Article

Induction of Apoptosis and Cell Cycle Arrest in two Human Cancer A549 and HT29 Cells Lines by Barhi Date Palm Kernels Extracts and Three Isolated Cytotoxic Compounds

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Abstract: *Background and objectives:* The initiation of apoptotic death in cancer cells is the aim of many chemotherapeutic strategies. As many anticancer agents have been discovered in natural plant sources, the search for novel phytochemicals displaying anticancer activities continues at a tremendous rate. This study was conducted to evaluate the effect of extractable polyphenols (EPP); non-extractable polyphenols (NEPP); nobiletin (NOB); tectorigenin (TEC) and persicogenin (PERS) from Barhi date palm kernels (BDPK) extracts on the induction of apoptosis in human lung cancer A549 and colon cancer HT29 cell lines. *Materials and Methods:* The anticancer activities were determined using MTT and trypan blue exclusion assays. Flow cytometry analysis was performed to study the cell viability, cell cycle arrest and apoptosis. The underlying mechanism of apoptosis induced by crude extracts and the purified compounds was investigated using caspase-3, -8 and -9 assays and the mitochondrial membrane potential assay. *Results:* The findings indicate that both BDPK extracts and the purified compounds do exert induced cell death on A549 and HT29 cells. The results observed from MTT- assay and trypan blue exclusion indicate that the cytotoxic effects of both plant extracts and the three isolated flavonoids are dose-dependent with higher cell death after 72 hours treatment. Treatment of human lung and colon cancer cells with EPP, NEPP, NOB, TEC and PERSI induced late stages of apoptosis as there was evidence of the DNA degradation and high percentage of the cells population was situated at sub-G_1 phase, indicating a high population of apoptotic cells. *Conclusions:* Study of the apoptotic mechanism demonstrated that EPP and NEPP exhibited dependent mitochondrial signalling pathway as seen with caspase-9 and induced receptor-mediated (extrinsic) apoptotic pathway as seen with caspase-8. Therefore, our results suggest that BDPK extracts and the three isolated flavonoids could be a good candidate for developing anticancer agents.

Keywords: apoptosis; Barhi date palm kernels (BDPK); extractable polyphenols (EPP); non-extractable polyphenols (NEPP); nobiletin (NOB); tectorigenin (TEC); persicogenin (PERSI).

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1. Introduction

Cancer is a term describing conditions characterized by unscheduled and uncontrolled cellular proliferation [1]. It is a very common disease, and its incidence is increasing at an average annual rate of 1.2% [2]. Lately, there has been an improvement in the treatment strategies for cancer, which has resulted in prolonged survival of patients with chronic cancer disease [3]. However, there is a growing need for additional means of cancer therapy, in the form of both palliative and curative treatments [4]. The strategies available today, are sophisticated and are only able to affect 50 to 60% of cancer patients, while the others will eventually die from the disease [5].

Chemotherapies have been being used for cancer treatment for more than five decades [6]; sometimes in combination with or parallel to surgery and radiotherapy [7]. After surgical ablation of progressive cancer, metastasized tumor cells continue to progress, and this is one of the faultiest associated with surgery [8]. On the other hand, radioactive rays and most anticancer chemotherapeutic agents damage DNA or suppress DNA duplication to kill the rapidly growing tumor cells [9]. At the same time, they also affect normal cells causing serious adverse effects, such as bone marrow function inhibition, bone necrosis, lung fibrosis, skin devascularization, ulceration, nausea, vomiting, renal damage and alopecia [7]. Thus, it is evident that a wide array of selective and potent components is needed to match the growing problems associated with cancer [10].

The extracts and purified compounds from botany and herbs displayed a vital role in biomedicine and exhibited s significant prototype for the design of new medication [11-13]. Natural products proposed a great source of phytochemicals with a wide variety of biotherapeutic effects and stable chemical structures. Numerous anti-cancer drugs had been isolated from natural sources; directly as pure native compounds, or as semi-synthetic analogues [14-17].

The traditions in the Middle East and Southeast Asia are particularly rich in medical plants that have been used by pioneer Arabic [18-20] and oriental physicians [21-24] to establish the basis for modern therapies. But a few of these plants have been examined scientifically. To this extent, we studied the potential effect of one Arabic traditional plant as anticancer agents. These were date palms *Phoenix dactylifera* that was mentioned in the holy Quran in more than one occasion which is traditionally used as a popular medication and foods.

وَهُزِّىٓ إِلَيْكِ بِجِذْعِ ٱلنَّخْلَةِ تُسَٰقِطْ عَلَيْكِ رُطَبًا جَنِيًّا (مريم - 25)

"Shake the trunk of this tree and fresh, ripe dates will fall down for you" (19:25) Holy Quran.

There have been studies that identified that the induction of programmed cell death in cancer cells occur in the presence of an extract from date palm seeds, some of which were compound mediated [25]. This verifies other research's findings that compounds such as those found in the *Phoenix dactylifera* extracts had inhibited allogenic solid Sarcoma-180 [26].

Some of the digested date extract and polyphenol-rich extract inhibits the oxidation of many harmful enzymes which leads to a decrease in the oxidation of certain fatty acids, have had adverse effects on human health [27]. There have been, however, indirect inhibitory effects on colon cancer growth by targeting some fermenter bacteria [27].

Paclitaxel stabilizes cellular microtubules, thus hindering any form of cellular functions that are important to the cell such as mitosis, cellular movement, cell cycle and spindle formation in DNA during these processes [28]. Paclitaxel was once widely used as an effective chemotherapeutic drug [29].

The purpose of this paper was to investigate the effect of extractable polyphenols (EPP); non-extractable polyphenols (NEPP); nobiletin (NOB); tectorigenin (TEC); persicogenin (PERS) from Barhi date palm kernels extract, and paclitaxel on human lung cancer A549 and human colon cancer HT29 cell lines and on the normal murine fibroblast 3T3 cell line using MTT crystal violet and trypan blue exclusion assays. Cell cycle progression using propidium iodide staining was investigated by flow cytometry. The apoptotic effect of the crude extracts and purified compounds were investigated using annexin V-FITC and propidium iodide staining. The underlying mechanism of apoptosis induced by crude extracts and purified compounds was investigated using caspase-3, -8 and -9 assays and the mitochondrial membrane potential assay. The current study focused on morphological

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alterations in both cancer cells and normal fibroblast after treatment with crude extracts and purified compounds.

2. Materials and Methods

2.1. Chemicals and Reagents

Fetal bovine serum (FBS), RPMI 1640 medium, Penicillin-streptomycin solution and phosphate buffer saline (PBS) were from Nacalai Tesque INC. (Kyoto, Japan). Accutase-EDTA (1×) were purchased from Innovative Cell Technologies, Inc. (San Diego, USA). Methanol and ethanol were from Merck Co. (Darmstadt, Germany). 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), RNase A, propidium iodide (PI), Dimethylsulfoxide (DMSO) and trypan blue were from Sigma (St. Louis, MO, USA). JC-1 Mitochondrial Membrane Potential Detection Kit (GeneCopoeia Inc., Rockville, MD, USA).

2.2. Sample Collection and Preparation

Barhi date fruits were purchased from the King of Dates market in Karrada-Dakhil, Baghdad, Iraq. The Barhi date palm kernels (BDPK) were collected at the "tamr stage" (full ripeness). BDPK were manually separated from the flesh rip fruit and soaked in water, washed to free them of any adhering date flesh, air-dried carefully under the shade at room temperature, then dried at 45°C in an air forced oven for two days to avoid deterioration and growth of unwanted microorganisms. The dried BDPK were finely ground into powder (1 mm) in a hammer mill (Retsch SM100, Germany) to generate a homogenous powder and then stored in an airtight container at -20^0 C until analysis.

2.3. Extraction Process

2.3.1. Preparation of Extractable Polyphenols (EPP) Extract

Extractable polyphenols (EPP) extraction was performed according to the method of [30]. Barhi date palm kernels powder (2 g) was extracted, under optimal conditions, in 109 ml of 75.39% methanol/ethanol-to-water concentration and at 43.23°C for 2.37 hours. Following the extraction process, the supernatants were collected, capped tightly and stored at -20°C until analysis. All the experiments were carried out in triplicate.

2.3.2 Preparation of Non-Extractable Polyphenols (NEPP) Extract

For the NEPP acid hydrolysis, the residue post-EPP extraction process under optimized conditions was collected and was used for the extraction of NEPP following the method described by [31]. The extraction process was conducted in the absence of a metal catalyst. Sirisena and her colleagues compared the yield of NEPP extracted from DPK in the presence and the absence of Fe3+ ions, and the results were obtained using spectrophotometric scan at absorption wavelength range 500 to 550 nm [31,32]. The scan for extraction without added Fe3+ showed a significant increase in absorbance at λ550 nm indicating the extraction of proanthocyanin. Based on these results, the NEPP extraction method was modified and performed without Fe3+ that could interfere with total flavonoid and antioxidant assays.

From the EPP residue, 2 g (dry weight) was mixed with 25-ml butanol/HCl (97.5:2.5, *v*/*v*) and heated at 100°C in a conical flask placed in the center of the magnetic stirrer, with stirring at a constant rate (at speed 7) for 3 hours (h). After the HCl-butanol depolymerization, the mixture was centrifuged at 7000 rpm for 15 min, the supernatant was collected, and the residue was subjected to two times of washings with 10 mL butanol. Supernatants were combined and evaporated at 60°C to a small volume, and the pH of the concentrated extract was adjusted to 4 with 1 M sodium hydroxide (NaOH). PH less than 4 and above 5 caused precipitation of the extract; hence PH ~4 was selected to

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maintain both intact extract and the compatibility with buffers. Using an amber reagent bottle, the dried extracts were collected, weighed to calculate the percent yield of the crude extract, capped tightly, freeze-dried and stored at -20°C until analysis. All the experiments were carried out in triplicate.

2.3.3. Fractionation and Isolation of Compounds

In this paper, which is based on unpublished data collected in the same experimental study [33]. Fractionation and isolation of compounds (nobiletin, tectorigenin and persicogenin) from Barhi date palm kernels (BDPK) was carried out following the method described by [33].

2.4. Cell Culture

The human lung carcinoma cells (A549) and human colon carcinoma cells (HT29) were obtained from the University Putra Malaysia (UPM). The murine fibroblast cell lines (3T3) were from the International Islamic University Malaysia (IIUM). They were chosen based on their high proliferation rates and availability. The cell lines (A549 and HT29) were routinely maintained as a monolayer in RPMI-1640 medium containing 10% fetal bovine serum, 5% penicillin-streptomycin, and 2 mM L-glutamine. 3T3 cell line was maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (inactivated at 56 °C for 30 min), 25mM HEPES (pH 7.4), penicillin (180 units/ml), streptomycin (100µg/ml) and amphotericin-B (0.2 µg/ml). The cells were grown to confluency in a humidified incubator with 5% CO2 in polystyrene culture flasks. They were subcultured by removing the medium and adding 1-3 ml of 0.05% accutase-EDTA solution. The cells were allowed to detach at (37˚C) for 5-10 min. About 1/6 of the detached cells or 1/4 of the other cells was passed twice a week to new flasks containing fresh-medium.

2.5. Cytotoxicity MTT Assay

Human colon cancer HT29, human lung cancer cells and murine normal 3T3 fibroblasts were seeded at a density of 5×10^3 cells/mL (200 µL/well) into 96-well plates and cultivated overnight in a humidified incubator with 5% CO_2 at 37°C. Then, the cells were treated with different concentrations of EPP; NEPP; NOB; PERSI from BDPK extracts as well as paclitaxel (as chemotherapeutic agent) for 24 hours. The cells of the control group were not treated with any compound. After 24 hours incubation, the numbers of cell proliferation were determined by the MTT "(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylte- trazolium bromide)" assay. The medium was removed and 1× PBS (200 µL/well) was used to gently wash the wells. Then, we mixed 5% MTT solution (Sigma-Aldrich, St. Louis, MO) with the medium in 1 to 9 ratio, and 200 µL mixture was added in each well. After incubated the cells for four hours at 37°C, the media was removed, and the MTT-formazan crystals formed by viable cells were dissolved in 200 µL of DMSO. Finally, the absorbance of the mixture at a wavelength of 570 nm was monitored with an enzyme-linked immunosorbent assay (ELISA) microplate reader (BioTek Instruments, Inc., Winooski, VT, USA).

2.6. "Trypan-Blue Dye Exclusion TBE" and Morphological Study

The "trypan-blue dye exclusion TBE" assay was used to determine the plant extract mediated cell death. 2 X 10^3 cells/well were seeded and grown in 6-well plates. After one day of treatment, the medium was replaced with different plant extracts and compounds (IC_{50} concentrations of EPP; NEPP; NOB; TEC and PERSI from BDPK extract, and chemotherapeutic drug (paclitaxel) in triplicates. After 72 hours incubation, the medium was discarded, and the cells were harvested by cell detachment solution (accutase) and washed twice with (PBS). A volume of 0.4% trypan-blue stain that is equal to the residual PBS was then added. After 5 min incubation, the cells were counted with a hemocytometer by compound light microscope. The unstained (viable) cells and the blue-stained (dead) cells were counted separately. Negative control cells were incubated with RPMI-1640 media

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without any extract and treated the same way. Each experiment was carried out in triplicate and repeated for one more time and the average of 3 wells was considered for each extract or compound. Before cells harvesting, general morphology and membrane changes in both cancer cells treated with extracts and compounds were examined via light inverted microscope (Olympus, Centre Valley, PA, USA).

