0±4.58
25	58.67±2.03	51.0±4.36	38.67±1.86	37.33±1.86
50	72.0±2.52	61.0±3.22	50.67±1.2	51.67±2.4
100	81.33±2.03	71.67±3.53	72.0±4.04	71.0±3.22

Each concentration in triplicate and the experiment were repeated twice

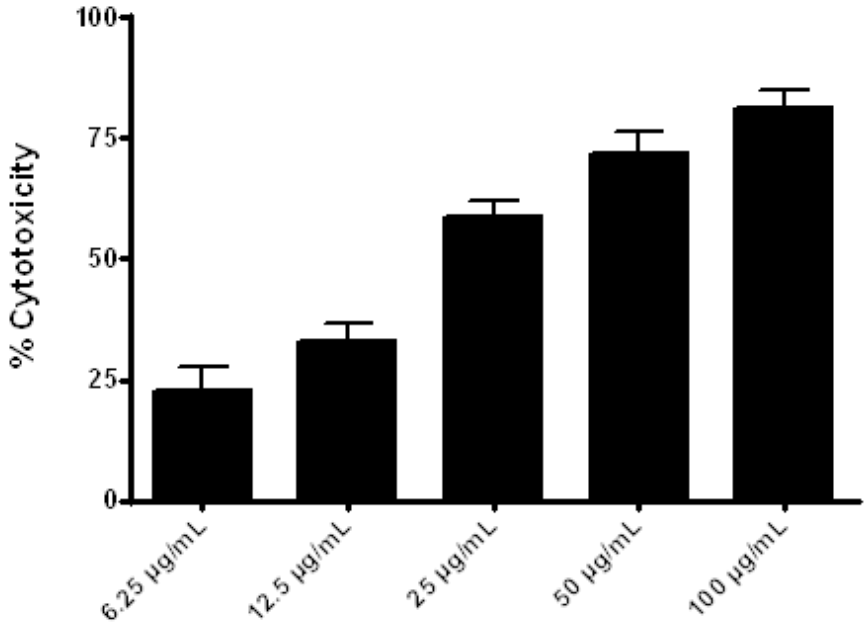

Figure 1. Cytotoxicity effect of Doxorubicin in AMJ13 cells. IC50=19.52 µg/mL

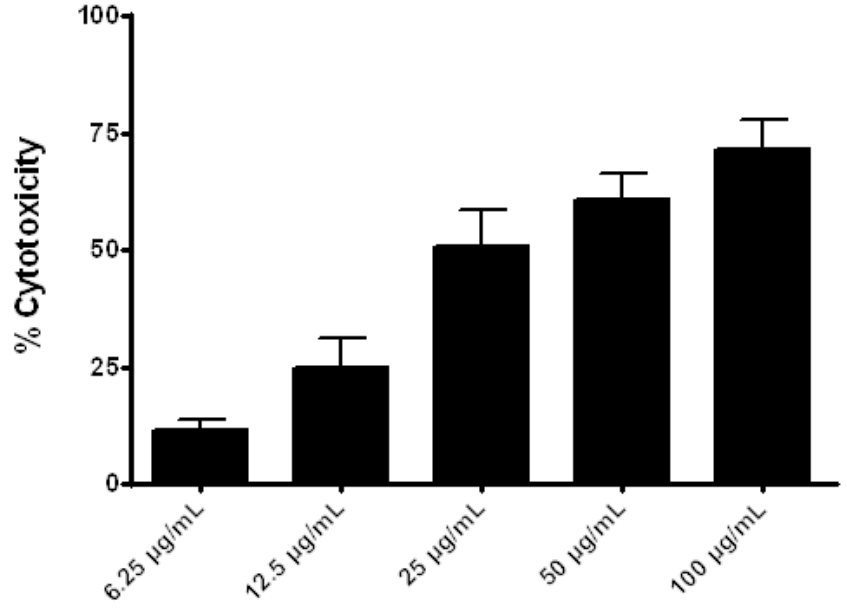

