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Anticancer Potential of Bee Venom and Propolis Combined Treatment on Human Breast Adenocarcinoma Cell Line (MCF-7)

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ABSTRACT:
Backgrounds: Natural remedies were used for cancer treatments, particular breast cancer. Also, the consumption of food products containing high amount of flavonoids and antioxidants had reported to lower the risk of various cancers. Bee venom (BV) and propolis were produced by honey bee. They were characterized by naturopathic formulation, affordability and containing high amount of antioxidants. Moreover, they were used safely since ancient times globally. Although that, there is no information about the synergistic or antagonistic anticancer effects of their combination. This study was designed to evaluate cytotoxic and pro-apoptotic effects of BV, propolis, and their combination on breast cancer (MCF-7) cells. **Materials and Methods:** As preliminary study, MCF-7 cells were treated with BV (5, 10, and 20µg/ml) and propolis (50, 150, and 450µg/ml) to specify the desired combination doses of each treatment with no anticancer effect individually. Consequently, doses of (5µg/ml BV+ 50µg/ml propolis and 5µg/ml BV+ 150µg/ml propolis) were chosen to evaluate the possible synergistic anticancer potential between them. All groups in this study were examined at 2, 4, and 12 hours intervals. The morphological changes were evaluated by acridine orange/ ethidium bromide dual fluorescent staining and Giemsa staining to reveal the formation of apoptotic bodies or nuclear condensation and cytoplasmic blebbing, respectively. DNA fragmentation assay was also carried out to record the reduction in DNA content and apoptosis. Bcl-2 expression, cytoplasmic anti-apoptotic marker, was used to prove the apoptotic properties, and autophagic cell death by florescent microscopy was evaluated also. **Results:** Morphological observation by inverted and florescent microscopy revealed apoptotic cell death under exposure to BV (10 and 20µg/ml) and propolis (450µg/ml). On the other hand, the results of combined treatments revealed significant morphological alterations after fluorescent and Giemsa staining. Apoptotic DNA fragmentation was clearly observed and Bcl-2 recoded significant down regulation which proved the apoptotic properties of combined treatments. Additionally, autophagic degradation results also supported the occurrence of stress on treated cells leading finally to cell death. All results of powerful anticancer potential were obvious among all combined-treated groups in dose and time dependent manner. This clear that, the combined treatments have possible synergistic effect which, propose it as potential candidates to be used in development of chemotherapy.
Keywords: Bee venom, propolis, combination, MCF-7, autophagy, and apoptosis.

INTRODUCTION

Cancer is the most common causes of mortality and creates many economic and health issues. The most common malignant tumors afflicting women worldwide is breast cancer [(1)] and ranked in the second place in mortality among cancer types [(2)].MCF-7, one of the breast cancer cell lines, is the acronym of Michigan cancer foundation-7[(3)]. It is a primary tumor [(1)], invasive breast ductal carcinoma [(4)].It is characterized by the presence of estrogen [(5)]and progesterone receptors [(6)], proliferative response to estrogens[(7)]and its phenotype is luminal epithelial[(8)].

Despite the presence of numerous clinical and traditional therapies which may be chosen according to the several factors and the curative role of chemotherapy in treatment of various cancers, most of them are not precise enough to distinguish between neoplastic and healthy cells which result in many serious side effects and others have hampered limited success on treatment[(9)]. So that, there is an imperative need to new safer and more successful type of cancer treatment.

Several studies reported that, many natural treatments exert anti-carcinogenic effects on different types of cancer. Up to 80%

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of cancer patients using alternative medicine [10], particularly breast cancer patients who favor the use of natural remedies [11].

Bee venom (BV) and propolis are considered as natural anticancer agents and BV is the most natural anticancer agent [12]. It contains at least of 18 active components [13], the principal one of them is melittin which about 50% of its dray weight [14]. While, propolis is a resinous hive product which is produced by honey bees using parts of plants. There are six main types of propolis according to the location of its botanical origin and collecting season as a result to types of plants around the hive [15], which lead to its variable chemical composition [16]. Egyptian propolis is classified as poplar-type [16], which composed predominantly from flavonoids and phenolic compounds which have antitumor activates [17].

However many studies reported the anticancer activity of crude BV and propolis against MCF-7 cells, there is no information about the synergistic or antagonistic anticancer effects of the combined treatment with BV and propolis. So that, the current study aims to evaluate the anticancer activities of BV and propolis as well as their combinations against MCF-7 cells and also to test which treatment is the most powerful one.

MATERIAL AND METHODS:

Propolis extraction:

The propolis used in the present study was Egyptian propolis. Propolis was extracted with 20X of 95% ethanol (w/v) at room temperature for 24 h. The ethanol suspension was filtered using Whatman filter paper (No. 1) under reduced pressure. The filtered liquid was then concentrated using rotary evaporator (Buchi, France) under reduced pressure at 40ºC until it reached a constant weight. Then it was stored under a dry condition at -20ºC until use. It has a brown color [18].

Bee venom:

Dried pure Egyptian honeybee venom (*Apis mellifera lamarckii*) was obtained and identified according to **Schmidt** [19] by the Bee Research Department, Plant Protection Institute, Ministry of Agriculture, Egypt.