2.7. Caspases 3, 8 and 9 Assays

Caspase activation remains the hallmark of apoptotic pathways. As such, bioluminescent caspase assays (Caspase-Glo, Promega) were used to determine the activation of caspases-3, -8 and -9 in A549 and HT29 cells after 24- and 27-hours exposure with IC_{50} concentrations calculated from MTT cytotoxicity assay. To evaluate the effect of 60 µg/mL of EPP, NEPP, NOB, TEC, PERSI and paclitaxel on the activities of caspases-3, 8 and 9 in A549 and HT29, 5×103 cells were seeded at every well in 96 well tissue culture plates (plating density determined following assay optimization for cancer cells to ensure maximum sensitivity for caspase detection). Clear cell culture plates were used to evaluate cell growth and confluence while white-walled plates (Nalge NUNC, VWR, Mississauga, ON) were used to measure the luminescent output of the assay. Cells were incubated for 24 hours and were then treated with either a 60 µg/mL of EPP, NEPP, NOB, TEC, PERSI or paclitaxel and treatment durations for 24 and 72hours were evaluated. Upon completion of the treatment, cell culture supernatant was carefully removed from each well and was replaced with room temperature l× PBS (50 µl/well). 50 µL of freshly reconstituted Caspase-Glo reagent was added to each well, and the plate was mixed on a plate shaker for 10 minutes, ensuring complete cell lysis. Plates were then incubated at room temperature for 3 hours prior to reading the luminescence in a plate-reading luminometer. Caspase activity of treated cells was expressed in optical density.

2.8. Cell Cycle Analysis

Flow cytometry is a technique that is used for the analysis of multiple parameters of individual cells within heterogeneous populations [34,35]. This method is used in various techniques, namely cell counting, immunophenotyping, green fluorescent protein expression as well as ploidy analysis [36]. When conducting this technique, a large number of cells (1000 cells/sec) pass through a laser beam, as these cells pass through the beam a light that emerges between the cells is then captured for analysis [37,38]. This data can be analyzed using flow cytometry software (BD Accuri), which can report on the cellular characteristics of each of the cells. These characteristics are namely the size, phenotype, health and complexity of the cell. The changes in the cell cycle of lung cancer A549 cells and colon cancer HT29 cells lines were monitored using a flow cytometer, propidium iodide was the stain used to detect the cells' DNA ploidy at different stages of the cell cycle. The histograms of cell cycle analysis illustrate cell progression and inhibition starting G0/G1 to G2/M phases through the S phase of the cell cycle. Both cancer cells were exposed to all treatments for 72 hours, fixed with 70% ethanol and stained with a DNA staining chemical; propidium iodide for DNA based cell cycle distribution analysis. Cells were allowed to grow to 70%- 80% confluence. This was followed by treatments of the cells with 60 µg/mL of EPP; NEPP; NOB and PERSI from BDPK extract, and chemotherapeutic drug (paclitaxel) for 72 hours prior to fixing.

The cancer cells were then washed with phosphate buffered saline (PBS) twice upon the removal of media and detached for two min with Accutase-EDTA. RPMI-1640 media was then added to detached cells then the cells were harvested by centrifugation (2054 ×g, 5 min, 4°C). Cells were re-suspended in 30 µL of ice-cold PBS and 70 µL of 100% ethanol which was chilled at -25°C. The chilled fixed cancer cells were kept at -25°C until they were required further experimental procedures. On the day of analysis, the fixed cells were washed with "ice cold phosphate buffered saline" and then stained with PI/RNAse staining solution (Sigma-Aldrich Co., St. Louis, MO, USA) and analysed by flow cytometry and analysed with the BD accuri™.

2.9. Annexin V/PI Staining Assay

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Cells were treated and incubated for 72 hours as described above with cell cycle analysis assay. Detached cells were harvested by centrifugation (2054 × g, 5 minutes, 25 °C). The supernatants were aspirated; the pelleted cells were washed with PBS then centrifuged (2054 × g, 5 minutes, 25 °C). The pelleted cells were re-suspended with 500 µL 1× Annexin V Binding buffer. The cells were then subjected to 5 µL Annexin V-FITC and 5 µL propidium iodide and incubated for 5 min at room temperature in the dark. Cells were then analysed using (Becton Dickinson, Franklin Lakes, NJ, USA).

2.10. Mitochondrial Transmembrane Potential

The depolarization role of our Barhi Date Palm Kernels extracts and purified compounds on the mitochondrial membrane potential was assessed through the efflux of rhodamine 123 dye. Cells were treated and incubated for 72 hours as described above with cell cycle analysis assay. Detached cells were harvested by centrifugation (2000 × g, 5 minutes, 25 °C). The supernatants were aspirated; the pelleted cells were washed with PBS then centrifuged (2054 × g, 5 minutes, 25 °C). The pelleted cells were re-suspended with 3 µL of a 5 mg/mL rhodamine solution in one mL of phosphate-buffered saline. The rhodamine 123 efflux or retention was analysed using FACSCalibur™ flow cytometry (Becton Dickinson, NJ, USA) and the data were analysed using CellQuest 3.3 software (Becton Dickinson).

2.11. Statistical Analysis

Statistical comparisons were made using Student's t-test. Results were expressed as means ± standard deviation (SD). P-values of less than 0.05 were considered significant.

3. Results

3.1. Cytotoxicity

The cytotoxicity of EPP, NEPP, NOB, TEC, PERS and paclitaxel on the A549, HT29, and 3T3 cells was determined by MTT assay. Figure 1A, B shows that EPP and NEPP have significant cytotoxic ($p < 0.05$) effects against lung cancer A549 and HT29 cells at all concentrations tested. In the case of the pure compounds (NOB, TEC, PERS), the lowest concentration (3.125 µg/L) had no significant effect ($p > 0.05$) on cell growth, where the lowest concentration (31.25 µg/L) of EPP and NEPP inhibited 97% and 90% (for A549) and 53% and 50% (for HT29) cells growth in the treated A549 and HT29 cells, respectively when compared to untreated cells (Figure 1A, B). Growth inhibition in cells was also observed to increase significantly (in a time-dependent manner) ($p < 0.05$) after the cells had been treated for 24-72 hours using the same extract or compound (Figure 1A, B).

The IC_{50} values of EPP, NEPP, NOB, TEC, PERS and paclitaxel calculated from the dose-response curves are shown in Table 1. The obtained results of MTT assay showed high significant changes ($p < 0.01$) in the viability of lung cancer A549 cells treated with NOB and TEC compounds at concentrations of 12.5 µg/mL and with PERS at 3.125 µg/mL for 24 hours when compared to cells treated with lower concentrations (Figure 1A).

On the other hand, the colon cancer HT29 cells were the least sensitive to all three compounds, with an IC_{50} values in the range 23-27 µg/mL, while the normal 3T3 fibroblast cells were found to be more resistant with an IC_{50} values of higher than 100 µg/mL (Table 1). The IC_{50} value of EPP in normal fibroblast 3T3 cells was 158 and 86 times higher than that shown in lung cancer A549 cells and colon cancer HT29 cells lines, respectively after 72 hours (Table 1).

Furthermore, compared with EPP, NEPP, NOB, TEC and PERS, paclitaxel showed lower selectivity against both cancer cells lines than seen with other treatments (Figures 1A, B). The cytotoxicity of paclitaxel was determined on cancer cells incubated for 24 and 72 hours at seven different concentrations 0.3125-10.0 µg/mL and on normal cells at seven different concentrations 0.3125-30.0 µg/mL (Figure 1A, B, C). The HT29 cells were the most sensitive to the anti-proliferative effect of chemotherapy (paclitaxel) when compared to other cells. The normal fibroblast 3T3 cells

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were less sensitive to paclitaxel than cancer cells (Figures 1C). Longer incubation time to paclitaxel led to greater cellular sensitivity effects on cell health.

Table 1. Cytotoxic activity (IC_{50}) of extractable polyphenols, non-extractable polyphenols, nobiletin, tectorigenin, and paclitaxel on A549, HT29 and 3T3 cells after 24 and 72 hours.

Treatment	IC_{50} (μg/mL)					
	A549		HT29		3T3	
	24h	72h	24h	72h	24h	72h
EPP	21.6±1.80	16.16±1.56	34.35±2.99	29.68±3.92	1423±67.51	2555±63.17
NEPP	26.06±2.35	17.4±1.44	40.05±3.70	31.4±4.00	811±19.08	980±20.00
NOB	11.54±1.87	8.56±0.85	27.6±1.90	23.12±1.58	148.7±14.19	153.35±10.23
TEC	19.29±2.15	10.48±1.50	22.93±1.68	13.32±1.55	171.5±13.23	209±24.98
PERS	8±1.00	5.75±0.50	23.15±2.36	11.26±1.01	135.3±8.78	107±11.53
TAXO	1.08±0.02	0.84±0.05	0.54±0.08	0.25±0.04	10.64±1.18	4.42±0.23

IC_{50}, the concentration of a substance/treatment required to inhibit cell growth by half (50%); EPP, extractable polyphenols; NEPP, non-extractable polyphenols; NOB, nobiletin; TEC, tectorigenin; PERS, persicogenin; TAXO, paclitaxel; hours, h.

3.2. Trypan Blue Exclusion

Trypan blue exclusion cell viability assay is measuring cell membrane integrity [39] in the treated cells and it is one of the most common ways to assess cell viability and cytotoxic effects [40,41]. In Figure 2, the inhibitory concentrations (calculated from MTT assay) were proportional to the cell viability of cancer A549 cell line shown with trypan blue exclusion after 72 h exposure to EPP, NEPP and paclitaxel, and the percentage of viabilities (42%, 57% and 45%, respectively) were nearest to 50% which were seen with MTT results (Figure 1A).

The cell viability was reduced by about 36%, 13% and 22%, at IC_{50} concentrations of NOB, TEC and PERSI, respectively. In comparison to paclitaxel, both Barhi Date Palm Kernels extracts; EPP and NEPP exhibited less inhibition to the growth of A549. At IC_{50} concentrations of NOB, TEC and paclitaxel, HT29 cancer cells recorded the most growth (viability) when compared to the untreated cells and displayed 75%, 69% and 64%, respectively while the inhibition was significant higher ($p < 0.05$) than 50% according to the paired student t-test (Figure 4).

A549 cell line had a 47%, 40% and 48% cells growth after 72 h exposure to EPP, NEPP and paclitaxel, respectively while the same concentrations displayed a 50% cell growth with MTT assay after the same treatment period (Figure 1). The cell viability of 3T3 fibroblast cell line was reduced by about 88%, 76%, 66%, 69%, 54% and 22% at IC_{50} concentrations of EPP, NEPP, NOB, TEC, PERSI and paclitaxel, respectively (Figure 2).

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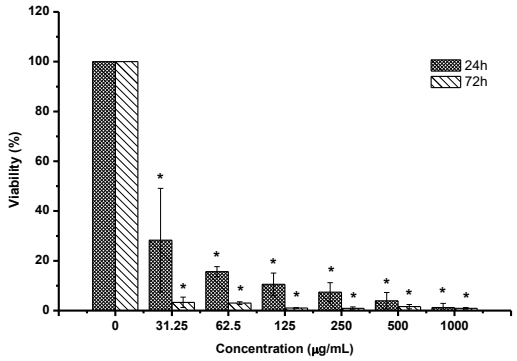
A

EPP



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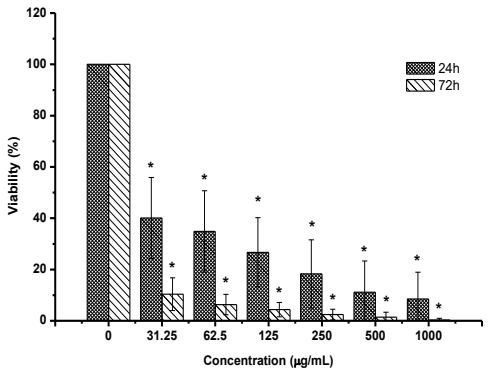

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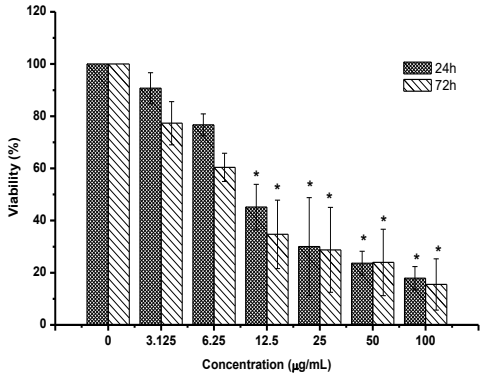

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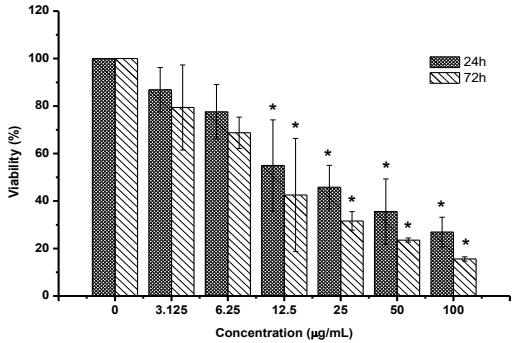

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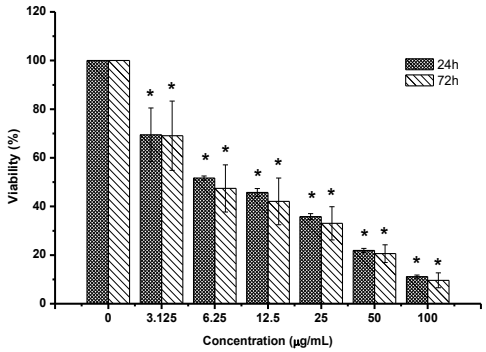

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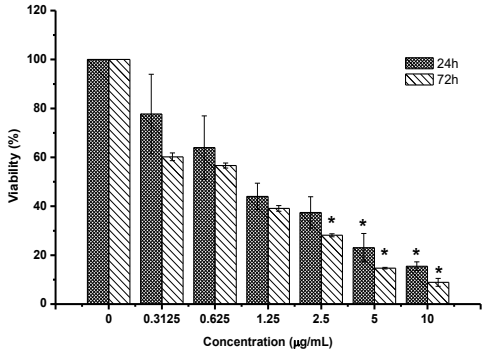

