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Effect of Bee Venom and Other Natural Product (Dates) Onlung Cancer: Invitro Trial

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Abstract: ***Bee venom has special pharmacological activity for its enzymes and peptides containment. Melittin is the bee venom's main constituent. The present work aimed to in vitro estimation of the anti-cancer potentials of bee venom (BV)in addition to evaluation of the synergetic potential of dates extract to bee venom against lung cancer (A_{549}). BV showed a higher toxicity toA549 cells accompanied with synergetic activity of its toxicity in combination with daters extracts where the IC50 was arranged in the order of BV> Mix> Dates compared with Doxorubicin as a positive control. Data revealed that BV/dates extract has a synergetic potential to BV that was accompanied with Anticancer activity via up/down regulation of pro and anti apoptotic genes compared with control; caspase-3, Bcl-2 andpro-apoptotic gene(p^{53}).Cell cycle analysis revealed that bee venom, dates and their mix induced pre G1 apoptosis and cell cycle arrest at G2/M phase.***

Keywords: Bee venom, dates (phoenixdactylifera), A549, apoptotic, anti-apoptotic gene, pro-apoptotic gene, cell cycle, apoptosis

1. Introduction

Cancer is a leading cause of death worldwide, Statistically about 8.8 million deaths in 2015. There are different categories of cancers that are termed as death cancers such as: Lung cancer (1.69 million deaths), Liver (788 000 deaths), Colorectal (774 000 deaths), Stomach (754 000 deaths) and Breast (571 000 deaths). Lung cancer has been diagnosed to be one of the most threatening cancers. [1]According to, [2]Egypt was lacking the calculation of the population infected by lung cancer, since its very rare. In 2007, it has been shown that 8.2% of the Egyptian population are those that are suffering from lung cancer, moreover it has been added that it is more common in males than in females. However, when contradicting with Somalia, the rates of lung cancer are about 2% of the entire population and will be increasing in the next few decades, this is due to involvements to accelerate smoking cessation and avoid initiation. [3]Cancer is caused by various chronic infections, it has been contributed that about 15% cancers are developed due to Helicobacter pylori, Human papillomavirus (HPV), Hepatitis B virus, Hepatitis C virus, and Epstein-Barr virus. [1]

Bee venom has a huge impact in the treatment of tumors. It inhibited growth of cancer cells through induction of apoptosis Bee venom is safe for most people when injected under the skin by a trained medical professional.[4]Some people might get local side effect like redness and swelling where the injection is given, and rarely systemic effect include itching, anxiety, trouble breathing, chest tightness, heart palpitations, dizziness, nausea, vomiting, diarrhea, sleepiness, confusion, fainting, and low blood pressure.[5] Recent reports point to several mechanisms of MEL cytotoxicity in different types of cancer cells such as cell cycle alterations, effect on proliferation and growth inhibition, and induction of apoptotic and necrotic cell death trough several cancer cell death mechanisms.[6]

It was reported that dates had the second-highest antioxidant value of 28 fruits commonly consumed in China, and the Hawthorn fruit showed the highest amount of antioxidants.[7]

The role of antioxidants in minimizing or preventing the risk several chronic diseases such as cancer, cardiovascular diseases, neurodegenerative diseases, and inflammation is well recognized [8,9 & 10]. Because of their high fiber content, certain minerals and vitamins as well as the presence of many antioxidant and antimutagenic phytochemicals, dates can potentially be considered as a functional food in human health. Dates have been used as additives for cancer patientsthrough its ability to induce apoptosis, and prevent metastasis and angiogenesis, cause cell cycle arrest, and even moderate deleterious side effects from chemotherapy.[11]

2. Materials

Lung cancer cell lines (A549) wereused purchased from VACSERA EGYPT. Bee venom were purchased from ANDI Coe in antivenom research, VACSERA EGYPT. Total RNA Isolation System, Reverse Transcription System, dNTPs Mixture, Taq DNA polymerase, Bench Top 1kb DNA Ladder and DNA loading buffer were all from Promega. 3-(4, 5-Dimethylthiazol-2- yl)-2, 5-diphenyltetrazolium bromide (MTT) and RPMI media were purchased from Sigma-Aldrich.

3. Methods

Preparation ofDates Phoenixdactylifera

Date seeds was purchased from Sigma – Aldrich USA and extracted in distilled water as 10-mg/ ml. The mixture was heated in a water bath 60ºC for 30 min followed by sterilization using 0.22-µm syringe filter (Millipore-USA).