Cells culturing:

MCF-7 cells were purchased from Holding Company for Biological Products and Vaccines (VACSRA), Giza, Egypt. Cells were trypsinized then subcultured into 25 cm^3 tissue culture flasks. 5x 10^5 cells were grown in each flask containing 7ml of complete growth medium, RPMI-1640 medium supplemented with 10% fetal bovine serum, 1% (100U/ml penicillin and 100 µg/ml streptomycin) at 37°C[20].When cells reached 70% confluency, the treatments were added in triplicates. All culture reagents were obtained from (Lonza) supplier, Egypt.

Study design:

MCF-7cells were divided into the following: **(Group I)** served as control without any treatments for 2h, 4h and 12 h; **(Group II)** cells were incubated with BV at dose of 5, 10, and 20 µg/ml for 2h, 4h and 12 h;**(Group III)** cells were incubated with propolis at dose of 50, 150 and 450 µg/ml for 2h, 4h and 12 h &**(Group IV)**cells were incubated with (5µg/ml BV + 50µg/ml propolis) and (5µg/ml BV + 150µg/ml propolis) for 2h, 4h, and 12 h. Each treatment was done in triplicates.

Determination of cell viability:

The visualization of MCF-7 cells viability was done by trypan blue stain method. It was counted using haemocytometer under light microscope.

Cells / ml = 10^4 x (Average count per square) x (Dilution factor) [21].

Giemsa staining:

Giemsa staining was originally designed to stain the cytoplasm with pink color and blue color for the nucleus**.**Cells were smeared on a glass slide and air-dried. Cells were fixed in (3 parts methanol: 1 part glacial acetic acid) for 5 minutes. Cells were washed with PBS for 1 minute then stained in Giemsa solution for 15 minutes and washed with PBS. Five hundred cells were examined (400 x) per slide using light microscope (Olympus BX 41, Japan) and digitally photographed [22].

Acridine orange/ ethidium bromide dual fluorescent staining:

Cells were smeared on a glass slide and air-dried, smeared cells were fixed in methanol/acetic acid (3:1) for 5 minutes, cells were hydrated with PBS for 1 minute, stained with a mixture (1:1) of acridine orange (50 µg/ml)/ethidium bromide (5 µg/ml) for 20 minutes and then cells were immediately washed with PBS and viewed under fluorescent

microscope (Olympus BX 41, Japan) with blue filter. Five hundred of cells per flask were evaluated (400x) and the damaged (apoptotic and necrotic) nuclei were recorded according to the affinity and pattern of fluorescent staining, and then the representative photos were digitally captured [23].

Total genomic DNA extraction and apoptosis detection:

DNA extraction and detection of apoptosis (DNA fragmentation assay) were done according to "salting out extraction method" of **Aljanabi and Martinez** [24] with some modifications by **Hassab El-Nabi and Elhassaneen**[25].The method of extraction is summarized as follows: cells were lysed with 0.5 ml lysing buffer (10 mM Tris base, 10 mM NaCl, 10 mM Na_2 EDTA, 0.5% SDS, pH 8.3) overnight at 37°C then; 4M NaCl was added to the samples. Centrifuge the mixture at 10,000 rpm for 10 minutes. The supernatant was transferred to a new tube then DNA was precipitated by 1 ml cold isopropanol by centrifugation for 5 minutes at 12.000 rpm. Wash the pellets with 70% ethanol. Resuspend the pellets in TE buffer (10 mM Tris, 1mM EDTA, pH 8). Incubate for 30 - 60 minutes with loading mix (0.1% RNase + loading buffer), and then loaded directly into the gel-wells. Gels were prepared using 1.8% normal melting electrophoretic grade agarose in 1X Tris borate EDTA buffer (89 mM Tris, 89 mM boric acid, 2mM EDTA, pH 8.3) for 1 h at 50 volts. The apoptotic bands of DNA fragmentation appeared and located at 180 bp and its multiples 360, 540 and 720 bp against DNA marker (100–3000 bp). The intensity of DNA fragmentation were measured by (ImageJ software) as a mean optical density values.

Immunocytochemical study:

Immunocytochemical reaction was performed using an avidin biotin complex immunoperoxidase technique on smeared cells of treated and control groups [26].Bcl-2 as a cytoplasmic marker for apoptosis was detected using an anti-human Bcl-2 monoclonal antibody (Glostrup). Cells were examined (400x) using light microscope (Olympus BX 41, Japan) and digitally photographed.

Evaluation of autophagic cell death:

Cells were grown on coverslip and the treatments were applied after reaching 70% confluency. After the desired treatment period, cells were stained in 1mg/ml acridine orange in PBS for 5 minutes then washed in PBS for 5 minutes and immediately examined using fluorescent microscope with blue filter (Olympus BX 41, Japan) and digitally photographed[27].

Statistical analysis:

Statistical analysis was performed using SPSS (Science Products to SYSTAT Software). Each experiment was repeated a minimum of three times and a mean value was derived in each case. The student's *t*-test was used to detect statistical significant differences between the susceptibility of different treatments.