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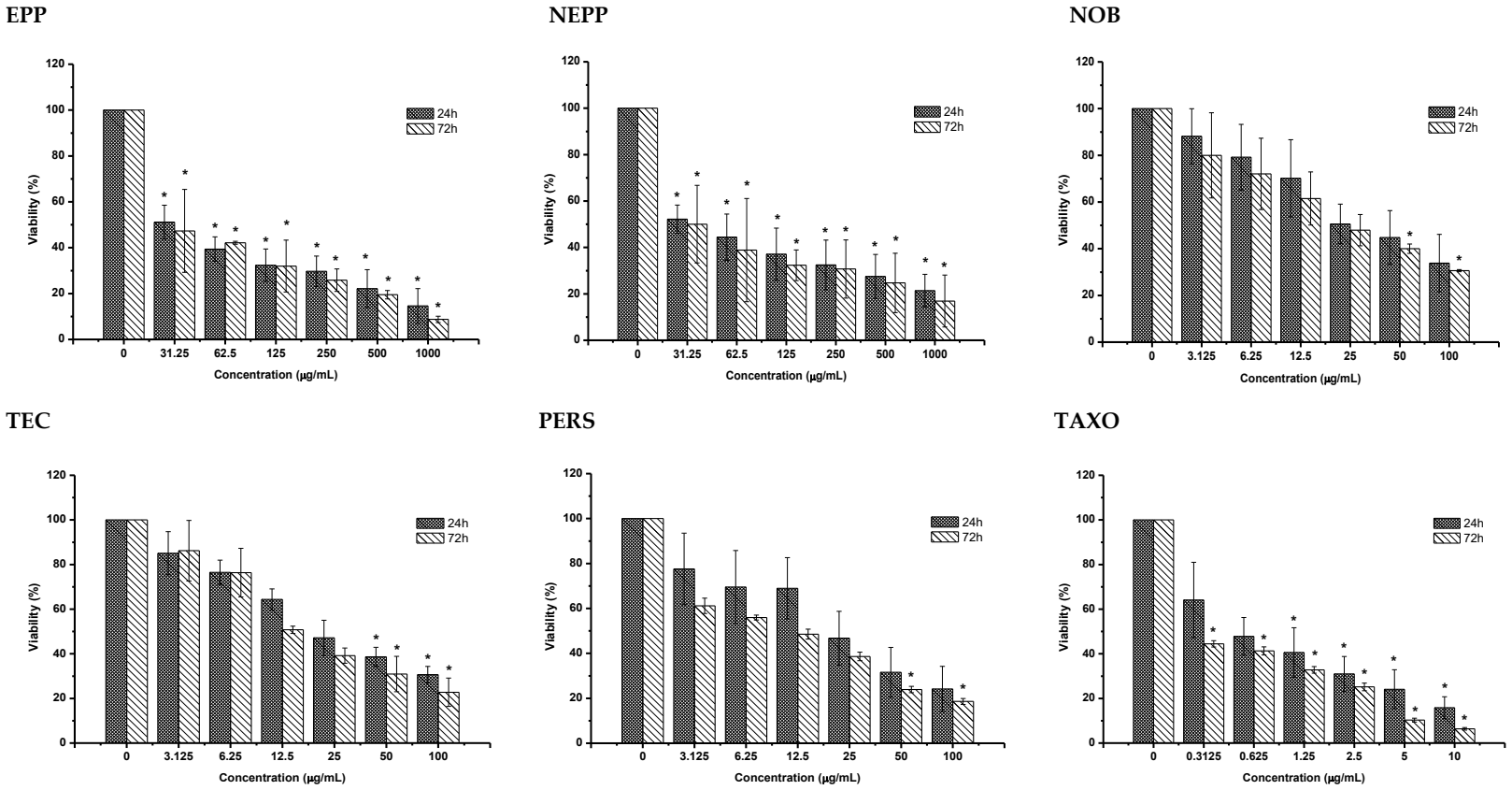
B



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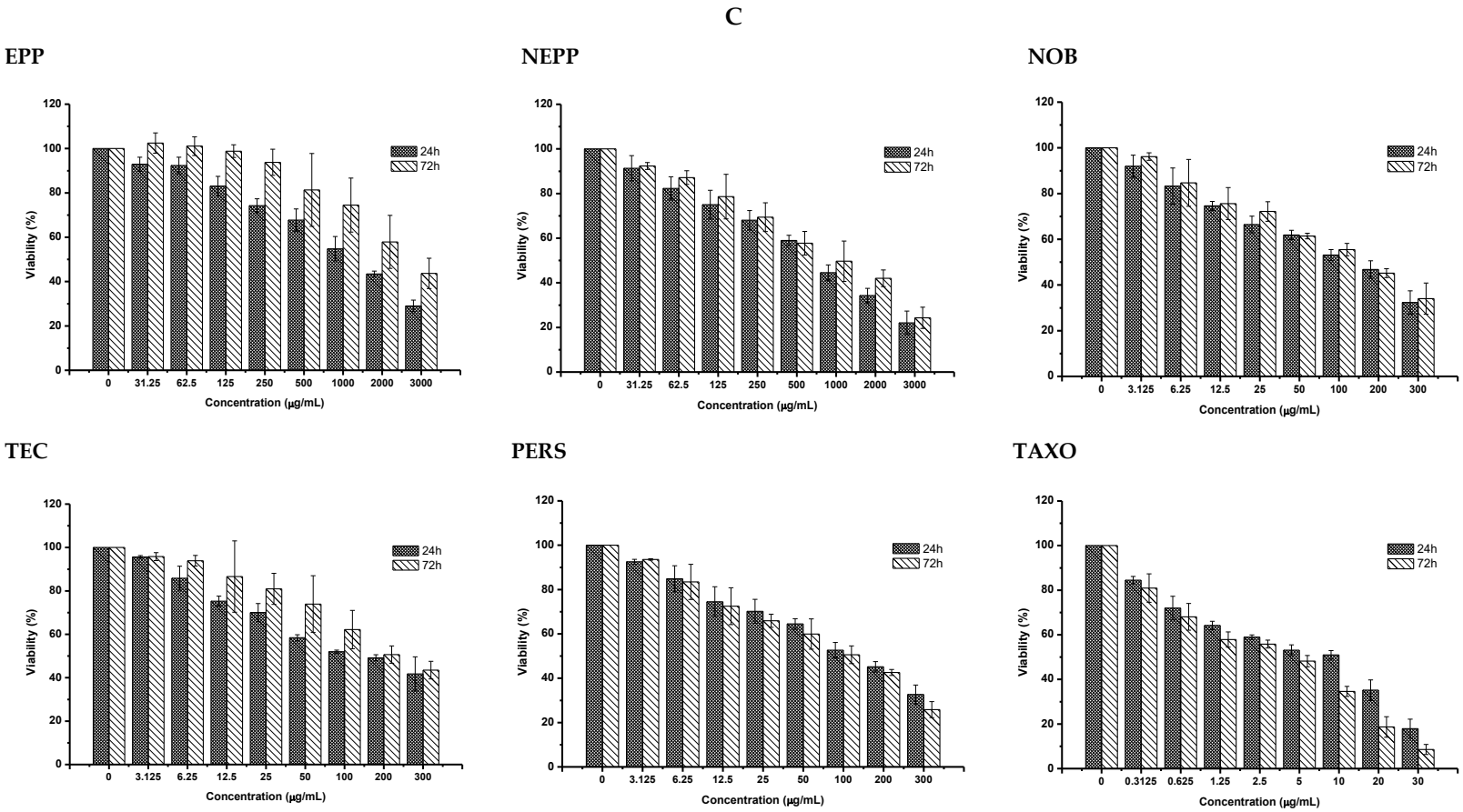

Figure 1. Effects of extractable polyphenols (EPP); non-extractable polyphenols (NEPP); nobiletin (NOB); tectorigenin (TE); persicogenin (PERS) and chemotherapeutic drug, paclitaxel (TAXO) on the viability of A549 (A), HT29 (B) and 3T3 cells (C). Cells were treated with different concentrations of Barhi date palm kernels extracts and their isolated flavonoids, and paclitaxel for 24 h and 72 h. Bars represent mean ± standard deviation (n=3 wells/treatment). *$p < 0.05$ compared with the untreated control cells.

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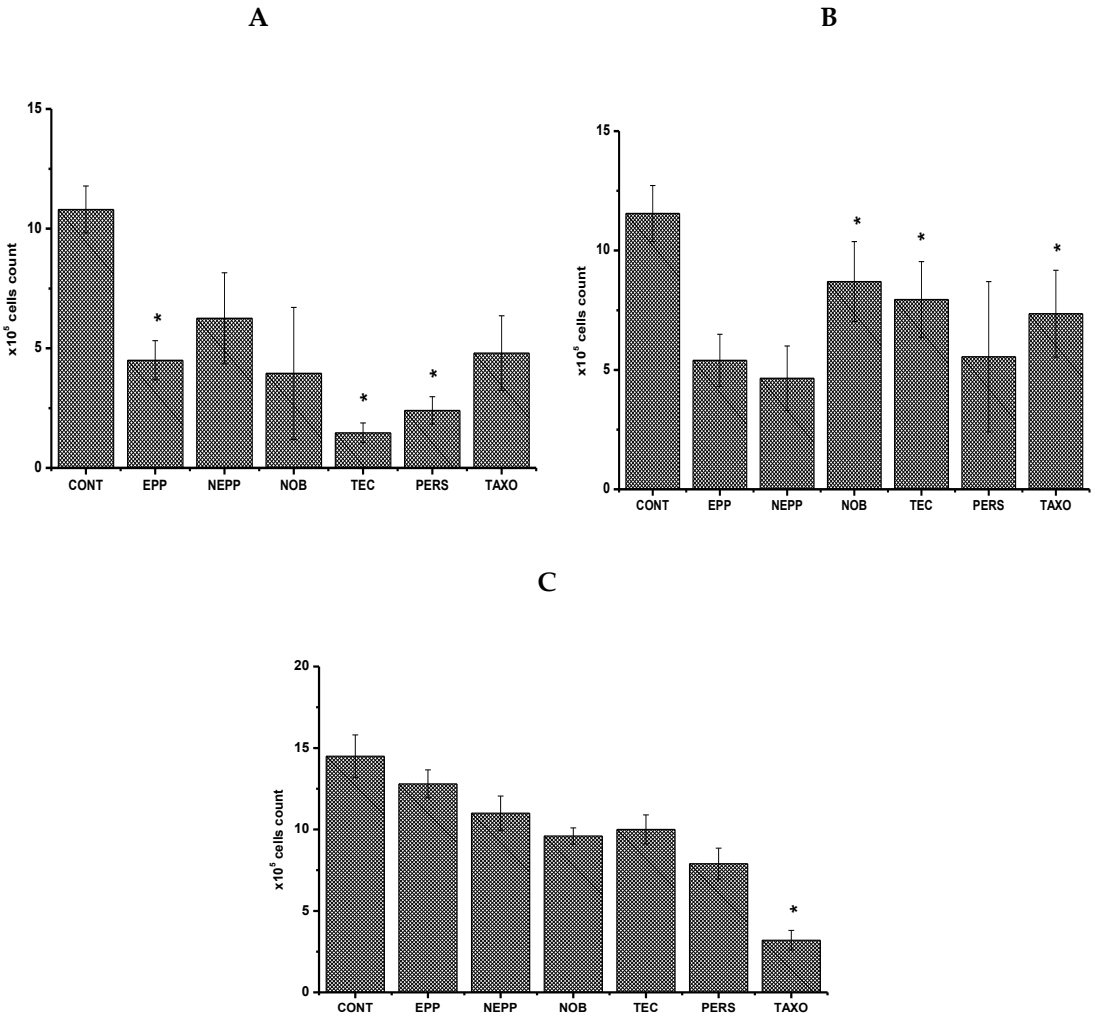

Figure 2. Cell viability of (A) A549, (B) HT29 and (C) 3T3 cancer and normal cells, respectively assayed by the trypan blue dye exclusion technique after 72 hours of treatment with IC50 concentration of Extractable polyphenols (EPP); Non-Extractable polyphenols (NEPP); Nobiletin (NOB); Tectorigenin (TE); Persicogenin (PERS) from Barhi date kernels extract, and Chemotherapeutic drug paclitaxel (TAXO). Mean ± standard deviation (n = 3 wells/treatment). *P< 0.05 compared with untreated cells. CONT, control untreated cells.

3.3. Morphological Alterations in the Treated Cells

The untreated lung cancer A549 and colon HT29 cells appeared to be morphologically intact and had a normal epithelial shape while untreated normal murine 3T3 are fibroblastic shaped cells. All untreated cells are relatively abundant in continuously attached monolayer. After the exposure to the EPP, NEPP, NOB, TEC and PERS, at a concentration of 60 µg/mL, the significant alterations in the morphology of A549 cancer cells were seen (Figure 3). NEPP and EPP extracts and TEC compound induced the most obvious changes to the A549 cells morphology such as, the rounding up and detachment of many cells (Figure 3).

On the other hand, the exposure of HT29 cells to 60 µg/mL of EPP, NEPP, NOB, TEC and PERS caused differences in the dramatic changes in the treated cells when a compared to untreated populations. Following the exposure to all BDPK extracts and compounds, HT29 became detached and rounded with accumulated objects at a single spot within the cellular cytoplasm as well as the rest of the cellular area evidently empty (Figure 5). These dramatic effects were observed mainly with NEPP extract (at 60µg/mL)–treated cells which is approved previously by MTT assay.