Cytotoxicity

Human lung (A_{549}) cancer cells were maintained in 75 cm^2cell culture flasks (TPP-Swiss) using RPMI-1640 medium (GIBCO-USA) supplemented with 10% (v/v) fetal bovine serum (G***IBCO***-USA) and incubated in 5% CO_2 incubator (Jouan-Franc) at a temperature of 37°C. Confluent cells were detached using 0.25% (w/v) trypsin solution and 0.05% (v/v) ethylenediaminetetraacetic acid ***(GIBCO-USA)*** for 5 min. Cells were plated at a concentration of 2 x 10^5cells/ml in 96-well cell culture plates and incubated at temperature of37°C for 24hours to achieve confluency. The medium was decanted and fresh medium containing various concentrations of BV and Dates extract for cytotoxicity determination using colorimetric MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) reduction assay (Sigma Aldrich-USA) [11]. MTT used as 0.05 mg/ ml used as 50 µl/well.

MTT stain was decanted and stained cells were washed twice using phosphate buffer saline (PBS) solution (ADWIA-Egypt). Developed formazan crystals were dissolved using 0.4% acidified Iso-propanol as 0.05 ml / well. Optical densityofdissolved crystals was measured at 570 nm using ELISA plate reader (ELx-800, Biotek, USA). OD was plotted against the concentration and the IC_{50} of BV and Dates extract was determined by using *MASTER PLEX* 2010 software [12]. The cell viability percentage was calculated using the following formula:

Cell viability (%)=OD of treated wells x 100
OD of control wells

Cell cycle analysis

A_{549} cells were 24 hrs. treated with the IC_{50}values of BV and Dates and their mix flasks were incubated at 37 oC.Affected cells were collected and residual no attached cells were collected using trypsin solution. Cells were pelleted using cold centrifuge (Jouan-KG-24 –Franc) and fixed gently with 70% Methanol (in PBS), maintained at 4ºC over night and resuspended in PBS containing 40µg/ml PI and 0.1% Triton X-100 in a dark room. After 30 min at 37ºC, the cells were analyzed using a flow-cytometer (Becton-Dickinson, San Jose, CA, USA) equipped with an argon ion laser at a wavelength of 488 nm [13].

Expression of apoptosis related genes using Real Time PCR

Total RNA was extracted from control and treated A_{549} cells using the GeneJET RNA Purification kit (Fermantus-UK) according to the manufacturer's protocol. The concentration and the integrity of RNA were assessed spectrophotometrically at 260/280 nm ratios and by gel electrophoresis, respectively. First-strand cDNA was synthesized with1 μg of total RNA using a Quantitect Reverse Transcription kit (Qiagen,Germany) in accordance with the manufacturer's instructions. These samples were subsequently frozen at a temperature of -80°C until use for determination of the expression levels of P^{53}, Casp-3 and Bcl-2 genes using real-time PCR. Quantitative real-time PCR was performed on a Rotor-Gene Q cycler (Qiagen, Germany) using QuantiTect SYBR Green PCR kits (Qiagen, Germany) and forward and reverse primers for each gene. Real-time PCR mixture consisted of 12.5 μL 2x SYBR Green PCR Master Mix, 1 μL of each primer (10 pmol/μL), 2 μLcDNA, and 8.5 μL Rnase-free water in a total volume of 25 μL. Amplification conditions and cycle counts were a temperature of 95◦ C for 15 min for the initial activation, followed by 40 cycles of denaturation at 94◦ C for 15 s, annealing at 60◦ C for 30 s, and elongation at 72◦ C for 30 s. Melting curves were performed after real-time PCR to demonstrate the specific amplification of single products of interest. A standard curve assay was performed to determine the amplification efficiency of the primers used. Relative fold changes in the expression of target genes (P53, casp-3 and Bcl-2) were accomplished using the comparative 2−ΔΔCt method with the β-actin gene as an internal control to normalize the level of target gene expression [14]. ΔΔCT is the difference between the mean ΔCT (treatment group) and mean ΔCT (control group), where ΔCT is the difference between the mean CT gene of interest and the mean CT internal control gene in each sample. Logarithmic transformation was performed on fold change values before being statistically analyzed, using the fold change values of three replicates for each gene measured.

Table 1: Primer sequences of apoptosis related genes and internal control.
(This report generated by Rotor-Gene 6000 Series Software 1.7 (Build 87)

ISO 9001:2000 (Reg. No. QEC21313).

