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Original Article

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Abstract

Phytochemicals are natural products which are chemically extracted from nature resources (such as fruits and vegetables). The natural existence of phytochemicals together with their potential potency attracted the attention of the scientific community as a promising therapeutic candidate for cancers. Regardless its down sides, chemotherapy remains the main therapeutic stream in combination with other treatments. In the present study, breast safeguard (BSG) and Docetaxel (DOC.), phytochemical and drug under study, were applied in a dose dependent manner on two different types of cell lines (HepG2 and HFs). MTT assay was used to evaluate the impact of BSG and DOC. at two time points (Day 2 and Day 4). The data revealed that the lowest dose of BSG were safe on HFs cells, while had antiproliferative effect on HepG2 cells, however the combination of DOC. with BSG manifested more inhibitory effect on HepG2 compared to a single treatment of DOC.

1. Introduction

Cancer is considered as a universal problem where is the second leading cause of death worldwide according to the statistics of world health organization (WHO), there is an estimation of 13 million death and more than 21 million cancer cases in the world. Hepatocellular carcinoma (HCC) is reported as the third most common cause of death from cancer. Surgical treatment is not suitable for some cases such as Patients who suffer from advanced tumor extension and/or insufficient liver functionality reservoirs. Although the success of eradication operations of HCC in some cases the therapists directed to the chemotherapy. Chemotherapy is an effective way for treatment, but it has some pitfalls. Lack of specificity lies as the main drawbacks of chemotherapy; chemical drugs cannot target the cancerous cell, so it may cause damage for the healthy cells. Scientists are trying to find an effective way for targeting only the cancerous cells without causing damage for normal healthy cells **(Rajeshkumar, 2016).** Accordingly, search for novel anticancer agents with higher efficacy and minimal side effects is continued**.(Ahmadian *et al.*, 2018)**.

Phytochemicals are non-nutritive compounds with disease-preventive properties that are found in plants. bioactive compounds can be extracted from plants such as fruits and vegetables including phenolics, carotenoids, anthocyanins, and tocopherols. In pharmaceutical studies, nearly 20% of known plants have been used in the healthcare system **(Altemimi *et al.*, 2017)**

It is suggested that most phytochemicals can target cancer cells through apoptosis pathway where they activate the proapoptotic proteins. Phytochemicals increase the sensitivity of cells for treatment, so they can inhibit cancer proliferation **(Mitra and Bhattacharya, 2020).**

Chemotherapy is suggested to be a good method for the treatment of cancer, but it has no specificity and is restricted by toxicity. So, the healthy cells are exposed to apoptosis. So, cancer chemotherapy needs targeted drug delivery, which is more effective and less harmful to healthy cells.

Chemotherapy is an effective way for treatment, but it has some defects. It is not specific enough; chemical drugs cannot target the cancerous cell, so it may cause damage for the healthy cells. Scientists are trying to find an effective way for targeting only the cancerous cells without causing damage for normal healthy cells **(Rajeshkumar, 2016).**

Drug resistance is defined as the decrease in the efficacy and potency of a certain drug to produce therapeutic merits. Drug resistance in cancer is a well-known phenomenon that occurs when cancer becomes tolerant to pharmaceutical treatment. The

problem of resistance to therapy in cancer is multifaceted **(Vasan *et al.*, 2019).**

Resistance to anticancer drugs arises from a wide variety of factors, such as genetic mutations and/or epigenetic changes, conserved but upregulated drug efflux, and various other cellular and molecular mechanisms. Based on the time when drug resistance is developed it can be categorized as intrinsic or acquired resistance. Intrinsic resistance exists before drug treatment while the acquired resistance is induced after therapy, each occurring in about 50% of cancer patients with drug resistance **(Wang *et al.*, 2019)**.

2. Materials and Methods

2.1.1. BSG preparation:

BSG powder 500mg was prepared in 100% (DMSO) purchased from (Serva Germany), vortexed for 5 minutes then resuspended in 4 ml of complete media in falcon 15 ml the suspension is vortexed for 30 min then in centrifuge at 2000 rpm, 0 acceleration, 20˚C for 30 min. A serial dilution was prepared from the produced supernatant **(El-deen *et al.*, 2021).**

2.1.2. Docetaxel preparation:

A stock of DOC powder (0.1 mg) was solubilized in 1ml in DMSO. A serial dilution was prepared from 25.9 to 77.8 nM ; IC50 of DOC as reported by (Al-Abd *et al*., 2011).