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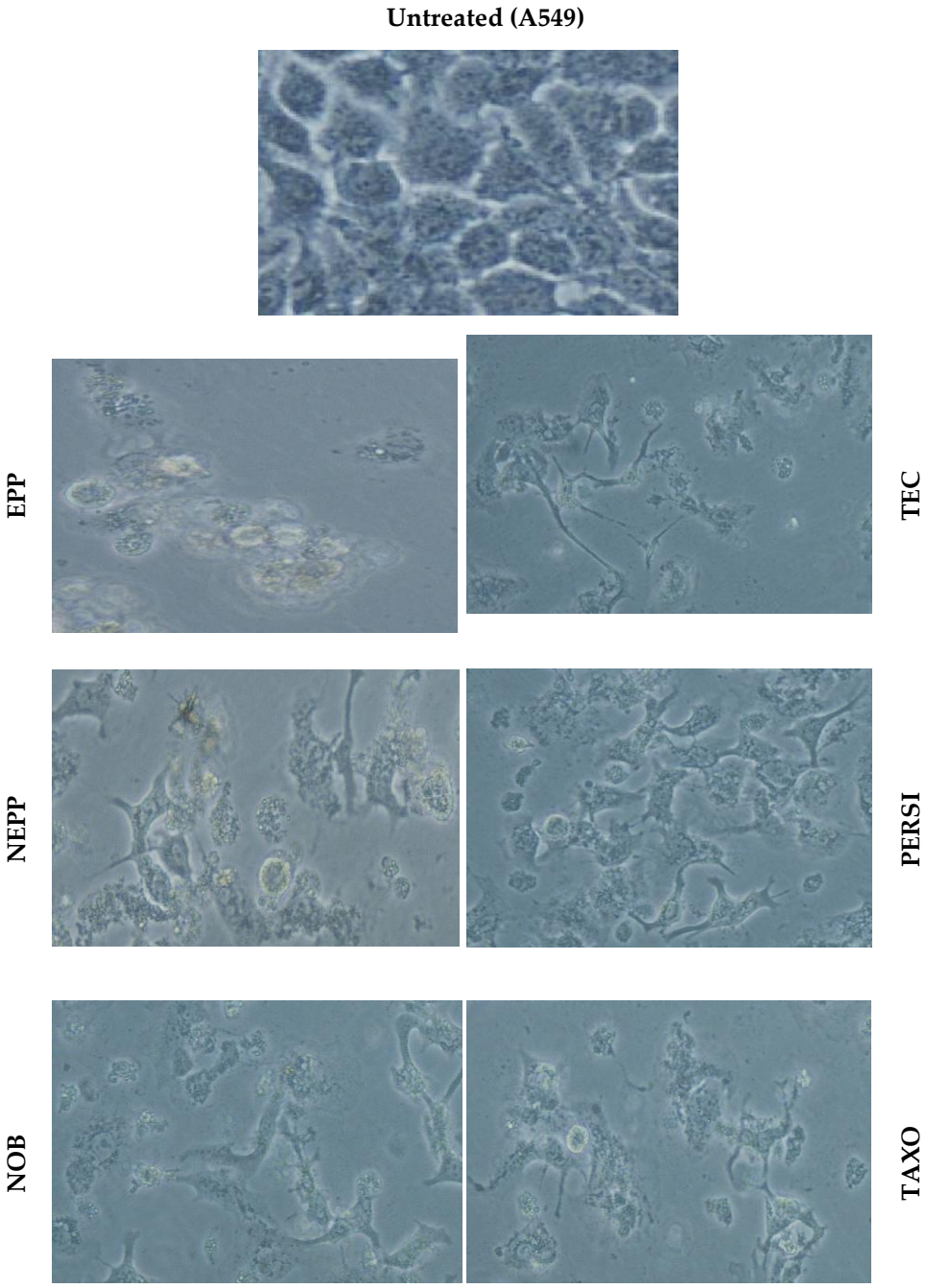

Figure 3. Morphological changes in A549 cells after treatment with extractable polyphenols (EPP); non-extractable polyphenols (NEPP); nobiletin (NOB); persicogenin (PERS) from Barhi date palm kernels extracts, and chemotherapeutic drug paclitaxel (TAXO). A549 lung cancer cells were pretreated with 60 μg/mL in culture medium for 72 h. Magnification (200×).

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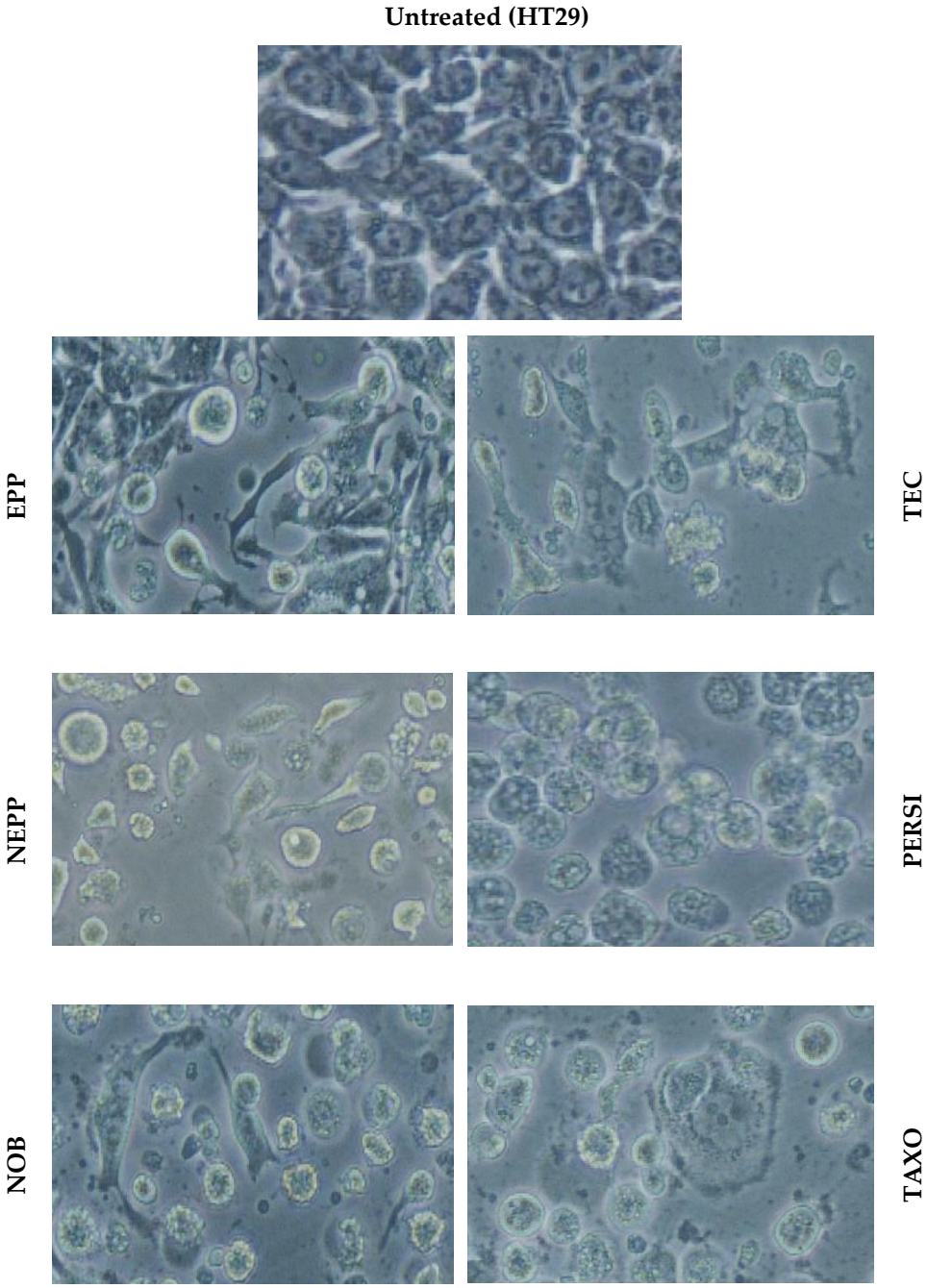

Figure 4. Morphological changes in HT29 cells after treatment with extractable polyphenols (EPP); non-extractable polyphenols (NEPP); nobiletin (NOB); persicogenin (PERS) from Barhi date palm kernels extracts, and chemotherapeutic drug paclitaxel (TAXO). HT29 colon cancer cells were pretreated with 60 µg/mL in culture medium for 72 h. Magnification (200×).

At 60µg/mL of BDPK extracts and compounds, the 3T3 cells' morphology was similar to that of cells from the untreated population. Therefore, 3T3 cells were exposed to the extracts and compounds at their IC50 value (Figure 5). Exposure of 3T3 cells with EPP and NEPP (at IC_{50} concentrations as

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calculated from MTT assay), the 3T3 cells showed morphological characteristics similar to the untreated cells with lower cells monolayer percentage (Figure 5).

The proliferation rate of 3T3 cells maintained in normal media was higher than HT29 and A549, as about 50% confluency was obtained after 72 hours of growth (Figure 5). Apparent alterations in cell morphology and few detachments of cells from the culture surface of 3T3 cells were observed after 72 hours of treatment with NOB, TEC and PERS at their IC50 concentrations (Figure 5).

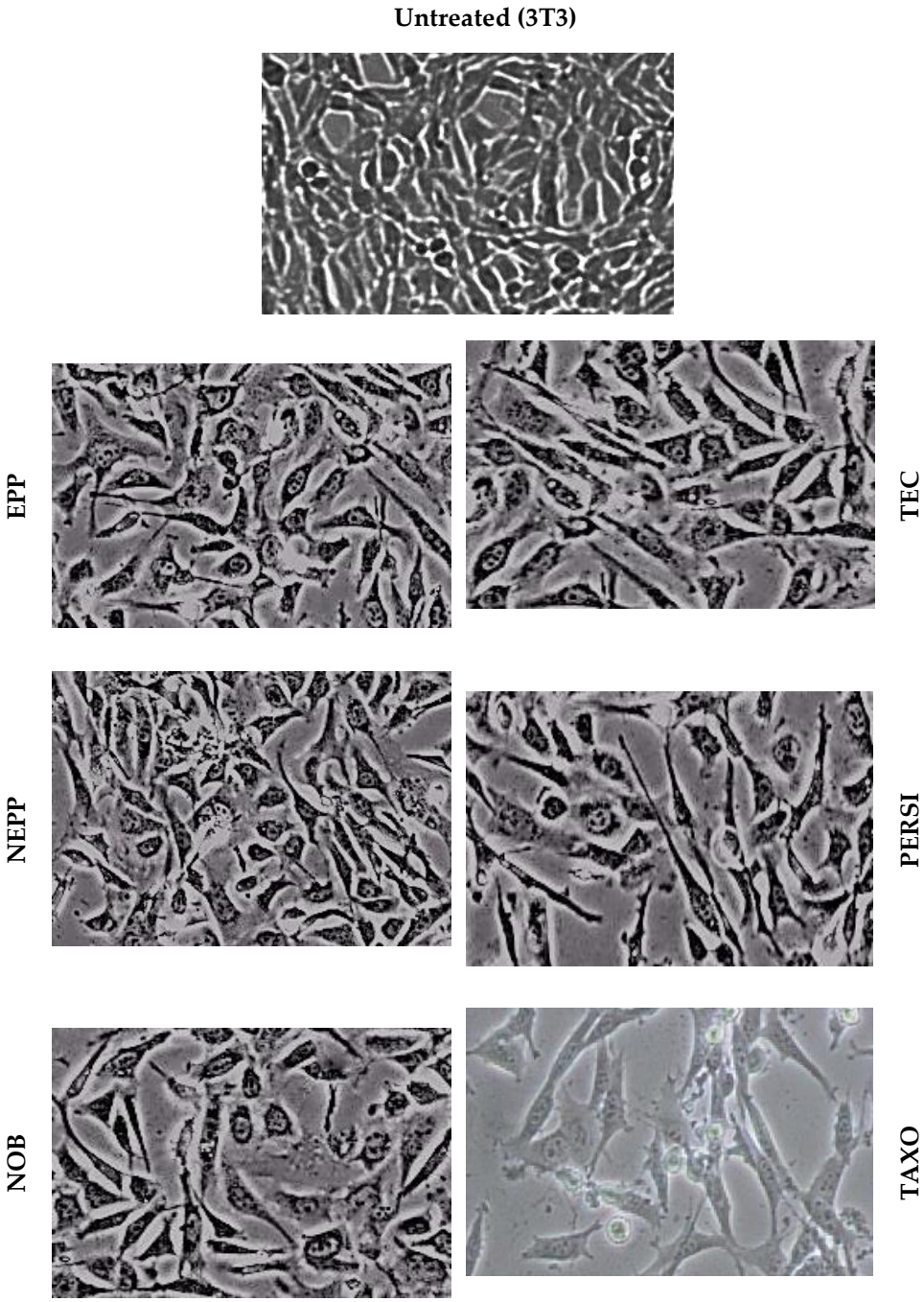


Figure 5. Morphological changes in 3T3 cells after treatment with extractable polyphenols (EPP); non-extractable polyphenols (NEPP); nobiletin (NOB); persicogenin (PERS) from Barhi date palm kernels extracts, and chemotherapeutic drug paclitaxel (TAXO). 3T3 normal fibroblast cells were pretreated with IC_{50} concentration in culture medium for 72 h. Magnification (200×).

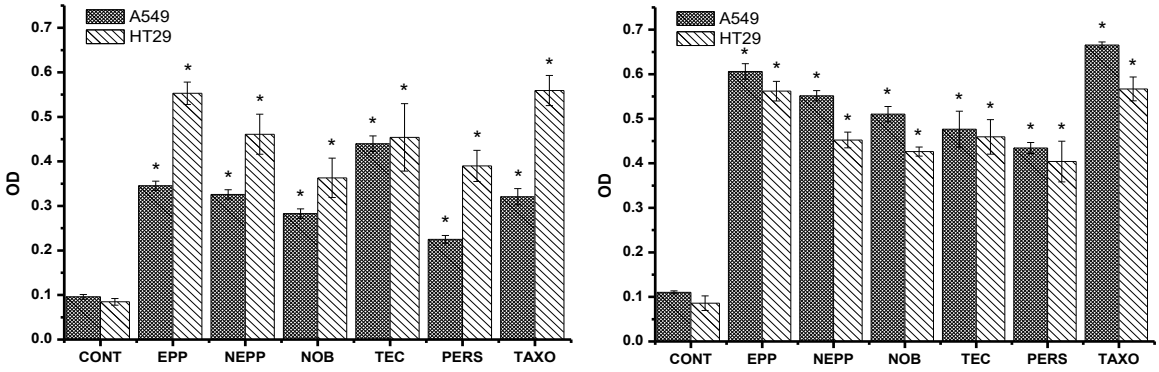
3.4. Caspase-3 -8 And -9

To define the effect of EPP, NEPP, NOB, TEC and PERS on the activities of the main caspases: caspase 3, 8 and 9 in human lung cancer A549 cells and human colorectal cancer HT29 cells, we treated them with 60 µg/ml for 24 and 72 hours. The caspases activities were evaluated from cell lysates using 96-well plates and a microplate reader. The results demonstrated that after treatment, cellular caspases activities of A549 cells have elevated in all BDPK extracts and compounds tested (Figure 6, 7, 8).