Figure 2. Cytotoxicity effect of crude in AMJ13 cells. IC50=25.18 µg/mL

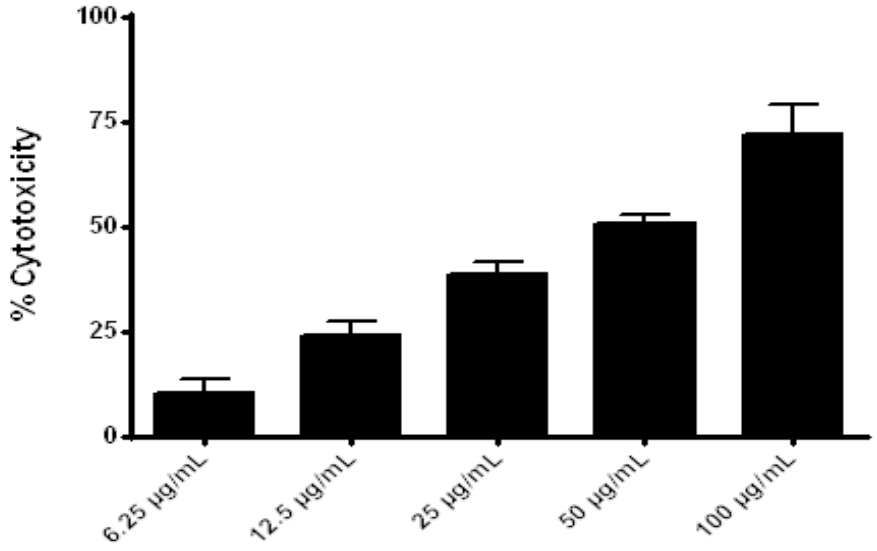

Figure 3. Cytotoxicity effect of flavonoids in AMJ13 cells. IC50=39.54 µg/mL

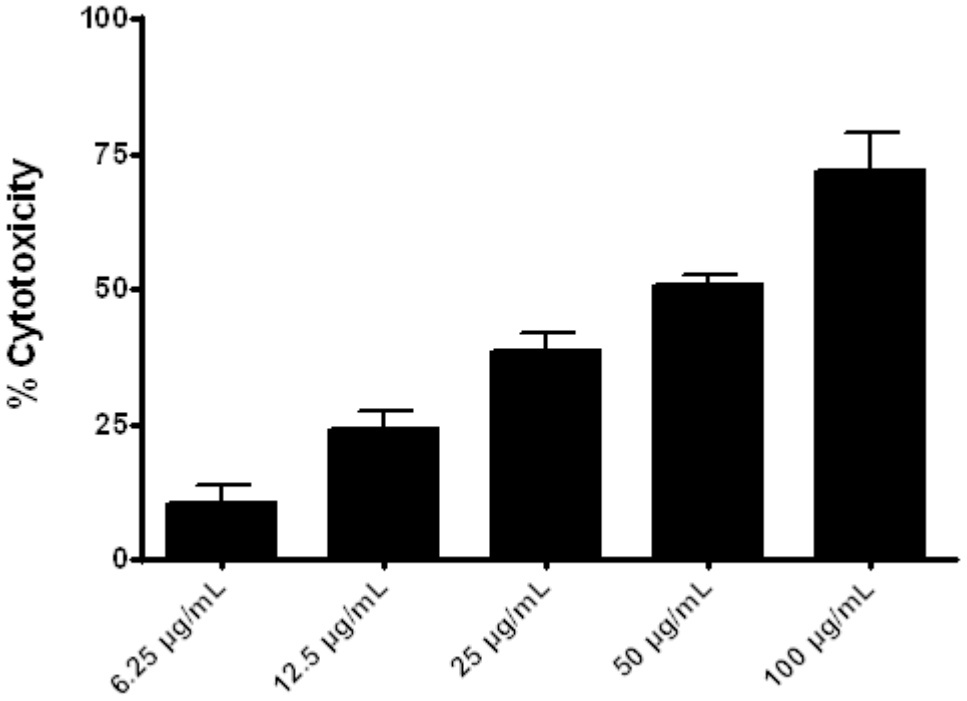

Figure 4. Cytotoxicity effect of phytosterols in AMJ13 cells. IC50=47.88 µg/mL

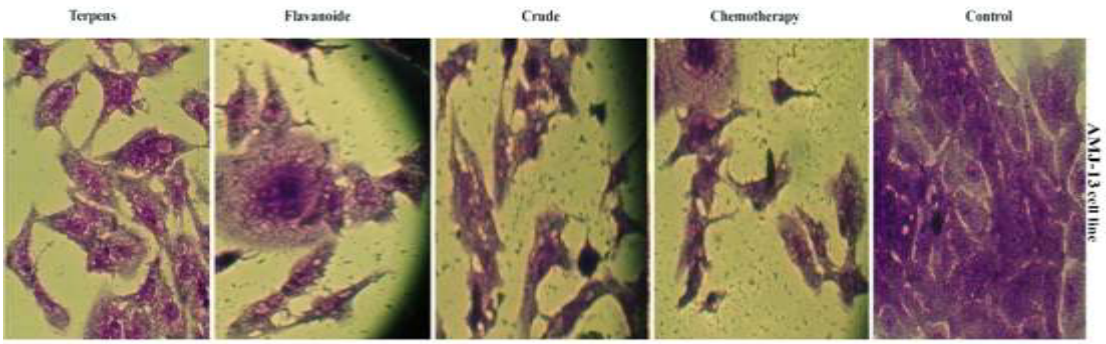

Figure 5. Cytotoxic activity of tested compounds against AMJ13 cells (40X)