RESULTS

The morphological changes in MCF-7 cells by acridine orange / ethidium bromide fluorescent method:

Acridine orange/ethidium bromide fluorescent staining revealed the formation of apoptotic bodies and nuclear condensation with bright orange color which is the characteristic features of dead and apoptotic cells in dose and time dependent manner among bee venom treated groups. Where, the groups treated with (10 & 20 µg/ml) recorded highly significant increase ($p \leq 0.01$) in both dead and apoptotic cells after 4 and 12hr when compared with control croups, but no changes were noticed after 2hr. While, the group treated with 5 µg/ml BV showed no significant changes at all intervals of treatments in comparison with untreated groups. At the same time, low doses of propolis did not cause any significant effects when compared with control cells which showed intact architecture with green color except the dose of 450µg/ml at 12 hours which recorded few non significant count of apoptotic cells(Figures:1&4).

Determination of total genomic DNA fragmentation in MCF-7:

As shown in Figure (2), the administration of (10 & 20 µg/ml) bee venom caused severe reduction in DNA content as a result of cell toxicity, while bee venom (5 µg/ml) and all propolis doses had no diagnostic DNA reduction except the highest dose (450µg/ml) which recorded a little effect when compared to control groups. This reduction in DNA content among treated groups reflects the

reduction of cells count and growth capability as a result of treatments.

At the light of pervious results, low dose of bee venom (5µg/ml) in additon to the low and middle doses (50µg/ml and 150µg/ml) of propolis did not exert antitumor potential with respect to control results. Consequently, we tried to investigate the possible synergestic potential of both extracts as a combined treatments.

Figures (3a,b&4) demonstrated that, the treatment with bee venom and propolis mixture afteracridine orange/ ethidium bromide fluorescent staining had apowerfull action on MCF-7 cells better than individual treatments. Where, it showed increasing percentage of apoptotic and deadcells, which is directly proportional to both dose and time.

Determination of total genomic DNA fragmentation in MCF-7 in control and combination treated groups:

The extracted total genomic DNA of control MCF-7 cells was found to be intact (undamaged) as seen in (lane: 1- Figure: 5) with no release of DNA. While, the treatments was indicated the presence of marked damage by the migration of released fragmented DNA (lane 2&3). So that, these treatments induced DNA damage as apoptotic laddering pattern of DNA fragmentation, at 180, 360, 540 and 720bp, throughout all time intervals of treatments as clear in Figure (5&6).

Evaluation of morphological changes of apoptosis by Giemsa staining method:

As shown in Figure (7 a, b&8) the cytological analysis of the combination treated groups of MCF-7 cells using Giemsa staining method indicated typical apoptotic morphology of membrane blebbing. Interestingly, the percentage of dead cells recorded no, slightly ($p \leq 0.05$) and highly ($p \leq 0.01$) significant changes at 2, 4, and 12hr respectively when compared with untreated cells. While, the percentage of blebbed cells showed inversely results. Where, it was recorded highly ($p \leq 0.01$), slightly ($p \leq 0.05$) and no significant changes at 2, 4 and 12 hr respectively when compared with control groups.

Evaluation of apoptosis by Bcl-2 expression:

The present data revealed that combination treated groups recorded highly significant decreasing ($p \leq 0.01$) in the percentage of positiveBcl-2 cells as compared with over expression of Bcl-2 in untreated MCF-7 cells at 2, 4 and 12 hr as appeared in Figures (9 & 10) .

Evaluation of autophagic cell death:

The morphological observations by inverted and florescent microscopy as shown in Figure (11) revealed autophagic cell death in MCF-7 cells under exposure to both combination doses at 2&4 hr. Where, the cells associated with accumulation of large numbers of acidic autophagic vesicles, which degrade organelles early in addition to cytoplasmic vacuolation while the cytoskeletons remain intact and functional until late in the process. Interestingly, after 12hr of treatments there are no autophagic vesicles were observed. Moreover, large number of cells was detached and undergoing cell death with a tremendous reduction in cells size and number.

DISCUSSION

In the light of the present results, BV treatment triggered both cytotoxic and anti-proliferative effects on MCF-7 cells and has inhibitory impact on their viability. In addition, its genotoxic effect which is proved by the generation of abundance fragments of DNA especially with high doses of treatments. There are many reports point to several mechanisms of BV cytotoxicity on cancer cells such as the effect on proliferation and/ or growth inhibition, and induction of apoptotic and necrotic cell death through many cell death mechanisms including the caspases and matrix metalloproteinases [(28)]. Furthermore, the antiprolifrative impact of BV on MCF-7 cells is caused by affecting Bax and Bcl-2 [(29)], while its apoptotic effect may be due to its ability to increase Fas receptors [(30)] which belongs to a family of receptors including tumor necrosis factors (TNF) and nerve growth factor (NGF) receptors that utilize related signaling pathways which regulate cell proliferation, differentiation or death by cross linking of Fas ligand (FasL) to trigger apoptosis in the target cells [(31)]. Other investigations returned the apoptotic effect of BV on tumor cells also to its ability in reduction of Bcl-2 expression [(29)] and Bax elevation [(30)] which leading to the activation of caspases-3[(32)], which is one of the protease enzymes with its critical role in executing programmed cell death to apoptosis [(33)] which

lead to DNA strand breaks. Where, apoptosis is involved in activation of endonucleases which causes DNA fragmentation as seen upon electrophoretic examination [(34)].