3.4.1. Caspase-3

The caspase-3 activity in the cancer cells treated by EPP, NEPP, NOB, TEC and PERS is represented in Figure 6A, B. Caspase-3 activities in both cells' lines, at all treatments, have increased when compared to untreated control cells after 24 and 72 hours. The optical densities were found to be significantly high ($p < 0.05$) at A549 (0.345 ± 0.010, 0.326 ± 0.011, 0.283 ± 0.011, 0.440± 0.017 and 0.225 ± 0.009), and HT29 (0.553 ± 0.025, 0.461 ± 0.045, 0.363 ± 0.044, 0.454 ± 0.076 and 0.390 ± 0.035) after one-day exposure to 60 µg/ml of EPP, NEPP, NOB, TEC and PERS, respectively when a compared to untreated A549 (0.096± 0.005) and HT29 (0.085± 0.008) cells, respectively (Figure 6A). The ability of paclitaxel to induce caspase-3 in lung cancer A549 cell line was the highest after 72 hours exposure when a compared to all compounds and extracts tested and colon cancer HT29 cell line (Figures 6B). The induction of caspase-3 reached the highest activity (659%) and (603%) as seen with HT29 and A549 cells lines, respectively when a compared to untreated cells after the applying of paclitaxel 2 µg/ml, respectively after 72 hours (Figures 6D).

(A) 24h (B) 72h



(C) 24h (D) 72h

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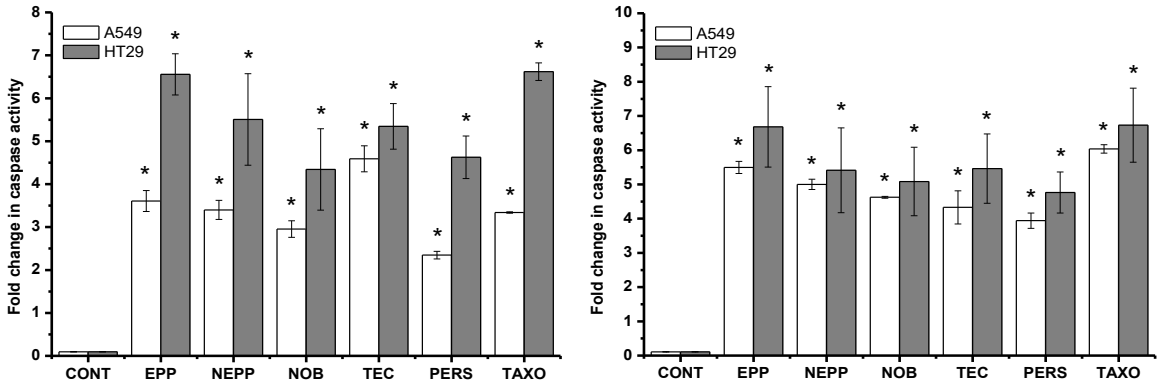
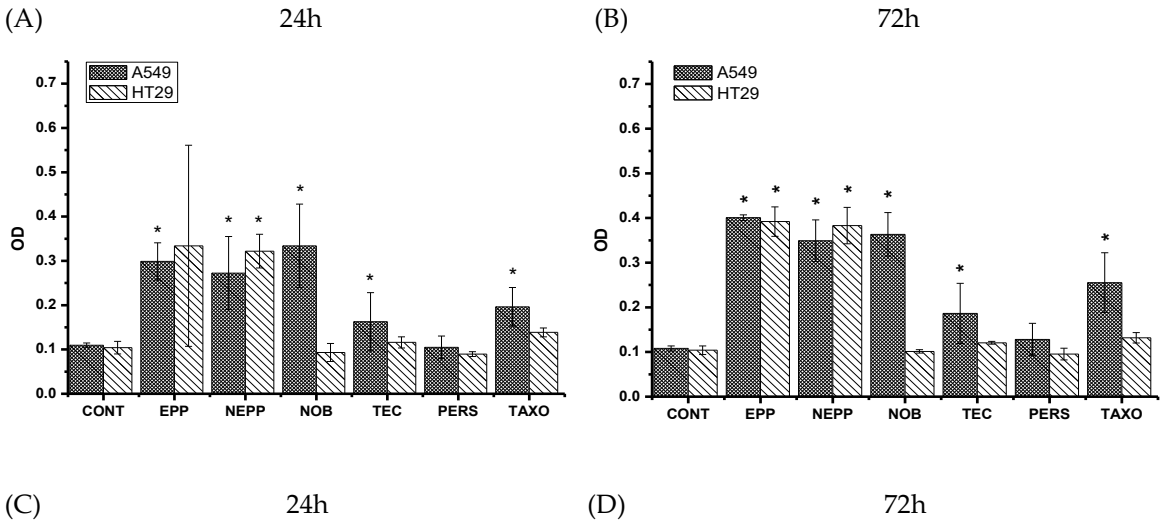


Figure 6. Cellular caspases and fold changes in caspase-3 activities of treated A549 and HT29 cells with respect to untreated control (CONT). Treatment with 60 µg/mL of BDPK extracts Extractable polyphenols (EPP) and Non-Extractable polyphenols (NEPP) and the three isolated flavonoids; Nobiletin (NOB); Tectorigenin (TEC) and Persicogenin (PERS), and the positive control paclitaxel (TAXO) was measured over 24 and 72 hours and their effects on caspase-3/7 (A) 24h, (B) 72h, and the fold changes in caspase-3/7 activates (C) 24h and (D) 72h were determined. Mean ± standard deviation (n = 3 wells/treatment). *P< 0.05 compared with untreated cells. OD, optical density. h, hours.

3.4.2. Caspase-8

In addition, both BDPK extracts; EPP and NEPP activated caspase-8 in a time-dependent manner, indicating the involvement of death-receptor pathway for apoptosis (Figure 7). We found that the levels of enzyme activities of caspase-8 in lung cancer A549 cell line have increased (3.0 and 1.5-fold for 24 h), and (3.4 and 1.7-fold for 72 h) more than untreated cells after exposure to 60 µg/ml of NOB and TEC, respectively in spite of that the same concentration have not exhibited a significant induction ($p < 0.05$) of caspase-8 after 24 hours and 72 hours in colon cancer HT29 cells (Figure 7C and 7D). No significant changes ($p > 0.05$) are observed in A549 and HT29 cells when the treatments with 60 µg/ml of PERS (96 % and 86%) are employed, respectively, when compared with untreated cells (Figures 7C and 7D).



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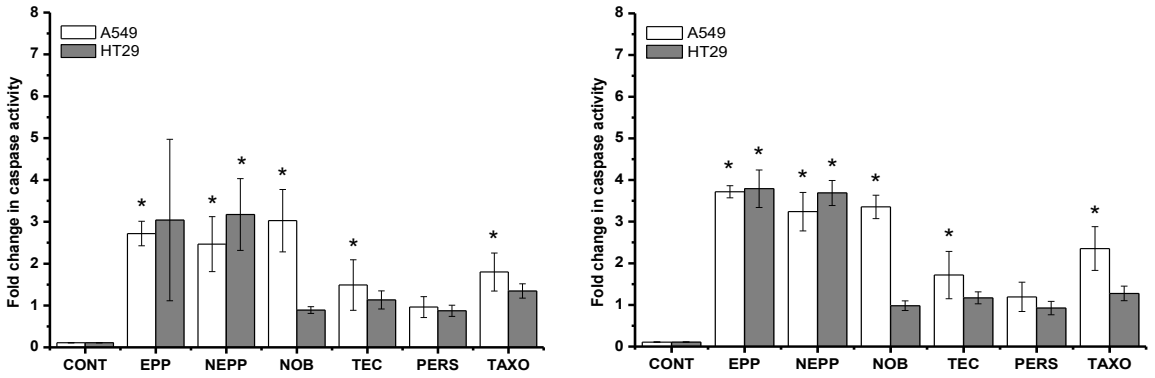
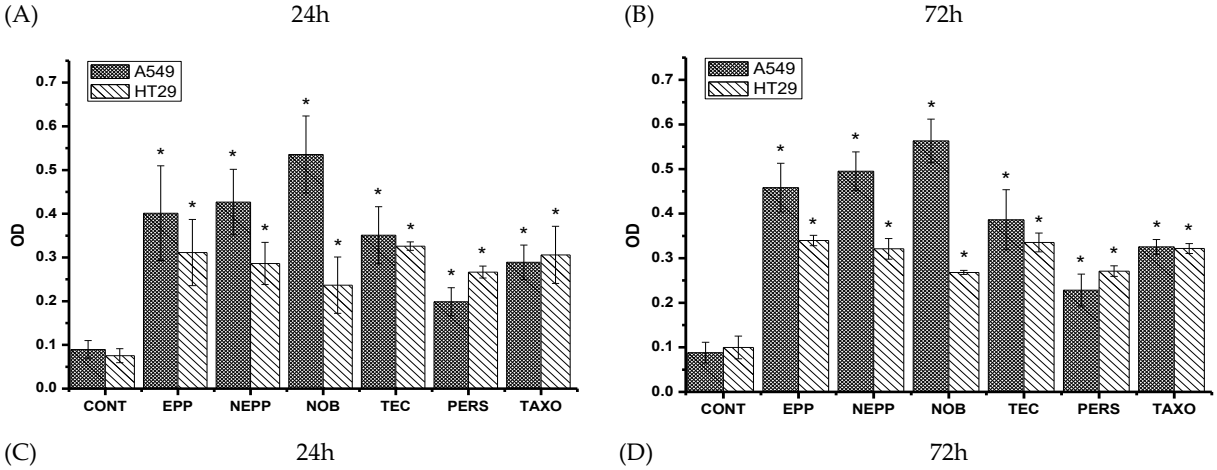


Figure 7. Cellular caspases and fold changes in caspase-8 activities of treated A549 and HT29 cells with respect to untreated control (CONT). Treatment with 60 µg/mL of BDPK extracts Extractable polyphenols (EPP) and Non-Extractable polyphenols (NEPP) and the three isolated flavonoids; Nobiletin (NOB); Tectorigenin (TEC) and Persicogenin (PERS), and the positive control paclitaxel (TAXO) was measured over 24 and 72 hours and their effects on caspase-8 (A) 24h, (B) 72h, and the fold changes in caspase-8 activates (C) 24h and (D) 72h were determined. Mean ± standard deviation (n = 3 wells/treatment). *$p < 0.05$ compared with untreated cells. OD, optical density. h, hours.

3.4.3. Caspase-9

The activities of caspase-9 after 24 and 72 h-exposure to EPP, NEPP, NOB, TEC and PERS have been investigated (Figure 8). The induction of caspase-9 has revealed that the effective inhibiting the growth of human colon carcinoma HT29 cells and human lung cancer cells, found in MTT assay as a result to exposure to both BDPK extracts and three purified compounds, was due to mitochondrion-mediated apoptosis as well as death-receptor pathway apoptosis (as seen with caspase-8) (Figure 7). In different way than seen with caspase-8 observations, the activity of caspase-9 in colon cancer HT29 cell line was increased in high significant way ($p < 0.01$) by 3.1-fold, 4.3-fold and 3.5-fold after 24 hours exposure to 60 µg/ml of NOB, TEC and PERS, respectively when compared to untreated cells (Figure 8C) while no such increase occurred with 72 hours of exposure same compounds when a compared to 24 hours. The activity of caspase-9 showed marked changes in A549 (448%, 476%) and HT29 (413%, 380 %), cells lines after 24 hours treatments by 60 µg/ml of EPP and NEPP, respectively, when compared with untreated cells (Figure 8C).



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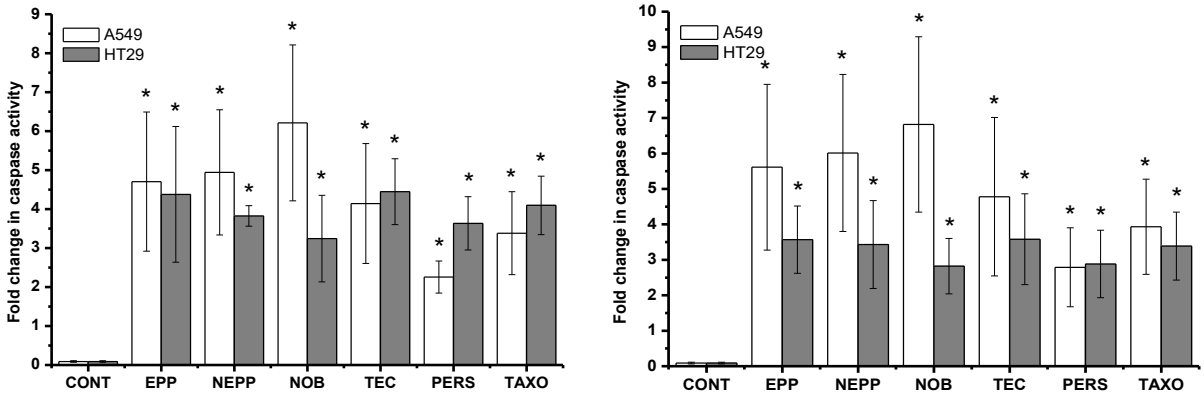

Figure 8. Cellular caspases and fold changes in caspase-9 activities of treated A549 and HT29 cells with respect to untreated control (CONT). Treatment with 60 µg/mL of BDPK extracts Extractable polyphenols (EPP) and Non-Extractable polyphenols (NEPP) and the three isolated flavonoids; Nobiletin (NOB); Tectorigenin (TEC) and Persicogenin (PERS), and the positive control paclitaxel (TAXO) was measured over 24 and 72 hours and their effects on caspase-9 (A) 24h, (B) 72h, and the fold changes in caspase-9 activates (C) 24h and (D) 72h were determined. Mean ± standard deviation (n = 3 wells/treatment). *P< 0.05 compared with untreated cells. OD, optical density. h, hours.