Gene	Primer sequences
Bcl-2	F: 5'-CCTGTG GAT GAC TGA GTA CC-3'
	R: 5'-GAGACA GCC AGG AGA AAT CA-3'
Casp3	F: 5'- CCCCTCCTGGCCCCTGTCATCTTC-3',
	F: 5'- CCCCTCCTGGCCCCTGTCATCTTC-3',
Bax	F: 5'-GTTTCA TCC AGG ATC GAG CAG-3
	R: 5'-CATCTT CTT CCA GAT GGT GA-3'

Statistical Analysis

The obtained results were processed statistically according to[12], where minimum, maximum, mean value, standard deviation, standard error, and range were presented. Comparison between groups for significance was done using *t*.test "Difference between two means".

4. Results and Discussion

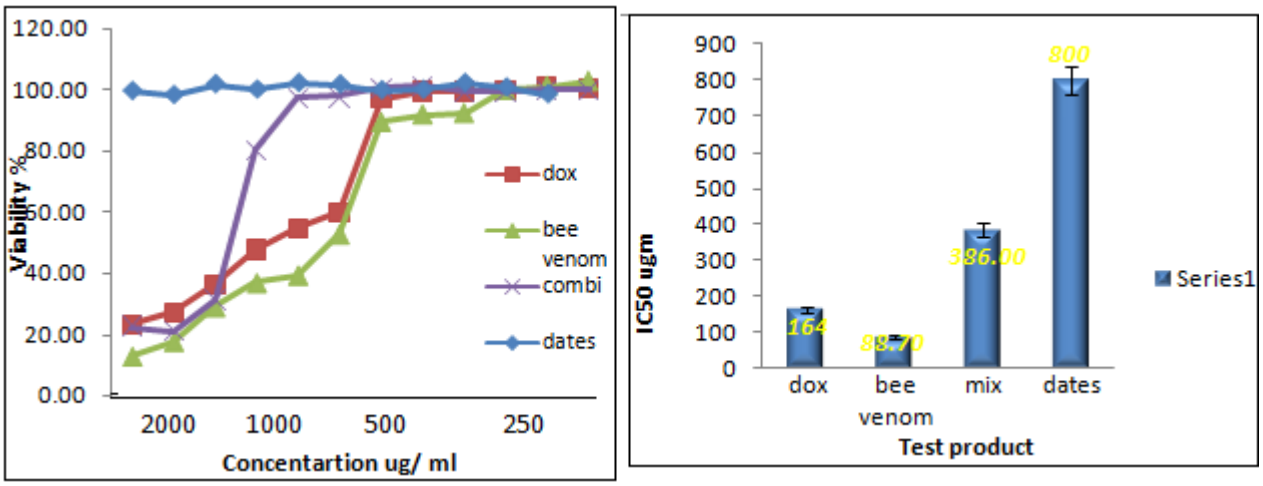


Figure 1: Cytotoxicity of tested products on A549 tissue culture cell

Data recorded in fig [1] revealed that toxicity of both BV and Dates extract in a single and combined forms were concentration dependent. Doxorubicin was the mostly toxic effective than the test products, as Mix of BV and dates extract showed a antagonistic behavior than that the single form application. BV was more toxic than Dox. ($P<0.05$) dates extract was the least cytotoxic effect. The IC50 values could be arranged in the order of BV>Dox>Mix> Dates.

Regarding the gene profile of treatedcells it was noticed that antiapoptotic genes Bcl-2 and casp-3 were down regulated in a significant way ($P<.0.05$) than that of control cells in the mean time there was a significant up regulation of Bax gene as pro apoptotic gene ($P<0.05$) Dos. Was mostly effective on gene up and down regulation than BV and Dates extract. The use of BV Dates mix showed the least up and down regulation of target genes [Fig.2]