2.2. Cell culture:

HepG2 and HFs were cultured in DMEM with 10% FBS, 1% Penicillin-Streptomycin. HepG2 and HFs cells were incubated in standard conditions of 37 °C and 5% CO2. Cells were regularly subculture by Trypsin-mediated dissociation (0.25%), during routine cell culture passaging.

2.3. cell proliferation and cytotoxicity assay:

HepG2 and HFs cells were cultured in DMEM supplemented with 10% FBS, 1% Penicillin/Streptomycin. HepG2 and HFs cells were incubated at standard conditions of 37 °C and 5% CO_2. Cells were regularly subcultured using Trypsin (0.25%), during routine cell culture passaging. For detecting IC50 of BSG, HepG2 cells were cultured in 96 well plate (3000 cell/well) in 100µl of complete media. In second day, different concentrations of BSG (5000, 2500, 1250, 625, 312.5, 156.25, 78.125, 39.06, 19.53 and 9.76 µg/ml) were added and untreated cells receives DMSO as a vehicle control. After 2 days of treatment, the IC50 of BSG and the inhibition at cell proliferation was measured at 570nm (Wilsher *et al.*, 2017). The inhibition percentage in cell proliferation were calculated from (Eq. (1)):

$$Inhibition\ (\%) = \frac{Control\ (OD) - Sample\ (OD)}{Control\ (OD)}\ \text{X}\ 100\ \%\qquad \text{Eq. (1)}$$

Where, (O.D) is the optical density.

For showing the significant effect of BSG and DOC. on normal cell line, HFs cells were cultured in 96 well plate (3000 cell/well) in 100µl of complete media. In second day, different concentrations of BSG (526, 263 and 131 µg/ml) were added and a group of cells receives DMSO as a vehicle control. After 2 days of drug exposure, The viability percentage in cell proliferation were calculated from (Eq. (2)):

$$viability\ (\%) = 100 - Inhibition\ (\%)\qquad \text{Eq. (2)}$$

2.4. Reverse transcriptase polymerase chain Reaction (RT-PCR):

It was used to determine the mRNA expression of MDR1 and GAPDH. The cells were treated with BSG IC_{50}, BSG 0.131 mg/ml, the combination between BSG 0.131 mg/ml + DOC0.125 µg/ml and DOC(0.125µg) for 24 hr Samples of cDNA were amplified in a conventional PCR using DreamTaq Green PCR Master Mix (2X) was purchased from (thermofisher scientific company). Running Thermocycles for 3 hr. at the detected annealing temperature of the defined primer followed by 30 cycles. The amplified products were separated by electrophoresis apparatus for 1 hr. at 300 V. A 50 base pair ladder was used as size standard (Indra et al., 2008).

The primers were used:

MDR1. 5' CCC ATC ATT GCA ATA GCA GG-3' (forward), 5' TGT TCA AAC TTC TGC TCC TGA-3' (reverse) with annealing temperature 58°C.

GAPDH 5'- ACC ACA GTC CAT GCC ATC AC -3' (forward) and (reverse) 5'- TCC ACC ACC CTG TTG CTG TA -3' With annealing temperature 60°C.

2.5. Statistical analysis

Data were expressed as mean ± SEM, Student's t-test was used to compare the mean differences between samples using Graphpad Prism software version 6.01 (GraphPad Software, CA, USA). For all analyses, $P < 0.05$ was considered statistically significant.

3. Results

3.1. The effect of BSG on HepG2 cell toxicity and proliferation

3.1.1. Estimation of IC50 of BSG by MTT (methyl thiazolyl tetrazolium) method

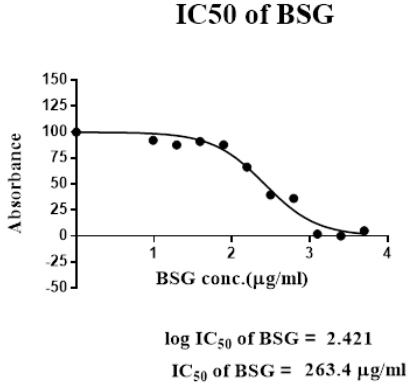

Fig. (1): Calculation of the IC_{50} of BSG: Estimation of the IC_{50} of BSG (263.4 µg/ml) by plotting log of the mean of absorbance Vs log of BSG concentration.