In addition, BV induced apoptosis in MCF-7 cells by mitochondrial-dependent mechanisms through its ability to increase the production of ROS and DNA damage by the role of Fas receptors in promoting caspase-8, change the ratio of Bcl-2/ Bax which may lead to mitochondria dysfunction followed by apoptosis, reduce MMP levels leading to release the cytochrome C and increase caspases-9 and caspases-3 activity and release Endo G and AIF from mitochondria [(35)].

At the same time, the current study indicated that, there is a dual effect of propolis on MCF-7 cells, where its high dose induced antitumor effect while low doses caused protective effect with no cytotoxicity, and its cytotoxicity increased at time dependant manner so it may needs more time to clear its acute apoptotic effect. Where, other study recorded a drastic toxicity of propolis when increasing the exposure time from 24 to 72hours[(36)].These results may be due to its dual role on ROS where propolis exerts a pro-oxidant effect at high concentration and acts as an antioxidant at low concentrations [(36)]. The high dose of propolis may elevate ROS levels leading to decrease mitochondrial membrane potential, which promote cytochrome C release leading to activate caspases to initiate apoptotic signaling pathway [(37)].In addition, it has a role in regulating ANXA7 and P53 proteins and mitochondrial membrane potential and inhibiting NF-ķB, so that, it has anti-carcinogenic effects [(20)]. Otherwise, the low dose of propolis acts as antioxidant which may be attributed to the flavonoids content in Egyptian propolis. Where, flavonoids have powerful antioxidant potential that can protect tissues from DNA damaging effects [(36)].

Interestingly, propolis has non/low toxicity to normal cells because of its selective toxicities to tumor cells as reported by **Rai *et al.*** [(20)].At the same time, BV possesses selective cytotoxic properties in both normal and cancerous cells [(38)]. Consequently, we tried to investigate the possible synergistic potential of propolis and BV as a combined treatment which may be had more cytotoxicity on malignant cells and more protective effect on normal cells.

Also in the light of the present data, the combination treated groups' demonstrated highly cytotoxic effect on MCF-7 cells. Where, they increased the percentage of autophagic, apoptotic and dead cells, induced DNA damage, and reduced the percentage of Bcl-2 expression. Moreover, both autophagic and apoptotic cell death resulted by combination treatment, although autophagy protect cells from undergoing apoptosis [(39)].We suggested that, this proapoptotic impact may be attributed to the role of combination in suppression of Bcl-2 activities shifting cells towards apoptosis. While, the activation of autophagic machinery occurred may be induced by MCF-7 cells as a trail to survive, where cancer cells enhanced autophagy to survive under metabolic and therapeutic stress [(40)].Also, autophagy may be induced by the treatment, where it may be contained the disaccharide trehalose which an MTOR-independent activator of autophagy [(41)] or by its ability for elevating the ROS level which increasing metabolic stress which enhanced autophagy. In addition, this autophagic degradation of catalase increases reactive oxygen species which enhance autophagic cell death [(42)].But after 12hr the cells haven't any ability to generate energy so they undergoing to apoptotic cell death mechanism only, where cells use autophagy to survive MOMP to prevent releasing of cytochrome C and other apoptogenic proteins and recover to continue and grow as long as energy can still be generated [(43)].

In the previous paragraph, we try to discuss the cause of presence of both autophagy and apoptosis in treated groups by expecting autophagy happened before apoptosis and suggested the mechanisms which may explain that. But it may be due to that two processes happened together, where both autophagy and apoptosis kill cells; one expects that there is coordination between their regulators. So, we suggested that the treatments enhanced apoptosis and autophagy together as a result of its ability to suppress Bcl-2 expression, whereBcl-2 / Bcl-xl inhibited apoptosis by binding to Bax and Bak and also inhibited autophagy by binding with Beclin 1/ Atg6 [(44)] or by blocking calcium release from the endoplasmic reticulum which leads to inhibition of MTOR to activate autophagy [(45)].

Collectively, the results of current study suggested that BV, propolis, and the combination exert antitumor effects mainly through inducing apoptosis in MCF-7 cells. Attractively, when the non effective individual doses of propolis and BV were used in combination, they gave sever autophagic and apoptotic cell death and DNA fragmentation in breast cancer cells which indicated a possible synergetic effect between BV and propolis. And also, may be attributed to its ability to enhance both autophagic and apoptotic (type I and II) cell death. This finding can be a promising agent for breast cancer therapy at both initial and devolved stages and can use as a conservative treatment. However, further research is needed to clarify its precise targets in breast cancer cells and its mechanisms of action.

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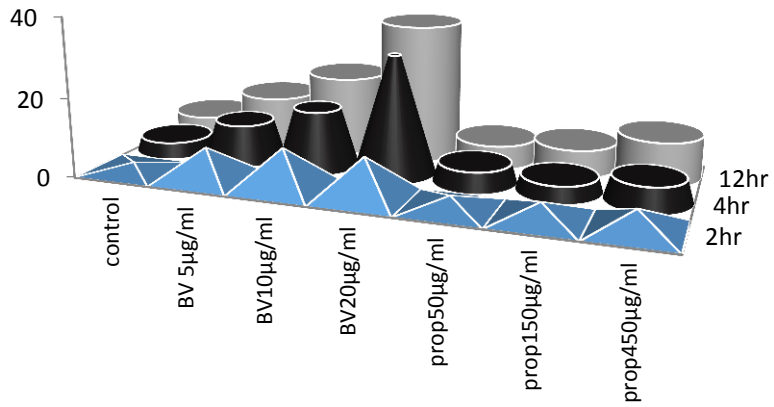
Figure (1): shows the percentage of dead MCF-7 cells after acridine orange/ ethidium bromide fluorescent staining for treated and untreated groups.