3.5. Cell Cycle Analysis

Propidium iodide is a stain used to bind to 4 base nucleotide of DNA thus identifying the stage that DNA is at different phases of the cell cycle [42,43]. This is conducted after the membrane has been permeabilized with the added fixative [44,45]. RNase is coupled with the propidium iodide dye to ensure that no RNA affects DNA integrity at each phase of the cell cycle [46].

To study the effect of EPP, NEPP, NOB, TEC and PERS on the cell cycle progression of cancer cell lines, two cancer cell lines (A549 and HT29) were selected. The selected cancer cell lines were incubated with two extracts and three compounds at a concentration of 60 µg/mL for 72 hours. After incubation, the cancer cells were stained with propidium iodide and analyzed by flow cytometer. The DNA flow histograms of A549 and HT29 cancer cells were plotted in Figures 9A, B and 10A, B, respectively and the percentage of change compared to control was presented in Table 2.

- A549

The effect of EPP, NEPP, NOB, TEC and PERS on the cell cycle progression of A549 cancer cell was shown in Figure 8. The total number of cells in G0/G1 increased notably from 82.78 ± 2.61 (for untreated cell) % to 93.75 ± 3.62%, 92.27 ± 1.62 %, 92.47 ± 4.34 % with the TEC, NEPP and PERSI, respectively. The total number of cells in G0/G1 changed slightly with NOB, EPP and paclitaxel when compared to untreated control cells.

However, the total number of cells in Sub-G$_1$ phase increased significantly ($p < 0.05$) from 3.83 ± 0.69 % with untreated cells to 77.12 ± 3.61 %, 80.86 ± 2.30 %, 76.48 ± 4.05 %, 72.97 ± 2.44 %, 83.41 ± 1.97 % and 88.73 ± 3.13 % after 72 hours exposure to 60 µg/mL of EPP, TEC, NEPP, PERSI, NOB and paclitaxel, respectively (Figure 9A, B). The change of sub-G$_1$ compared to untreated cells was increased to 21.78 and 21.11fold with NOB and TEC, respectively when a compared to the untreated cell, which was the second largest increase in Table 2 after paclitaxel (which exhibited 23.17-fold increase higher than control). The total number of cells in S phases decreased significantly ($p < 0.05$) from 7.19 ± 0.26 % with untreated cells to 4.59 ± 0.08 %, 4.95 ± 0.12 %, 4.60 ± 0.36%, 5.38 ± 0.21 %, 5.91 ± 0.16 % and 3.99 ± 0.36 % after 72 h exposure to 60 µg/mL of EPP, TEC, NEPP, PERSI, NOB and paclitaxel, respectively (Figure 9B).

Besides, the total number of cells in M/G2 phase decreased significantly (P < 0.05) from 10.03 ± 0.09 % with untreated cells to 4.72 ± 0.05 %, 1.33 ± 0.01 %, 3.13 ± 0.06 %, 2.15 ± 0.01 %, 3.60 ± 0.01% and

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6.68 ± 0.08 % after 72 hours exposure to 60 µg/mL of EPP, TEC, NEPP, PERSI, NOB and paclitaxel, respectively (Figure 9A, B). This indicated that the inhibitory effect of our extracts and compounds on cell cycle progression of A549 was mainly due to apoptosis and G0/G1 arrest (Figure 9A).

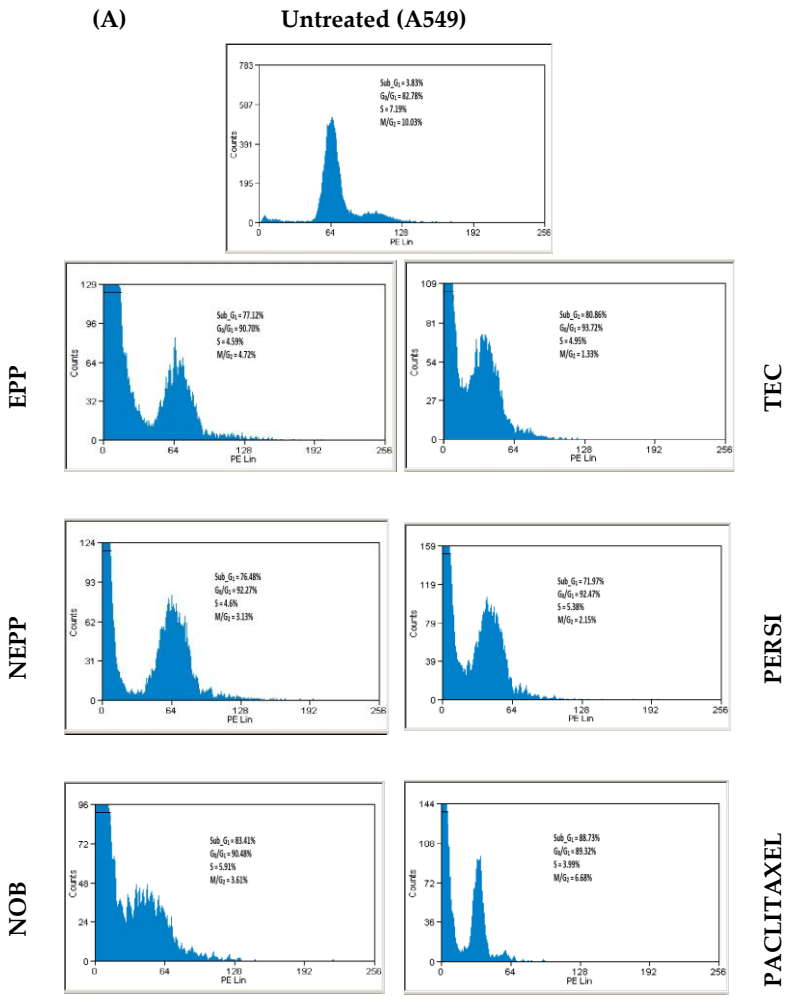

Figure 9. Histograms show DNA content analyses for A549 cells treated with 60 µg/mL of Extractable polyphenols (EPP); Non-Extractable polyphenols (NEPP); Nobiletin (NOB) and Persicogenin (PERSI) from BDPK extract, and chemotherapeutic drug paclitaxel (TAXO) for 72 h by flow cytometric analysis. Treated cells were stained with propidium iodide (PI) and processed for cell cycle analysis. flow cytometry analysis was performed on treated and untreated A549 cells. (A) Representative flow cytometry graph for each treated or untreated groups; (B) Histograms represent the percentage of cells in various phases. * $p < 0.05$ as compared with the untreated control cells. Data represent the mean ± SD of triplicate.

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(B)

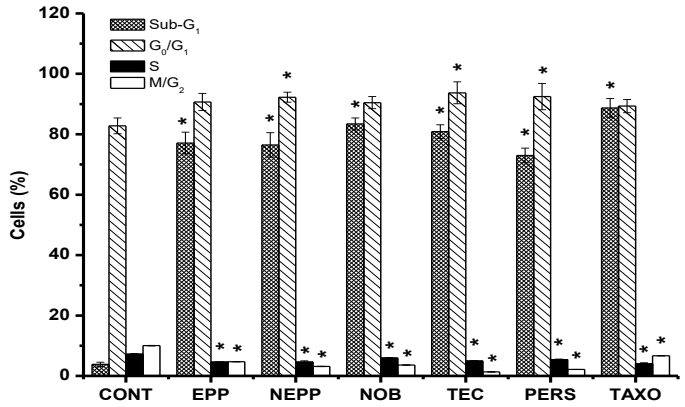

Figure 9. Continued.

Table 2. Effect of Barhi date palm kernels extracts and the three isolated compounds and paclitaxel on cell cycle distribution of lung A549 cancer cells determined by flow cytometry and fold changes in cells numbers with respect to untreated control.

Treatment	**A549**							
	Sub-G1	**Fold change**	**G_0/G_1**	**Fold change**	**S**	**Fold change**	**M/G_2**	**Fold change**
CONT	3.83±0.69	____	82.78±2.61	____	7.19±0.26	____	10.03±0.09	____
EPP	77.12±3.61*	20.14	90.70±2.84	1.10	4.59±0.08*	0.64	4.72±0.05*	0.47
NEPP	76.48±4.05*	19.97	92.27±1.65*	1.11	4.60±0.36*	0.64	3.13±0.06*	0.31
NOB	83.41±1.97*	21.78	90.48±1.99	1.09	5.91±0.16*	0.82	3.60±0.01*	0.36
TEC	80.86±2.30*	21.11	93.72±3.62*	1.13	4.95±0.12*	0.69	1.33±0.01*	0.13
PERS	72.97±2.44*	19.05	92.47±4.34*	1.12	5.38±0.21*	0.75	2.15±0.01*	0.21
TAX	88.73±3.13*	23.17	89.32±2.16	1.08	3.99±0.36*	0.55	6.68±0.08*	0.67

The table depicts the mean percentage of each cell cycle phase ± S.D. from three independent experiments. *$p < 0.05$ representing a significant difference from the control/untreated. Fold changes was calculated based on the untreated control. Abbreviations: CONT, control/untreated cells; EPP, extractable polyphenols; NEPP, non-extractable polyphenols; NOB, nobiletin; TEC, tectorigenin; PERS, persicogenin; TAX, paclitaxel.

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- HT29

The effect of EPP, NEPP, NOB, TEC and PERSI on the cell cycle progression of HT29 cancer cells was shown in Figure 9A. After 72 hours treatment with PERSI and both BDPK extracts EPP and NEPP (at 60 µg/mL), the total number of cells in G0/G1 phase increased significantly ($P < 0.05$) from 71.11 ± 0.63 % with untreated cells to 87.00 ± 0.14 % , 91.58 ± 1.06 % and 80.12 ± 1.03, respectively, on the other hand, the total number of cells in G0/G1 phase decreased significantly ($P < 0.05$) to 50.33 ± 0.31 % after 72 hours exposure to paclitaxel, with no significant changes ($P \geq 0.05$) in G0/G1 phase were observed with HT29 cells after exposure to TEC and NOB (Figure 10A, B).

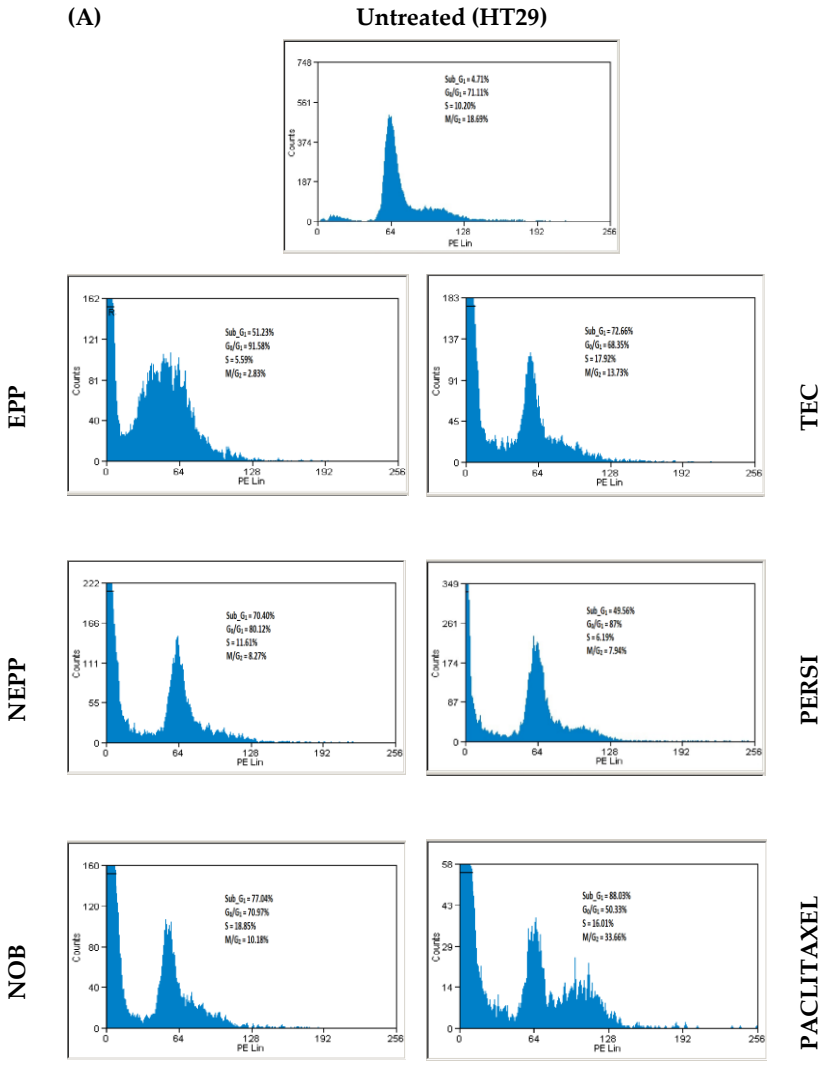

Figure 10. Histograms show DNA content analyses for HT29 cells treated with 60 µg/mL of Extractable polyphenols (EPP); Non-Extractable polyphenols (NEPP); Nobiletin (NOB) and Persicogenin (PERS) from Barhi date kernels (BDPK) extract, and chemotherapeutic drug paclitaxel (TAXO) for 72 h by flow cytometric analysis. Treated cells were stained with propidium iodide (PI) and processed for cell cycle analysis. Flow cytometry analysis was performed on treated and untreated HT29 cells. (A) Representative flow cytometry graph for each treated or untreated groups; (B) Histograms represent the percentage of cells in various phases. * $p < 0.05$ as compared with the untreated control cells. Data represent the mean ± SD of triplicate.