Apoptosis assay

In our study, the *Althaea officinalis* extracts induced apoptosis in AMJ13 cells with non-significant effects on normal cells. The most extensive apoptotic effects was shown by crude extract which is comparable to Doxorubicin effects on AMJ13 breast cancer cell line followed by flavonoids fraction and then phytosterols fraction which has the least effect where the viable cells are stained green while the apoptotic cells are stained orange or red (Figure 6).

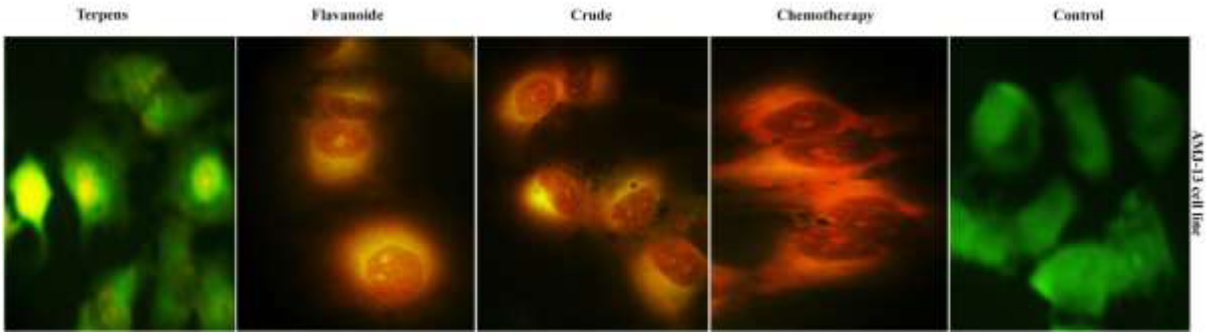

Figure 6. Effect of test substances on AMJ13 cells by apoptosis mechanism using fluorescent microscope

Discussion

The cytotoxic and antiproliferative activity of Doxorubicin and *Althaea officinalis* extracts were tested by the MTT assay, which is a colorimetric assay for evaluating cell viability [14]. In this study, in vitro cytotoxicity of Doxorubicin, crude, flavonoids, and phytosterols were evaluated with AMJ13 human breast cancer cell line to determine if these agents have any cytotoxic effect against breast cancer cell line. The results showed that the three extracts of *Althaea officinalis* had significant cytotoxic effect in a concentration dependent manner after 72 h exposure period on AMJ13 breast cancer cell line that was used and could reduce the viability of cancer cell line that were used to a variable extent. Crude extract exert the highest cytotoxicity effect, followed by flavonoids, and phytosterols fractions according to the threshold proposed by Suffness and Pezzuto where they establishes that naturally derived compounds showing a growth inhibitory effect (IC50 ≤ 100 µg/mL) can be considered to be cytotoxic and selected for further studies, whereas the most promising ones are those with an GI50 lower than 30 µg/mL [15] where the IC50 of crude, flavonoids, and phytosterols were 25.18 µg/mL, 39.54 µg/mL, 47.88 µg/mL respectively compared to IC50 of Doxorubicin, which is equal to 19.52 µg/mL. The naturally derived compounds and the bioactive constituents could influence the developing and progressing of carcinoma in several manners, for instance, inhibit the development and metastasis of cancer cells, immune-modulation, defensive against carcinogens and improving chemotherapy [16]. This study is the novel study all over the world to study the cytotoxic activity of *Althaea officinalis* extracts on AMJ13 cancer cell line. The results agreed with other previous studies that carried out in vitro and confirmed that some flavonoids could inhibit the cell growth of colon, prostate, liver, and breast cancer [17]. Previous study showed that scopoletin of *Althaea officinalis* produced dual action on tumoral lymphocytes exhibiting both a cytostatic and a cytotoxic effect on the cell, and also exert apoptosis [9,18]. Flavonoids are a class of compounds that have antioxidant, anti-inflammatory, antimutagenic, and anticarcinogenic properties [19,20]. The anticancer activity of flavonoids is related to their modulation of signal transduction pathways within cancer cells [21]. As a result, flavonoids can inhibit cell proliferation, angiogenesis, and metastasis, while also promoting apoptosis.