Figure(2): shows percentage of total DNA content in treated and untreated MCF-7 cells after three different periods.

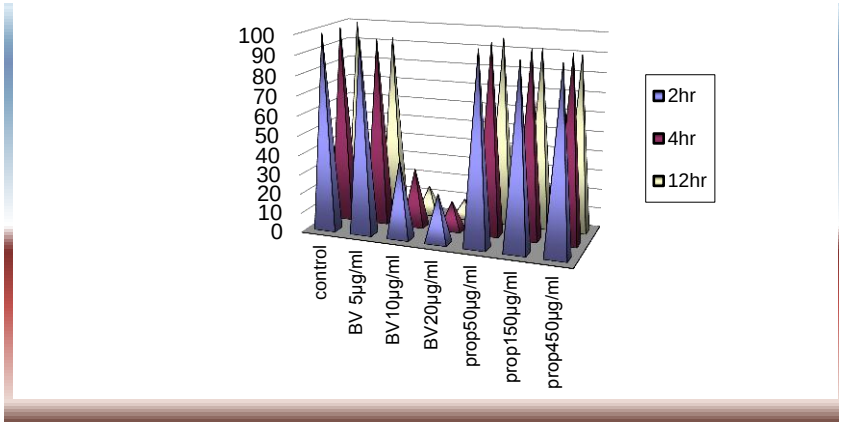
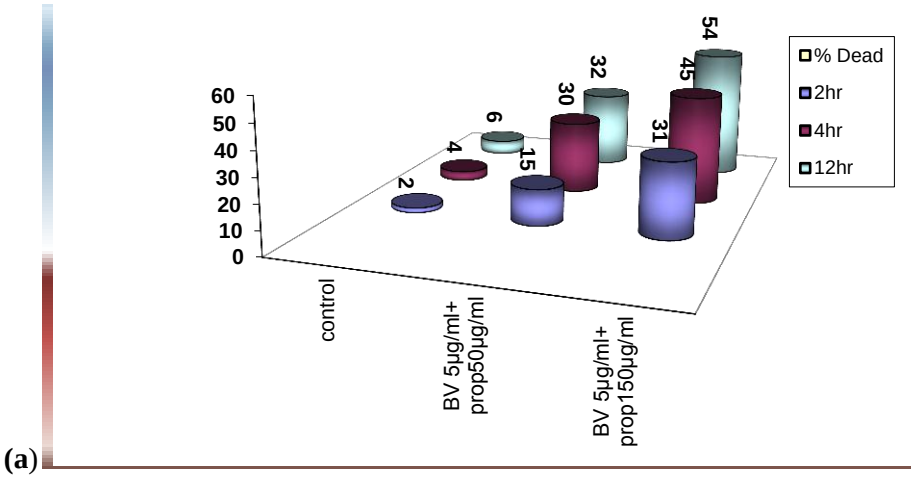
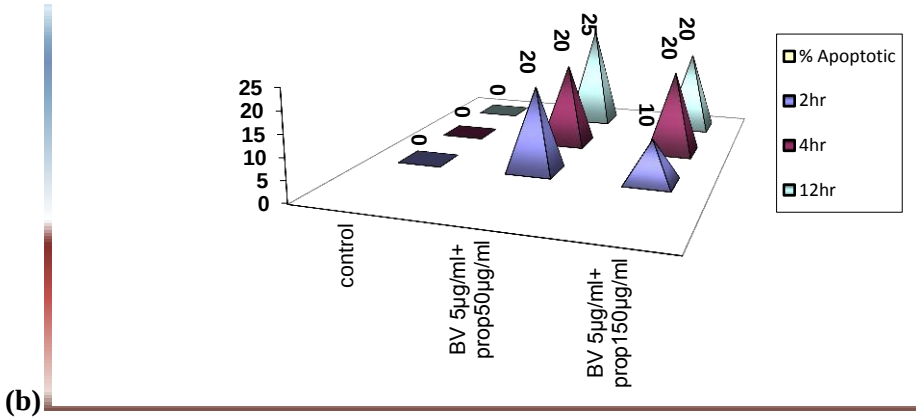


Figure (3): shows the morphological changes in MCF-7 cells after acridine orange/ ethidium bromide fluorescent staining for control and combination treated groups. **(a)** represents % dead cells and **(b)** represents% apoptotic cells.