The total number of cells in S phase decreased significantly ($P < 0.05$) from 10.20 ± 0.02% with untreated cells to 5.95 ± 0.02 and 6.19 ± 0.07, respectively % after 72 hours treatment with EPP and

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PERSI (at 60 µg/mL), while the total number of cells in S phase increased significantly ($P < 0.05$) to 17.92 ± 0.20 %, 18.85 ± 0.14 % and 16.01 ± 0.01 after 72 hours exposure to TEC, NOB and paclitaxel, respectively with no significant changes ($P \geq 0.05$) in S phase were observed with HT29 cells after exposure to NEPP (Figure 10A, B).

Besides, the total number of cells in M/G2 phase increased significantly ($P < 0.05$) from 18.69 ± 0.13 % in untreated HT29 cells to 33.66 ± 0.14 % after 72 hours exposure to paclitaxel, while the exposure to EPP, TEC, NEPP, PERSI and NOB decreased the total number of cells at M/G2 phase to 2.83 ± 0.03 %, 13.73 ± 0.08 %, 8.27 ± 0.04 %, 7.94 ± 0.0.8 % and 10.18 ± 0.08 %, respectively (Figure 10A, B).

Furthermore, the total number of sub-Gl cells increased significantly ($P < 0.05$) from 4.71 ± 0.04 % with untreated cells to 51.23 ± 0.67 %, 72.66 ± 0.70 %, 70.40 ± 0.54 %, 49.56 ± 0.85 %, 77.04 ± 1.37 % and 88.03 ± 1.67 % after 72 hours exposure to 60 µg/mL of EPP, TEC, NEPP, PERSI, NOB and paclitaxel, respectively. The change of sub-Gl population in HT29 cells was increased 16.36-fold, 15.43-fold and 14.95-fold higher than untreated cells, which were the largest increase in Table 3 after paclitaxel-treated cells. This indicated that the inhibition of cell proliferation of all BDPK extracts and purified compounds were mainly due to the apoptosis and cell cycle arrest.

The cell cycle arrest was at G0/G1 phase with both extracts EPP and NEPP as well as PERSI. The accumulation of cells population was at S phase after exposure to TEC and NOB (Figure 9A, B). Paclitaxel-treated HT29 cells exhibited cell cycle arrest at both S and M/G2 phases (Figure 10A, B).

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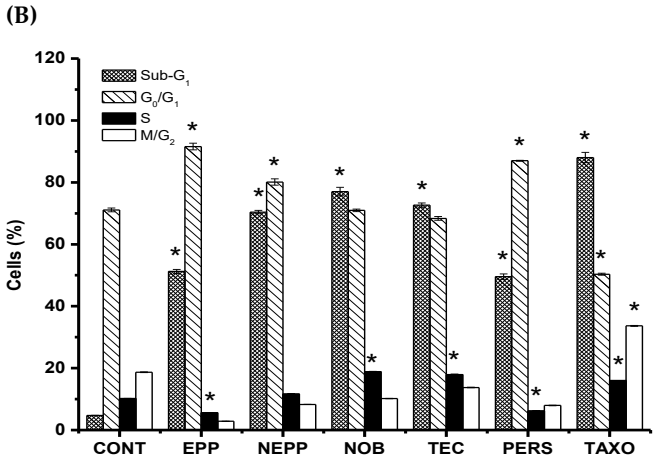

Figure 10. Continued.

Table 3. Effect of Barhi date palm kernels extracts and the three isolated compounds and paclitaxel on cell cycle distribution of lung HT29 cancer cells determined by flow cytometry and fold changes in cells numbers with respect to untreated control.

Treatment	HT29							
	Sub-G1	Fold change	G_0/G_1	Fold change	S	Fold change	M/G_2	Fold change
CONT	4.71±0.04	____	71.11±0.63	____	10.20±0.02	____	18.69±0.13	____
EPP	51.23±0.67*	10.87	91.58±1.06*	1.29	5.59±0.02*	0.55	2.83±0.03	0.15
NEPP	70.40±0.54*	14.94	80.12±1.03*	1.13	11.61±0.20	1.14	8.27±0.04	0.44
NOB	77.04±1.37*	16.34	70.97±0.42	0.99	18.85±0.14*	1.85	10.18±0.08	0.54
TEC	72.66±0.70*	15.42	68.35±0.61	0.96	17.92±0.20*	1.76	13.73±0.08	0.73
PERS	49.56±0.85*	10.51	87.00±0.14*	0.82	6.19±0.07*	0.61	7.94±0.0.8	2.35
TAX	88.03±1.67*	18.68	50.33±0.31*	0.71	16.01±0.01*	1.57	33.66±0.14*	1.80

The table depicts the mean percentage of each cell cycle phase ± S.D. from three independent experiments. *$p < 0.05$ representing a significant difference from the control/untreated. Fold changes was calculated based on the untreated control.Abbreviations: CONT, control/untreated cells; EPP, extractable polyphenols; NEPP, non-extractable polyphenols; NOB, nobiletin; TEC, tectorigenin; PERS, persicogenin; TAX, paclitaxel.

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3.6. Annexin V/PI Staining Assay

- A549

Human lung cancer A548 cells that were treated with BDPK extracts EPP and NEPP, NOB, TEC, PERS and paclitaxel were analyzed, using a flow cytometer, to measure the degree of apoptotic activity after treatment over a 24 hours period (Figure 11). The scatter graph was set up with four quadrants, lower left quadrant which represented viable cells (Annexin-V and Pi negative), upper left quadrant which represents dead cells, these were either necrotic cells or a cell death (PI positive), upper right quadrant which represents cells that underwent late apoptosis (Annexin-V and PI positive) and cells that underwent the early stages of apoptosis were represented by the lower right quadrant (Annexin-V positive). The data of cell death recorded in the upper right and lower right quadrants were added together in order to sum up the total percentage of apoptotic cells that were observed during the assay (Table 4).

The level of total apoptosis in lung cancer A549 cells are as follows: 0.18% (untreated cells), 97.88% (EPP), 96.89 % (NEPP), 61.4% (TEC), 73.39 % (PERSI), 44.33 % (NOB) and 64.7% (paclitaxel) (Figure 11). The data was collected in duplicates (n = 3) and the standard deviation was calculated from the mean (mean±SD), the varying degrees of apoptosis were plotted on a bar graph (Figure 12). The data were statistically analysed using Student's t-test and a high degree of significance ($P < 0.05$) was observed.

The analysis of untreated A549 cells indicated that 99.82% of cells located in the lower left quadrant, remained viable; 0.18% of cell death was observed with 0% in the upper left quadrant, 0.03% in the upper right quadrant and 0.18% were found in the lower right quadrant (Figure 11). A549 cells that were treated with both Barhi Date Palm Kernels extracts EPP and NEPP indicated that 2.12% and 3.10 % of cells located in the lower left quadrant remained viable; 97.88% and 96.9% cell death was also observed with 0.0% and 0.01% cells in the upper left quadrant, 91.89%, 86.28% in the upper right quadrant, and 5.99% and 10.61% in the lower right quadrant, respectively (Figure 11).

A549 cells treated with NOB, TEC and PERSI (Figure 11) displayed 55.67%, 12.20% and 17.69 % viable cells in the lower left quadrant; 44.33%, 87.80% and 82.31% cell death was also observed with 0.00%, 26.40% and 8.92% in the upper left quadrant, 1.15%, 40.41% and 56.31% in the upper right quadrant and 43.18%, 20.99% and 17.08% in the lower right quadrant, respectively. In Figure 11, data indicated that 12.12% of cells treated with paclitaxel remained viable (lower left quadrant); 87.88% cell death was recorded with 31.81% in the upper left quadrant, 27.18% in the upper right quadrant and 36.89% in the lower right quadrant (Figure 11).

Untreated (A549)

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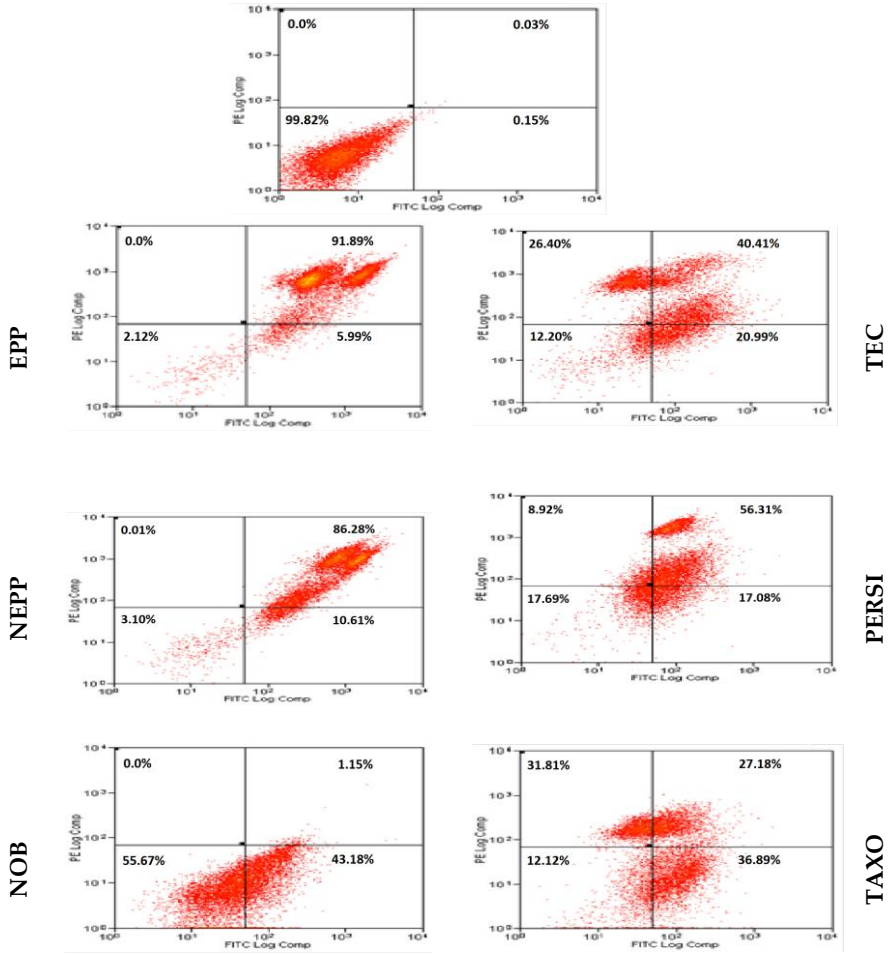

Figure 11. Scatter graph depicting Annexin V/PI double-staining assay of A549 cells treated with extractable polyphenols (EPP); non-extractable polyphenols (NEPP); nobiletin (NOB); tectorigenin (TEC); persicogenin (PERS) at 60 μg/mL and paclitaxel (TAXO) at its respective IC50 concentration for 24 h. The Y-axis represents the PI-labeled population, whereas the X-axis represents the FITC-labeled Annexin V positive cells. The lower left portion of the fluorocytogram (An−, PI−) shows viable cells, whereas the lower right portion of the fluorocytogram (An+, PI−) shows early apoptotic cells. The upper right portion of the fluorocytogram (An+, PI+) shows late apoptotic cells.

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Table 4. Total percentage of apoptotic cells and percentages of FITC-labelled annexin-V and PI accumulation after 24 hours of exposure of A549 to 60µg/mL Barhi date palm extracts and isolated compounds.

Treatment	A549				
	Necrosis	**Late Apoptosis**	**Available**	**Early Apoptosis**	**Total Apoptotic Cells (%)**
CONT	0.00±0.01	0.15±0.01	99.82±0.03	0.03±0.01	**0.18±0.01**
EPP	0.00±0.01	5.99±0.01	2.12±0.02	91.89±0.91	**97.88±0.92**
NEPP	0.01±0.01	10.61±0.07	3.10±0.01	86.28±0.67	**96.89±0.69**
NOB	0.00±0.00	43.18±0.16	55.67±0.06	1.16±0.01	**44.34±0.17**
TEC	26.40±0.04	20.99±0.10	12.20±0.05	40.41±0.52	**61.40±0.50**
PERS	8.92±0.07	17.08±0.07	17.69±0.10	56.31±0.35	**73.39±0.28**
TAX	31.81±0.05	36.89±0.08	12.12±0.06	27.18±0.37	**64.07±0.32**

CONT, control/untreated cells; EPP, extractable polyphenols; NEPP, non-extractable polyphenols; NOB, nobiletin; TEC, tectorigenin; PERS, persicogenin; TAX, paclitaxel.

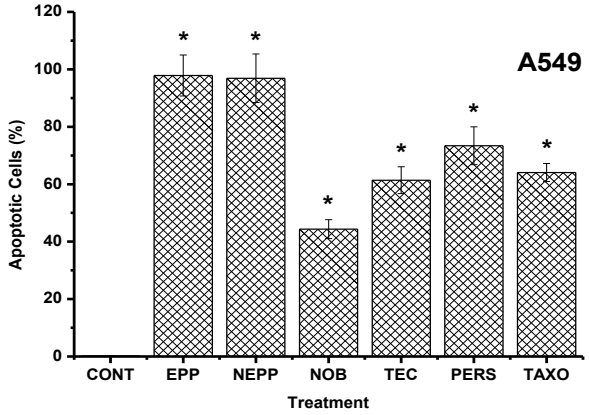

Figure 12. Statistical representation of apoptotic cells after treatment in comparison with untreated/control cells. Plots illustrate results of apoptotic studies of untreated/control cells of A549 together with cells treated with EPP, extractable polyphenols; NEPP, non-extractable polyphenols;NOB, nobiletin; TEC, tectorigenin; PERS, persicogenin; TAX, paclitaxel, respectively. The data were statistically analyzed using Student's t-test and a high degree of significance ($p < 0.05$) was found. $P < 0.05$ was considered statistically significant.

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- HT29

Figure 13 represents the percentage of viability and degree of apoptotic activity of the treatments on HT29 cells.