3.2. The effect of BSG on HFs cell viability and proliferation

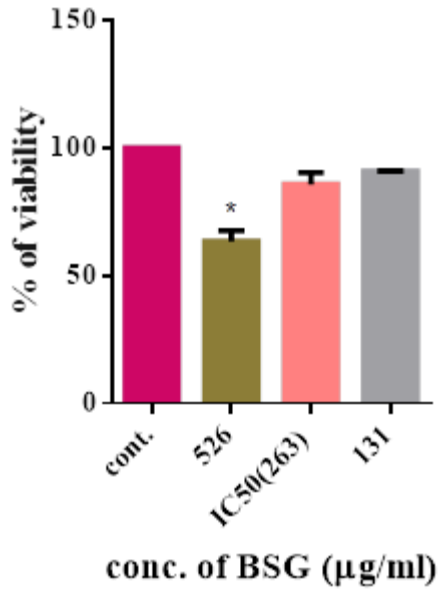

Fig. (2): Histogram of dose dependent effect of a serial dilution of double BSG IC50 (526 µg/ml) till dose 131 µg/ml on HFs cell proliferation for 2 days of drug exposure: The data revealed significant effect in cell viability of HFs cell were exposed to BSG 526 µg/ml. Significance was denoted as (*) $P < 0.05$ in comparison with the control group.

3.3. The effect of Doc. on HFs cell viability and proliferation

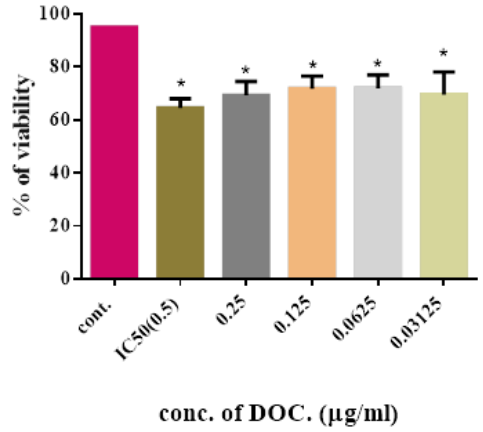

Fig. (3): Histogram of dose dependent effect of a serial dilution of DOC. IC50 (0.5 µg/ml) till dose 0.03125 µg/ml on HFs cell proliferation for 2 days of drug exposure: The data revealed the same significant effect in cell viability of HFs cell were exposed to all doses of DOC. Significance was denoted as (*) $P < 0.05$ in comparison with the control group.

3.4. Effect of BSG and/or Docetaxel on HepG2 cell proliferation after 2 days of treatment

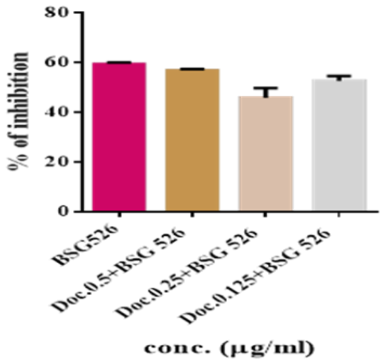

Fig.(4): Histogram between % of inhibition Vs doses of the drugs under study after 2 days of treatment: illustrating the comparision between the inhibitory effect of BSG double BSG IC50 (526 µg/ml) and its three combinations of DOC (0.5, 0.25, 0.125 µg/ml).

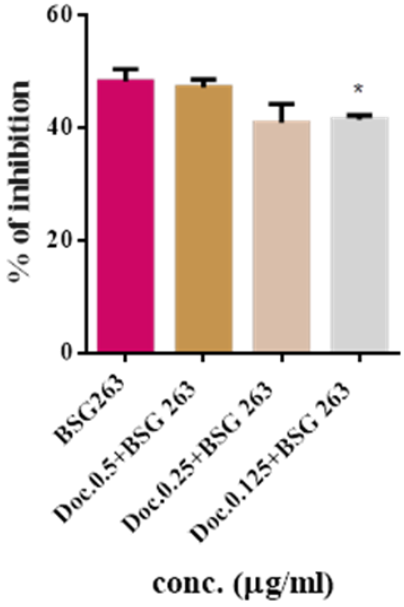

Fig.(5): Histogram between % of inhibition Vs doses of the drugs under study after 2 days of treatment: illustrating the comparision between the inhibitory effect of BSG IC50 (263 µg/ml) and its three combinations of DOC (0.5, 0.25, 0.125 µg/ml). The data revealed significant effect of the combination of DOC (0.125 µg/ml) with BSG IC50 (263 µg/ml). Significance was denoted as (*) $P < 0.05$ in comparison with HepG2 treated with BSG without DOC.