(a)



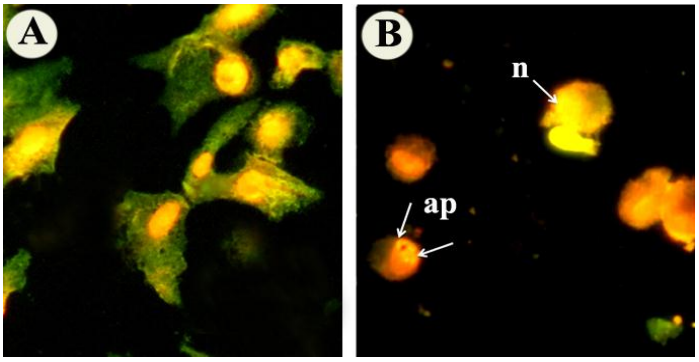


Figure (4): representative photomicrograph for MCF-7 cells nuclear morphology after ethidium bromide / acridine orange fluorescent staining, (400x). Where (A): normal control cells, (B): dead cells, where **ap**: apoptotic chromatin condensation and **n**: necrotic nuclei with high intensity of fluorescence.

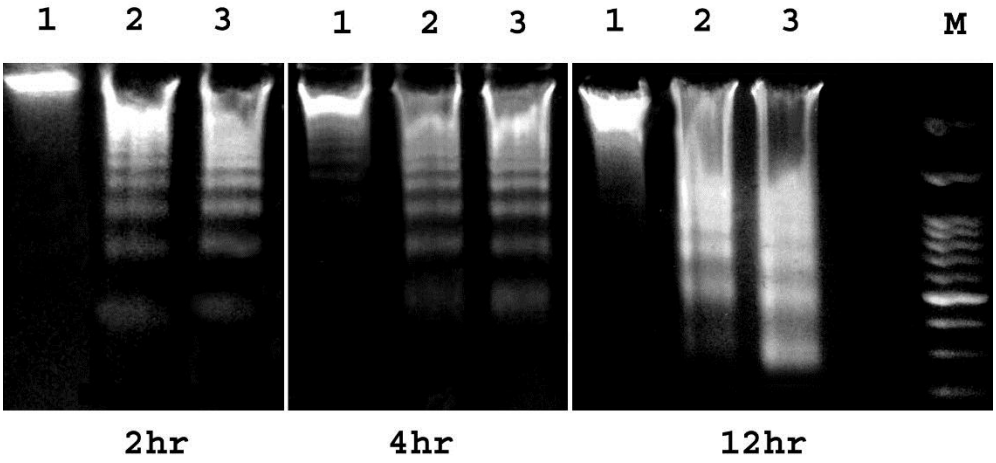


Figure (5): representative photograph of total genomic DNA electrophoresis showing the effect of treatments on MCF-7 cells. Where, lane: 1 resembles control cells, lane: 2: 5µg/ml of BV + 50µg/ml of propolis, lane: 3: 5µg/ml of BV + 150µg/ml of propolis and M: 100bp DNA marker.

Figure (6): shows optical densities of total genomic DNA fragmentation in treated and untreated MCF-7 cells after three different periods.

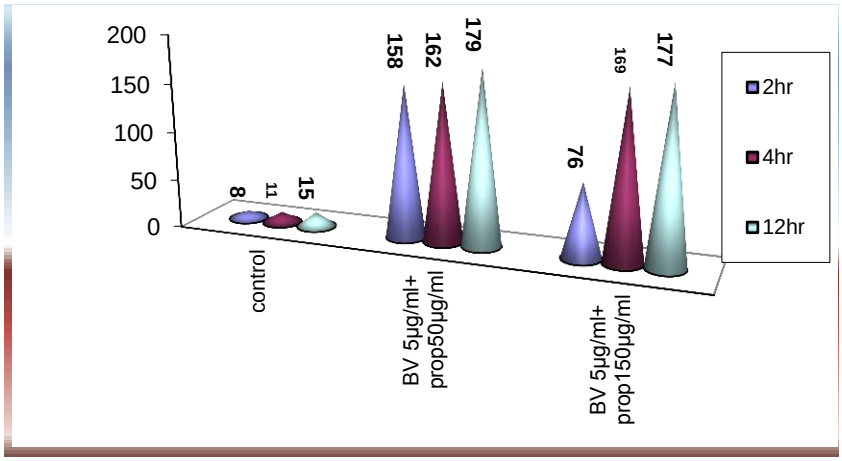
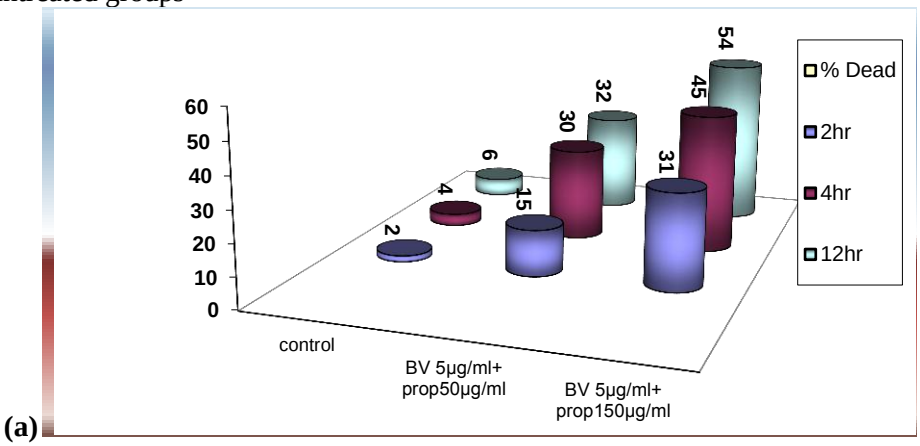
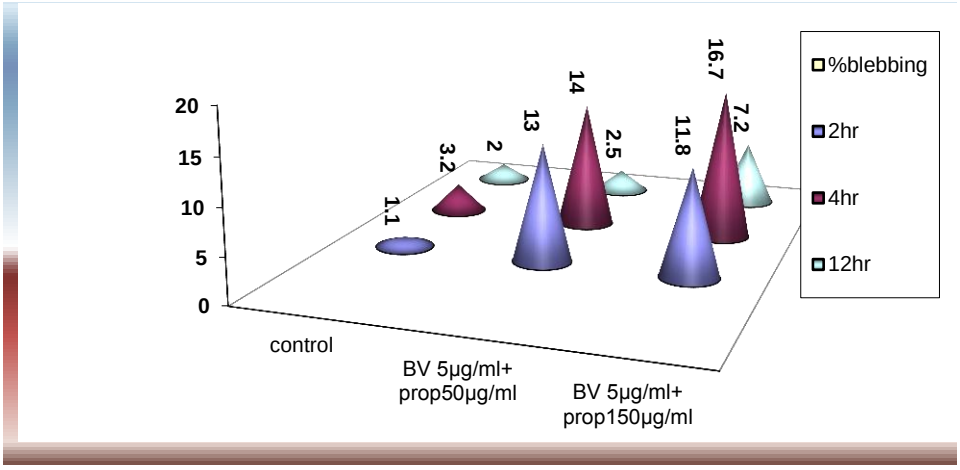