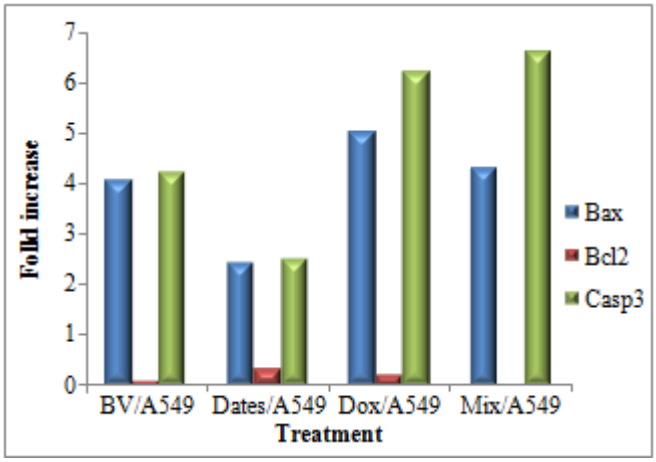
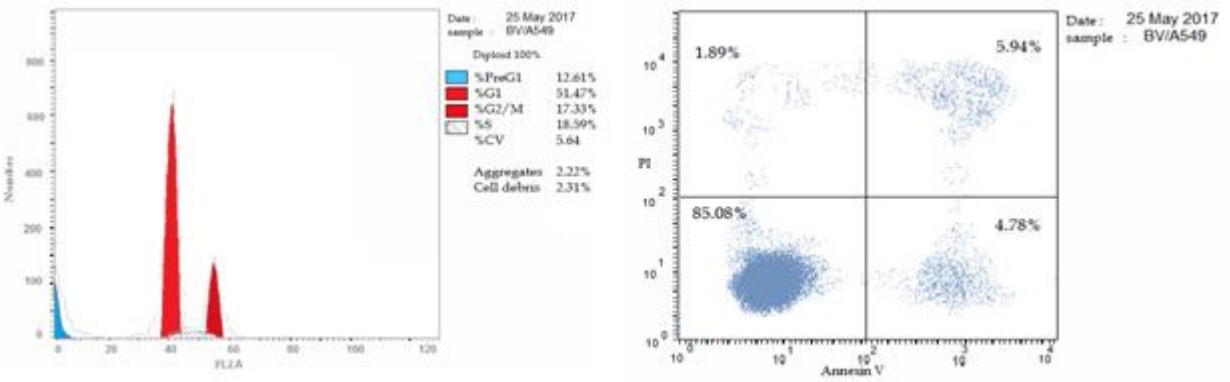


Figure 2: Effect of tested products on gene expression by RT-PCR

Date recorded revealed that the main arrest of treated cells was in the G2/M phase the % of arrest was significantly occur post cellular treatment with Dox. Followed by BV, the least % of arrest was post dates extract treatment. Despite the antagonistic effect of BV-Dates E mix on gene profile and toxicity there was a significant elevated arrest insignificantly compared with that induced post treatment of cells with Bv and Dox ($P>0.05$) accompanied with significant elevated % of apoptosis than that induced post cell treatment with BV,Dox and BV-Dates mix ($P<0.05$). [Fig.3]