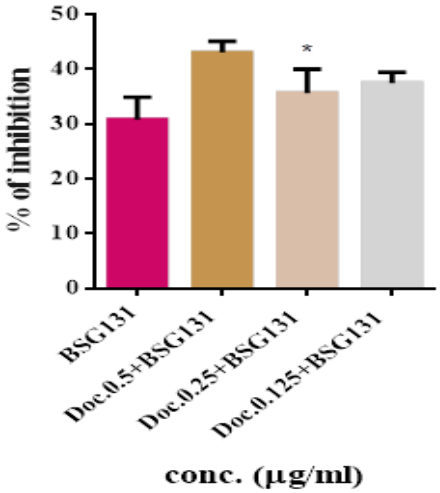

Fig.(6): Histogram between % of inhibition Vs doses of the drugs under study after 2 days of treatment: illustrating the comparision between the inhibitory effect of BSG (131 µg/ml) and its three combinations of DOC (0.5, 0.25, 0.125 µg/ml). The data revealed significant effect of the combination of DOC (0.25 µg/ml) with BSG. Significance was denoted as (*) $P < 0.05$ in comparison with HepG2 treated with BSG without DOC.

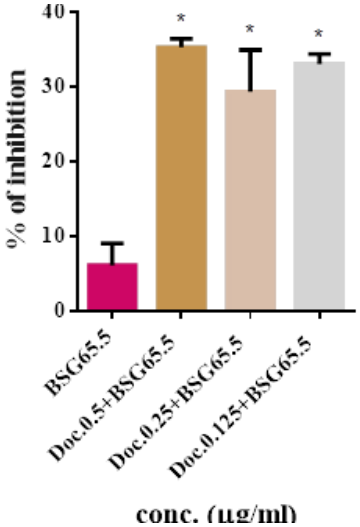

Fig.(7): Histogram between % of inhibition Vs doses of the drugs under study after 2 days of treatment: illustrating the comparision between the inhibitory effect of BSG (65.5 µg/ml) and its three combinations of DOC (0.5, 0.25, 0.125 µg/ml). The data revealed significant effect of the combination of all DOC doses with BSG. Significance was denoted as (*) $P < 0.05$ in comparison with HepG2 treated with BSG without DOC.

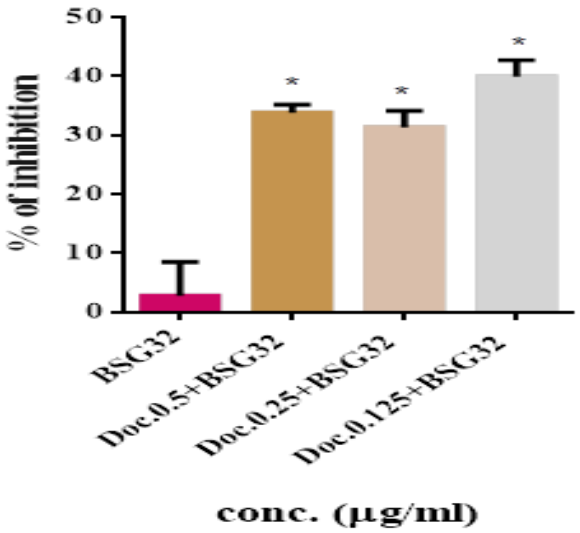

Fig.(8): Histogram between % of inhibition Vs doses of the drugs under study after 2 days of treatment: illustrating the comparision between the inhibitory effect of BSG (32 µg/ml) and its three combinations of DOC (0.5, 0.25, 0.125 µg/ml). The data revealed significant effect of the combination of all DOC doses with BSG. Significance was denoted as (*) $P < 0.05$ in comparison with HepG2 treated with BSG without DOC.

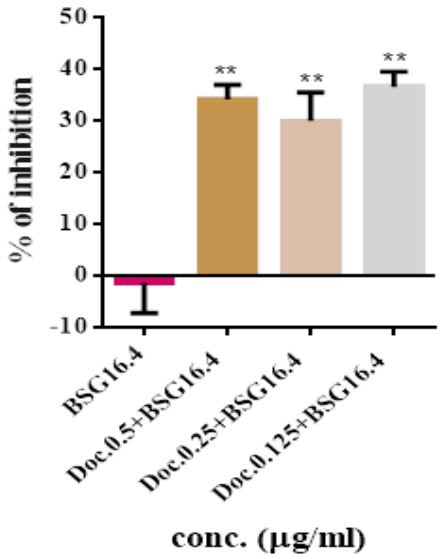

Fig.(9): Histogram between % of inhibition Vs doses of the drugs under study after 2 days of treatment: illustrating the comparision between the inhibitory effect of BSG (16.4 µg/ml) and its three combinations of DOC (0.5, 0.25, 0.125 µg/ml). The data revealed significant effect of the combination of all DOC doses with BSG. Significance was denoted as (**) $P < 0.01$ in comparison with HepG2 treated with BSG without DOC.