Figure (7 a, b): shows the percentage of blebbing and dead MCF-7 cells after Giemsa staining for treated and untreated groups



(a)



(b)

Figure (8): representative photomicrographs for Giemsa-stained MCF-7 cells showing morphological changes under light microscope (400x). Where (A): normal control cells, (B): cells with blebbing cytoplasm as a feature of apoptosis (arrows) and (C): dead cells with dark blue staining.

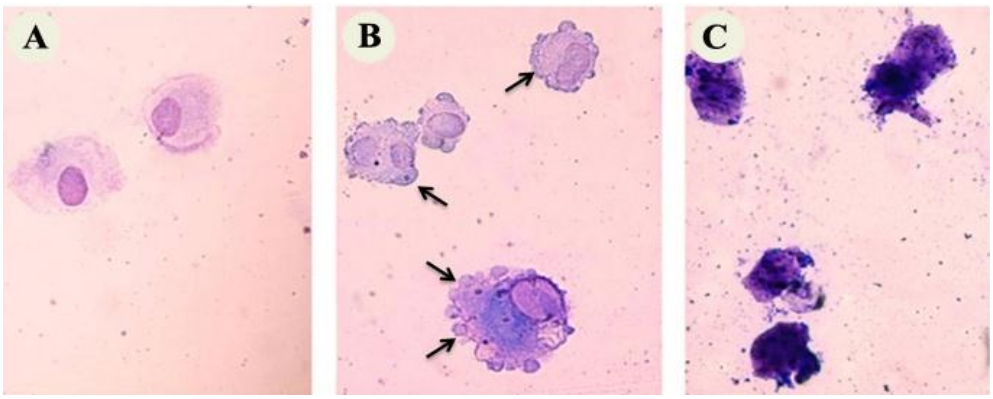
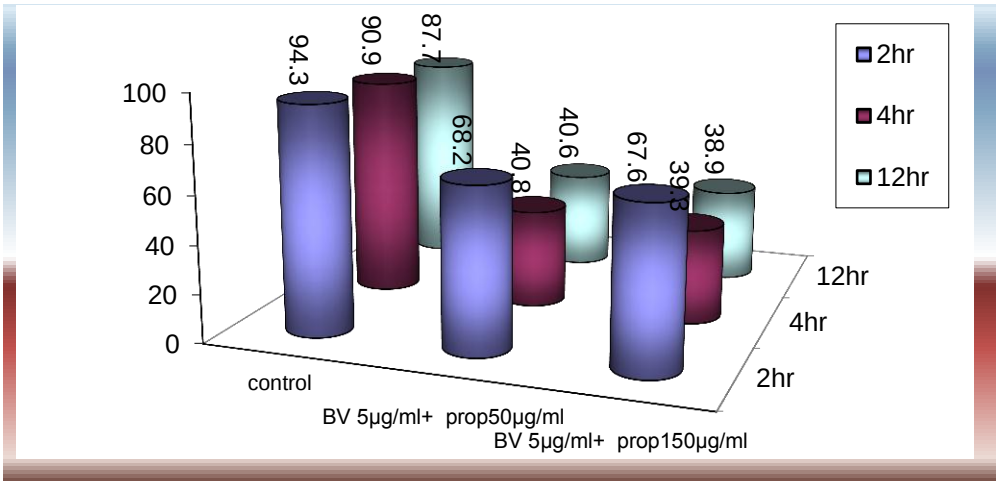


Figure (9): shows the percentage of positive Bcl-2 in MCF-7 cells after treatment with the combinations and untreated MCF-7 cells.