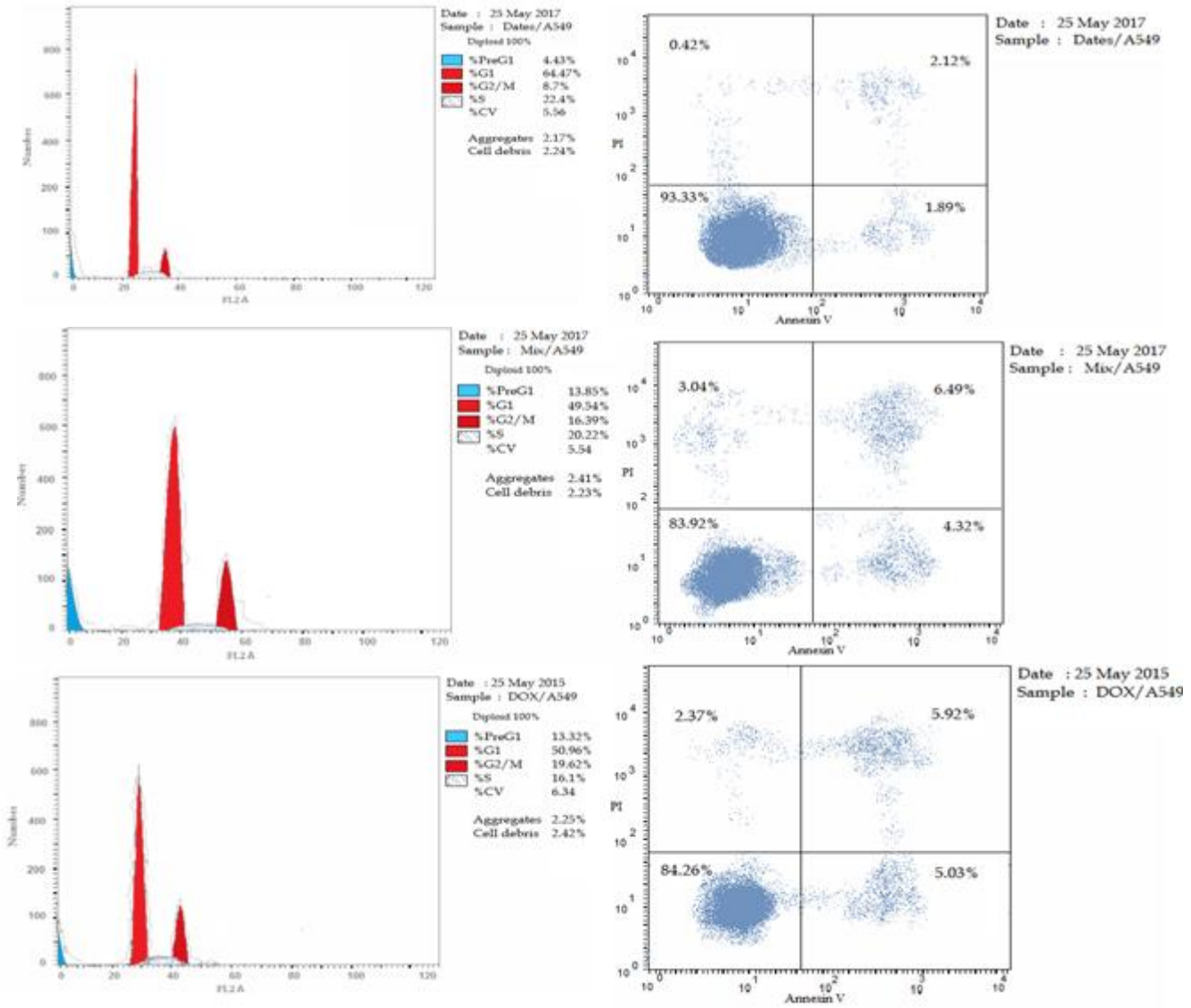


Figure 3: Cell cycle analysis of tested products

The present study aimed to estimate the anti-cancer potentials of bee venom, dates, and dates bee venom mixturevia inducing apoptosis in human lung cancer (A549 cell line). To investigate whether the dates, and bee venom have an anti-cancer effect, lung cancer cell line A549 was treated with different concentration of tested products for 24 hr. The results showed that the cell viability of A549 cell lines was inversely proportional with the three substances different concentrations, Egyptian bee venom showed high cytotoxicity more than the others on the lung cancer cell line and this illustrated atfig. (10). Regarding the previous results we found that The Egyptian bee venom results that inhibited the cell proliferation and induced morphological changes arein agreement with the results of a study done by[13], and,[14] which confirms that the bee venom has an inhibitory effect on A549 cell line after the treatment for 24 hr. Dates show the most viability %,While the mixture show high viability than both bee venom and dox, these result maybe due to the synergistic effect between bee venom and dates as the mixture increase viability and decrease cytotoxicity of the cell.This result may be due to the therapeutic potential of the extract,as it showed no significant difference from the control, Accordingto a study done by. [15]

Concerning APOPTOSIS and cell cycle arrest (fig-3), Egyptian bee venom, dates and dox. induce preG1apoptosis and cell cycle arrest at G2M in the lung cancer cell line. This means that our new products resemble to the effect of dox on apoptosis and cell cycle. Concerning bee venomthis result may be due to activation of intrinsic and extrinsic pathways according to a study done by.[14] Bee venom affected death receptors (DR) leading to apoptosis in A549 through the interaction with TNF cytokine family such as tumor necrosis factor (TNF) with death receptor 1 (DR1), Fas Ligand (FASL) with death receptor 1 (DR2), and Apo3 ligand (Apo3L) with death receptor 3 (DR3).[14]Concerning dates this results may be due to its Antioxidant, Anti-inflammatory, Antimutagenic, and Anticancer Activities, [16,17]Because of their high fiber content, certain minerals and vitamins as well as the presence of many antioxidant and antimutagenic phytochemicals, dates can potentially be considered as a functional food in human health. Further research is therefore needed to study the various date varieties in terms of their total antioxidant capacity, free radical scavenging activities, reducing power, anti-inflammatory properties, and protection against chronic diseases as cancer.[10]Alsodates can be considered a good source of phenolics[7,18] reported that dates had the second-