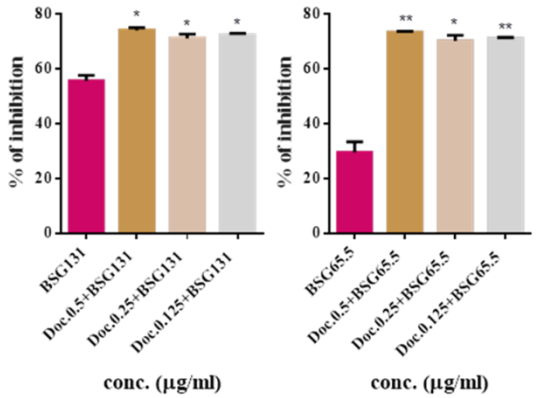

Fig.(12): Histogram between % of inhibition Vs doses of the drugs under study after 4 days of treatment: illustrating the comparision between the inhibitory effect of BSG (131 µg/ml) and its three combinations of DOC (0.5, 0.25, 0.125 µg/ml). The data revealed significant effect of the all DOC doses with BSG. Significance was denoted as (*) $P < 0.05$ in comparison with HepG2 treated with BSG without DOC.

Fig.(13): Histogram between % of inhibition Vs doses of the drugs under study after 4 days of treatment: illustrating the comparision between the inhibitory effect of BSG (65.5 µg/ml) and its three combinations of DOC (0.5, 0.25, 0.125 µg/ml). The data revealed significant effect of the combination of DOC (0.25 µg/ml) with BSG. Significance was denoted as (*) $P < 0.05$, while combination with DOC (0.5, 0.125 µg/ml) Significance was denoted as (**) $P < 0.01$ in comparison with HepG2 treated with BSG without DOC.

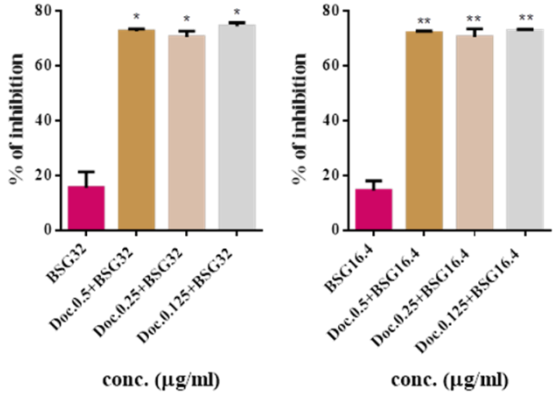

Fig.(14): Histogram between % of inhibition Vs doses of the drugs under study after 4 days of treatment: illustrating the comparision between the inhibitory effect of BSG (32 µg/ml) and its three combinations of DOC (0.5, 0.25, 0.125 µg/ml). The data revealed significant effect of the all DOC doses with BSG. Significance was denoted as (*) $P < 0.05$ in comparison with HepG2 treated with BSG without DOC.

Fig.(15): Histogram between % of inhibition Vs doses of the drugs under study after 4 days of treatment: illustrating the comparision between the inhibitory effect of BSG (16.4µg/ml) and its three combinations of DOC (0.5, 0.25, 0.125 µg/ml). The data revealed significant effect of the all DOC doses with BSG. Significance was denoted as (**) $P < 0.01$ in comparison with HepG2 treated with BSG without DOC.

3.6. Effect of BSG and/or Docetaxel on HepG2 Drug Resistance (Expression of MDR1 by RT PCR):

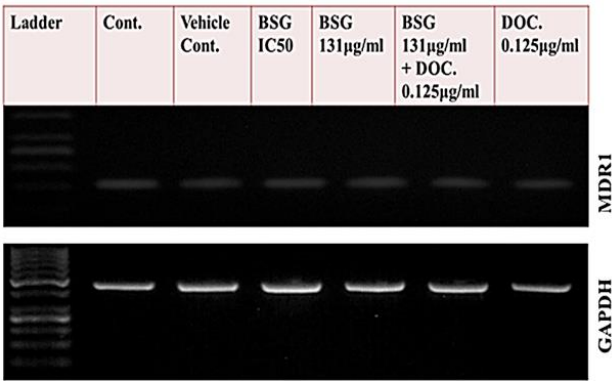

Fig. (16): RT PCR of MDR1 expression: the data did not reavel any marked effect between groups under study.