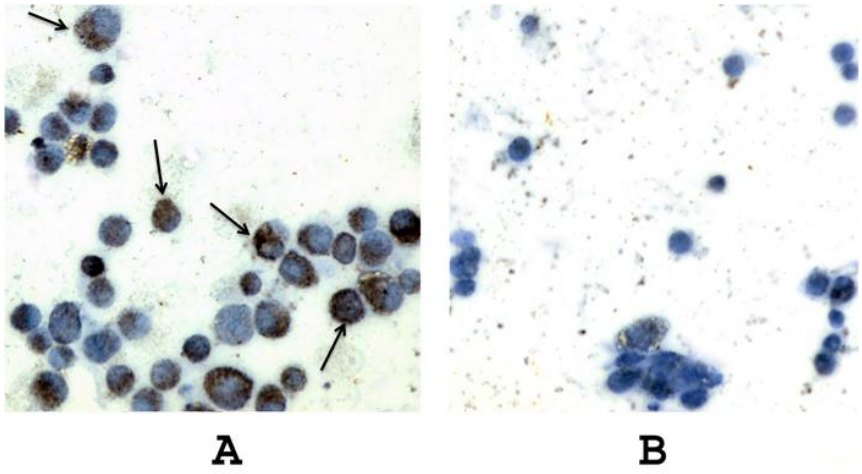


Figure (10): representative photomicrographs for Bcl-2 expression in treated and control groups. The positively stained cells were visualized under (400x) magnifications using light microscope. Where A: control, positive stained cells cytoplasm (arrows), and B: treated cells with very low expression of Bcl-2.

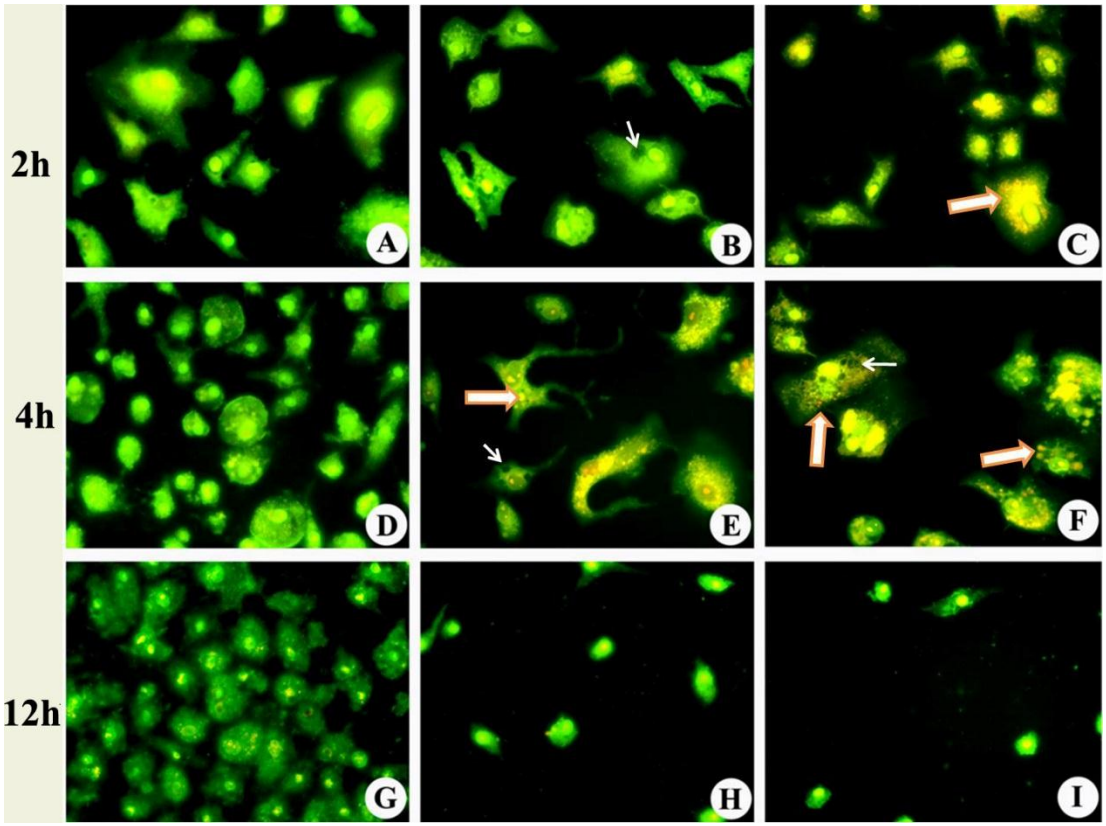


Figure (11): representative fluorescent photomicrograph showing the effect of BV and propolis combined treatment after 2, 4, & 12 hours on MCF-7 autophagy (400 x). Where, **A**: control 2hr, **B**: 5µg/ml of BV + 50µg/ml of propolis after 2hr, **C**: 5µg/ml of BV + 150µg/ml of propolis 2hr; **D**: control 4hr, **E**: 5µg/ml of BV + 50µg/ml of propolis after 4hr, **F**: 5µg/ml of BV + 150µg/ml of propolis 4hr; **G**: control 12hr, **H**: 5µg/ml of BV + 50µg/ml of propolis after 12hr, **I**: 5µg/ml of BV + 150µg/ml of propolis 12hr. (Thick arrows: acidic lysosomal vesicles) and (Thin arrows: cytoplasmic vacuoles).