Phytosterols are natural products, showing anticancer activity. Campesterol, β-sitosterol, and stigmasterol are the three most common sterols [22]. It has been reported that phytosterols have protective effects on various chronic ailments including cardiovascular diseases [23,24] and diabetes [25]. Moreover, it is suggested that diets rich in phytosterols can reduce the risk of cancer by 20% [26,27]. Phytosterols and their oxy-derivatives may offer protection to the human body and inhibit cell proliferation and metastasis [28], as well as

the induction of apoptosis [29,30], all of which have been experimentally verified. In addition, phytosterols may also be important in host systems and exert antitumor effects by improving the immune system's identification of cancer, affecting hormone-dependent endocrine tumor growth, and regulating sterol biosynthesis [31,32]. Stigmasterol is antiangiogenic compound that inhibit endothelial cell proliferation, migration, and capillary network formation through the disruption of the TNF-α-VEGFR-2 axis, and it effectively suppress the growth cholangiocarcinoma xenografts by downregulating inflammatory cytokine production, macrophage recruitment and tumor angiogenesis [33]. The antiproliferative activity of stigmasterol also reported in human vascular smooth muscle cells A7R5 [34], hepatoma cells HepG2 [35], and human monocyte cell line U937 [36]. In the most recent study done by Al-Fatlawi at 2019, stigmasterol exerts anti-proliferative activities against the breast (MCF-7) and liver cancer (HepG2) cells. This study also found that stigmasterol helped to regulate apoptotic regulatory genes in cancerous cells where it led to high expression levels of pro-apoptotic genes (bax, p53), and negative expression of antiapoptotic genes (bcl-2). In addition, the stigmasterol treated both cancerous cell lines showed an increase in expression of the gene of caspase-9 and caspase-3. According to the gene expression analysis results, stigmasterol probably activates the apoptosis signaling pathway, and hence genomic DNA fragments were observed through gel electrophoresis. Therefore, this compound may be beneficial therapy without possible side effects on normal cells [36].

The changes in the nuclear morphology of AMJ13 cells after treated with crude, flavonoids, and phytosterols fractions of *Althaea officinalis* and Doxorubicin were studied using AO/EtBr dual staining method. The apoptotic cells were evaluated based on DNA damage. The AO-EtBr dual staining method was employed to stain specific parts of the cell and to determine the distinct apoptotic signs of nuclear alternations. The viable and non-apoptotic cells are stained green while the apoptotic cells are stained orange or red. The results in our study showed the exposure of the cells to the tested compounds caused an increase in membrane disruption and formation of lysosome vacuoles compared to the untreated control cells. The results showed the ability of test fractions to cause cell death due to their ability to penetrate the cell membrane and cause the mRNA expression levels of p53, bax, bcl-2, caspase-3, and caspase-9 (apoptosis is controlled through these pathways) [37]. The most extensive apoptotic effects were shown by crude extract which is comparable to Doxorubicin effects on AMJ13 breast cancer cell line followed by flavonoids fraction and then phytosterols fraction which has the least effect. The results of our study were coincided with preceding studies in that flavonoids and phytosterols have anticancer properties through induction of apoptosis mechanism in cancerous cells [38]. The majority of flavonoids compounds can promote apoptosis, induce cell cycle arrest, and promote cellular differentiation [19]. Alvarez-Sala and his colleagues stated that phytosterols including β-sitosterol produced apoptotic cell death with the induction of DNA fragmentation through the appearance of a sub-G1 cell population [39]. The antitumor activity of stigmasterol might be mediated through the activation of protein phosphatase 2A by ceramide causing apoptosis, as is shown by structurally similar phytosterols [40].

In conclusion, crude, flavonoids, and phytosterols extracts of *Althaea officinalis* exert a significant cytotoxicity and have the ability to cause apoptosis on AMJ13 breast cancer cells. Besides that, crude extract has the highest significant cytotoxic activity on AMJ13 cells.

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Author contribution

Dr Kadhum and Dr Al-shammari: collection, analysis of data, interpretation and discussion

of results. Dr Abd: concept, supervision and revising the manuscript.

Conflict of interest

Authors declare no conflict of interest.

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