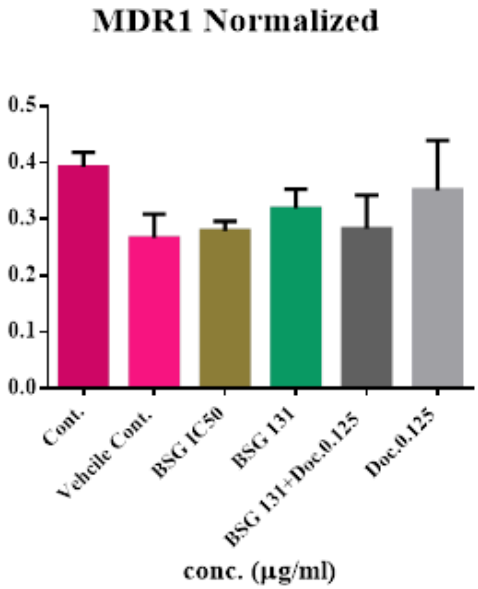

Fig.(17): Histogrm of Normalized MDR1 expresion: the data did not reveale any significant effect in comparing with vehcile control.

4. Discussion

The rationale of the present study is to use a cocktail of phytochemicals to target several signaling pathways to hurdle breast cancer cell proliferation, metastasis and survival. Relying on this data, a commercial product named "breast safeguard" was developed as a nutrient supplement to support breast health. Recently, BSG showed a profound effect in

sensitizing liver cancer cell line HepG2 to x-ray radiations. This work was directed to assess the best chemical extraction approach that shows potent antioxidant activity and significant inhibitory effect on HepG2 cell proliferation and migration. **(Abdraboh *et al.*, 2020)**

The effect of BSG on HepG2 was estimated by MTT assay, and the BSG ic50 was determined as 263 mg/ml. as reported by **(Al-Abd *et al.*, 2011)** the IC50 of DOC. was 0.5 µg/ml.

The chemical assessment of BSG extract antioxidant activity was first conducted using DPPH assay (antioxidant assay) and the data revealed a significant effect of the BSG extract in scavenging DPPH generated free radicals in a dose dependent manner. This potency may be referred to the marked increase at the flavonoid, polyphenols and tannin content of the BSG extract (18.1025 mg Catechin equivalent/gm BSG, 109.365 mg gallic acid equivalent/gm BSG and 30.944 mg gallic acid equivalent/gm BSG) compared to catechin and gallic acid as reference standards, respectively **(El-deen *et al.*, 2021).**

This work aimed to study the effect of BSG and Docetaxcel drugs on hepatocellular carcinoma and HFs cells.

After two days of BSG and/ or DOC. treatment on HepG2 cells, The data of MTT assay revealed that the inhibitory effect of BSG with DOC. was more than the inhibitory effect of the same BSG concentration without adding DOC.

Moreover, HepG2 cells were treated with the lowest concentration of BSG (16.4mg/ml) showed proliferation after two days of treatment.

After four days of BSG and/ or DOC. treatment on HepG2 cells, the data of MTT assay revealed more inhibitory effect on HepG2 cells.

In the present study,The effect of BSG and DOC. on HFs cells was determined by MTT assay. The data of MTT assay on HFs cells for BSG revealed that, low doses of BSG did not have toxic effect on HFs cells as shown in fig. (2), but when HFs cells were exposd to DOC. the data of MTT assay revealed low viability of cells comparing with the unexposed cells as shown in fig. (3).

Several studies showed the relation between cancer cells and drug resistance by treating with natural product as supplement intake or combined with other chemicals **(Wang *et al.*, 2015)**. Cancer cells targeting by mixed products even naturally present or chemically prepared could reduce the cancer cell to drug resistance, but as **(Chatterjee and Bivona, 2019)** reported in their study treatment with monotherapy compound did not effect on drug resistance for treated cancer cells. In present study, by showing the expression of MDR1 there was not any marked change upon treatment with either BSG and/or DOC. as is shown in fig.(16).

In conclusion, the combination of BSG and DOC showed a profound effect in halting HepG2 cell proliferation. Meanwhile, no marked effect of the combination was recorded on cell apoptosis or drug resistance. BSG IC50 and Doc separate treatment showed profound effect on induction of cell apoptosis. Moreover, BSG in low doses had safe effect on normal cell viability.

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