highest antioxidant value out of 28 fruits commonly consumed in China. Date varieties contain different concentrations and patterns of phenolic acids, which are mostly present in the bound form. [19]reported that the average contents of total phenolics in fresh and dried dates were 193.7 and 239.5 mg/100 g, respectively. They also detected a total of nine free and bound phenolic acids of which five consisted of hydroxylated derivatives of benzoic acid (gallic acid, protocatechuic acid [PCA], p-hydroxybenzoic acid, vanillic acid, and syringic acid) and four were cinnamic acid derivatives (caffeic acid, p-coumaric acid, ferulic acid, and o-coumaric acid), ad hoc PCA is a polyphenol antioxidant with mixed effects on normal and cancer cells. PCA has been reported to induce apoptosis in human leukemia cells, as well as in malignant HSG1 cells taken from human oral cavities. Depending on the amount of PCA and the time before its application, PCA can reduce or enhance the tumor growth. [20,21&22],[17]observed that the preparations made from the extracted polysaccharides (glucans) from Lybian dates exhibited a dose-dependent anticancer activity by suppressing the growth of Sarcoma 180 tumor cells in female CD1 mice.

The expression level of apoptotic gene caspase-3, pro-apoptotic gene Bax and anti-apoptotic gene Bcl-2 was measured using PCR after 24 hr treatment with dates, bee venom, and mixture on lung cell line. Our data showed that the expression level of Bax was upregulated cell line after the treatment with bee venom. However, the Bcl-2 oncogene is downregulated. These results are in agreement with a study done by. [13,14]The upregulation of the tumor suppressor gene Bax, and caspase-3 and down regulation of anti-apoptotic gene Bcl-2 contributes in the induction of apoptosis. These result may be owed toTNFR1 induces receptor trimerization and recruitment of TNFR1-associated death domain protein (TRADD), which is responsible for recruiting receptor-interacting protein 1 (RIP1), Fas-associated death domain protein (FADD), TNF-receptor-associated factor 2 (TRAF2). FADD and RIPK recruitment by TRADD results in caspase cascade activation that leads to induction of apoptosis via activation of initiator caspases caspase-2, -8,and -10 by the activated
receptor TNFR1. Initiator caspases cleave and activate effector caspases-3, -6 and -7.[23]Fas bind to Fas ligands (FasL) and FADD forming death-inducing signaling complex(DISC). DISC is responsible for recruiting initiator caspases as caspase8 which in turn cleaves and activate caspase 3 and 7 to induce cell apoptosis.[14]Apo-3 ligand will bind to the death receptor 3 resulting in induction of FADD which in turn recruit procaspase 8. Procaspase 8 is then activated via autocatalysis resulting in active caspase 8. Apoptosis is stimulated via activated caspase 8 through two different cascades. The first cascade is induction of apoptosis directly via cleavage and activation of caspase 3 by caspase 8.[14]

The second cascade depends on the cleavage of Bid (a Bcl-2 family protein) by caspase 8. Then, the truncated Bid (tBid) is translocated to mitochondria and induces the release of cytochrome C which activates caspase 9 and 3 and thus induces apoptosis. Mitochondrial membrane permeability that results in the release of cytochrome C is regulated by Bax/Bcl2. Thus, after the release of cytochrome C and activation of caspase 9 and 3, the expression of apoptotic regulatory protein will increase. Anti-apoptotic Bcl2 activity will decrease while pro-apoptotic Bax expression will increase indicating that apoptosis of the cells take place. [14]Through the extrinsic apoptotic signaling, BV induced apoptosis via Fas receptor. This results in the release of cytosolic proteins as Bcl-2. These proteins will target the mitochondria and change its membrane potential causing it to swell and make it leaky. This will lead to release of the apoptotic effector proteins in the cytosol including secondary mitochondria derived activator of caspase (SMAC) and cytochrome C. [13]The cytochrome C will bind to Apaf-1 and procaspase-9 forming apoptosome which is the final irreversible stage of apoptosis. The initiator caspase-9 will activate the effector caspase-3 and induce apoptosis of the cells via damaging mutated DNA. [13]

5. Conclusion

Anti-cancer potentials could be confirmed by upregulation and downregulations of apoptotic, pro and anti-apoptotic genes.

6. Recommendations

More cancer cell lines could be tested for verification of anti-cancer potentials of target test materials (dates and bee venom). Also, in *vivo* application of anti-cancer potentials of tested materials using murine models, and monitoring of biochemical changes pre and post treatment with anti-cancer agent regarding the antioxidant parameters (GTH, MDA, NO, ROS etc.)

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