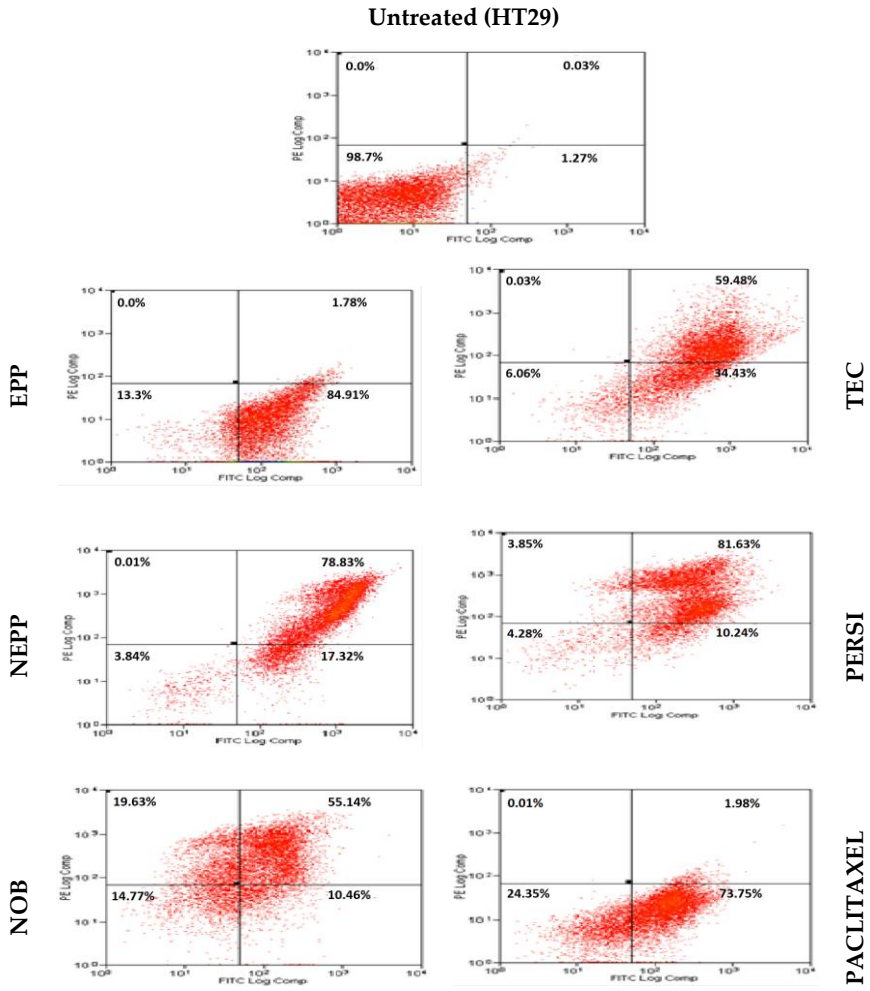

Figure 13. Scatter graph depicting Annexin V/PI double-staining assay of HT29 cells treated with extractable polyphenols (EPP); non-extractable polyphenols (NEPP); nobiletin (NOB); tectorigenin (TEC); persicogenin (PERS) at 60 µg/mL and paclitaxel (TAXO) at its respective IC50 concentration for 24 h. The Y-axis represents the PI-labeled population, whereas the X-axis represents the FITC-labeled Annexin V positive cells. The lower left portion of the fluorocytogram (An−, PI−) shows viable cells, whereas the lower right portion of the fluorocytogram (An+, PI−) shows early apoptotic cells. The upper right portion of the fluorocytogram (An+, PI+) shows late apoptotic cells.

A histogram plot demonstrates the treatment of HT29 cells with NOB, TEC, PERS and paclitaxel the extracts of BDPK EPP and NEPP at 24 hours (Figure 14). The data represent independent experiments and each experiment was performed in triplicate. The total percentage of apoptotic cells were observed during the assay (Table 5).

The level of total apoptosis in colon cancer HT29 cells are as follows: 1.3% (untreated cells), 86.69% (EPP), 93.91% (TEC), 96.15 % (NEPP), 82.87 % (PERSI), 65.60 % (NOB) and 75.73% (paclitaxel). The data was collected in duplicates (n = 3) and the standard deviation was calculated from the mean (mean±SD), the varying degrees of apoptosis were plotted on a bar graph (Figure 14). The analysis of untreated HT29 cells indicated that 98.70% of cells located in the lower left quadrant, remained viable;

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1.30% of cell death was observed with 0% in the upper left quadrant, 0.03% in the upper right quadrant and 1.27% were found in the lower right quadrant (Figure 13).

HT29 cells that were treated with both extracts EPP and NEPP indicated that 13.30% and 3.84 % of cells located in the lower left quadrant remained viable; 86.70%, 96.16% cell death was also observed with 0.0% and 0.01% cells in the upper left quadrant, 1.78%, 78.83% in the upper right quadrant, and 84.91% and 17.32% in the lower right quadrant, respectively (Figure 13).

HT29 cells treated with NOB, TEC and PERSI displayed 14.77%, 6.06% and 4.28 % viable cells in the lower left quadrant; 85.23%, 93.94% and 95.72% cell death was also observed with 19.63%, 0.03% and 3.85% in the upper left quadrant, 55.14%, 59.84% and 81.63% in the upper right quadrant and 10.46%, 34.43% and 10.24% in the lower right quadrant, respectively (Figure 13).

Cytotoxicity of paclitaxel was higher on A549 cell than HT29 cells and most dead HT29 cells were at an early stage in apoptosis. In Figure 13, data indicated that 24.35% of cells treated with paclitaxel remained viable (lower left quadrant); 75.65% cell death was recorded with 0.01% in the upper left quadrant, 1.98% in the upper right quadrant and 73.75% in the lower right quadrant (Figure 13).

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Table 5. Total percentage of apoptotic cells and percentages of FITC-labelled annexin-V and PI accumulation after 24 hours of exposure of HT29 to 60µg/mL Barhi date palm extracts and isolated compounds.

Treatment	HT29				
	Necrosis	**Late Apoptosis**	**Available**	**Early Apoptosis**	**Total Apoptotic Cells (%)**
CONT	0.00±0.00	1.27±0.02	98.70±0.03	0.03±0.01	**1.30±0.03**
EPP	0.00±0.00	84.91±0.11	13.30±0.06	1.78±0.02	**86.69±0.10**
NEPP	0.01±0.00	17.32±0.03	3.84±0.02	78.83±0.28	**96.15±0.29**
NOB	19.63±0.04	10.46±0.02	14.77±0.04	55.14±0.05	**65.60±0.07**
TEC	0.03±0.01	34.43±0.10	6.06±0.02	59.48±0.08	**93.91±0.17**
PERS	3.85±0.03	10.24±0.04	4.28±0.02	81.63±1.13	**91.87±1.15**
TAX	0.01±0.00	73.75±0.05	24.35±0.02	1.98±0.02	**75.73±0.04**

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CONT, control/untreated cells; EPP, extractable polyphenols; NEPP, non-extractable polyphenols; NOB, nobiletin; TEC, tectorigenin; PERS, persicogenin; TAX, paclitaxel.

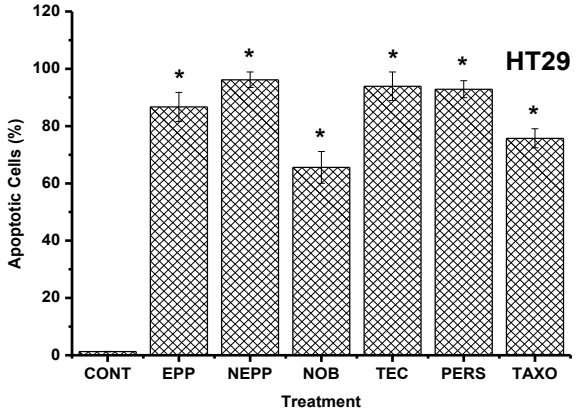

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Figure 14. Histogram profiles of HT29 cells representing apoptotic cells after treatment in comparison with untreated/control cells. Plots illustrate results of apoptotic studies of untreated/control cells of A549 together with cells treated with EPP, extractable polyphenols; NEPP, non-extractable polyphenols; NOB, nobiletin; TEC, tectorigenin; PERS, persicogenin; TAX, paclitaxel, respectively. The data were statistically analysed using Student's t-test and a high degree of significance ($p < 0.05$) was found.

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692 *3.7. Mitochondrial Transmembrane Potential*

693 • A549

694 It is well-known that the collapse of mitochondrial membrane potential ($\Delta\psi$) leads to the
695 discharge of cytochrome c from the mitochondrion to the cytoplasm, in turn, activates caspase
696 cascades that trigger in cell death processes [47,48]. EPP, NEPP, NOB, TEC, PERS and paclitaxel
697 induced a great drop in the mitochondrial transmembrane potential in lung cancer A549 cell line
698 (Figure 14). Both extracts; EPP and NEPP at IC50 concentrations as calculated from MTT assay,
699 caused the highest dissipation of the mitochondrial membrane potential in the cancer A549 cells, that
700 were challenged for 72 hours, and decreased to 14.88% and 9.16% as seen in Figure 14, respectively
701 when compared to 97.51% shown with untreated control cells (Figure 15).

702 Exposure of human lung cancer A549 cells with NOB, TEC, PERS and paclitaxel (at IC_{50}
703 concentrations as calculated from MTT assay) significantly ($p < 0.05$) decreased the percentage of cells
704 negative for rhodamine-123 from 97.51% in untreated cells to 17.23%, 19.23%, 16.65% and 39.38%,
705 respectively (Figure 16).
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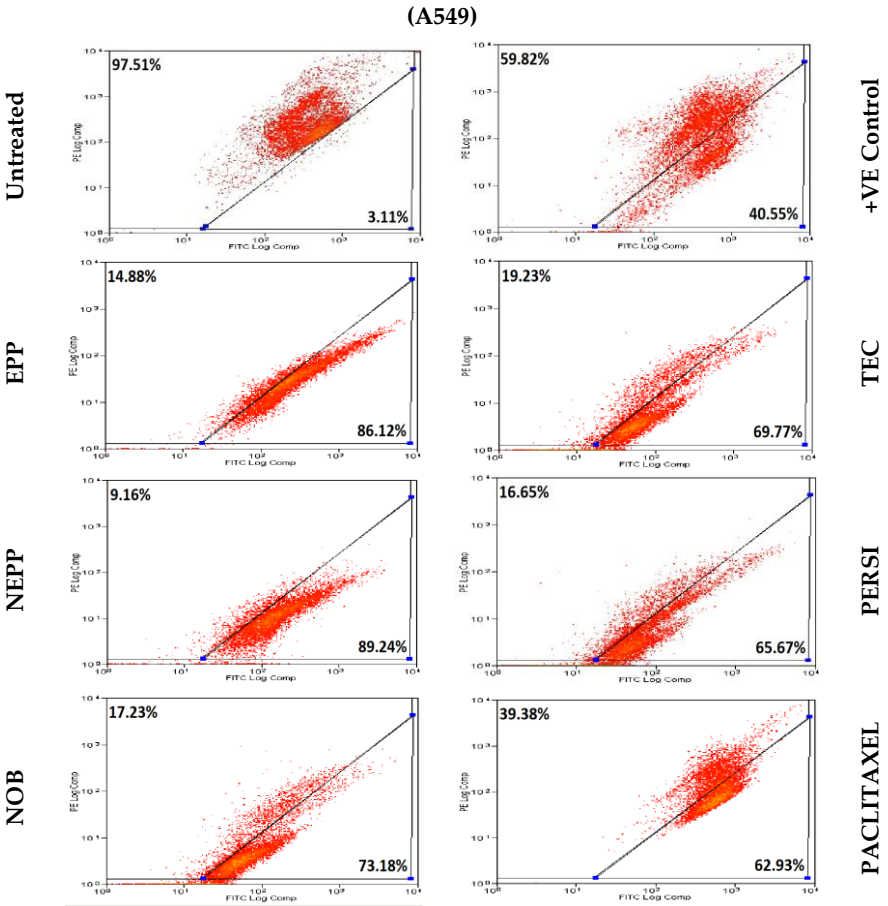

707 **Figure 15.** Effect of Extractable polyphenols (EPP); Non-Extractable polyphenols (NEPP); Nobiletin (NOB)
708 and Persicogenin (PERS) from Barhi date kernels (BDPK) extract, and chemotherapeutic drug paclitaxel
709 (TAXO) on the integrity of mitochondrial membrane of A549 cells. A549 cells were treated at the respective
710 IC_{50} concentration of EPP, NEPP, NOB, TEC, PERS, and TAXO, and with the positive control (50 µM CCCP)
711 and incubated for 72h and analyzed by flow cytometry. Data are presented the fluorescence intensity.
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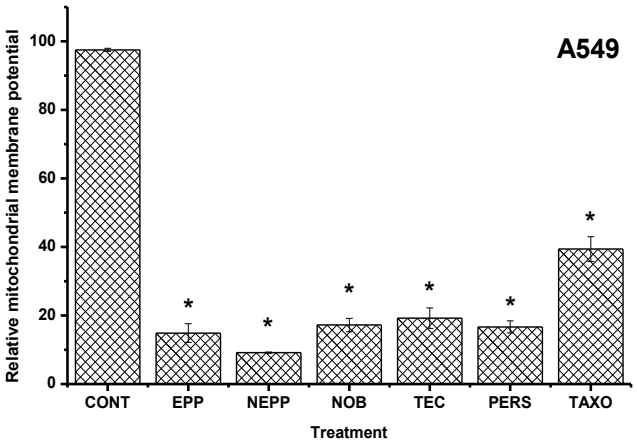

Figure 16. Extractable polyphenols (EPP); Non-Extractable polyphenols (NEPP); Nobiletin (NOB) and Persicogenin (PERS) from Barhi date kernels (BDPK) extract, and chemotherapeutic drug paclitaxel (TAXO) induced dissipation of mitochondrial membrane potential in A549 cells using JC-1 fluorescence dye. A549 cells were treated at the respective IC_{50} concentration of EPP, NEPP, NOB, TEC, PERS, and TAXO, incubated for 72hr and analyzed by flow cytometry. Data are presented the fluorescence intensity. * represents significant results ($p < 0.05$) compared with untreated/controlled (CONT) A549 cells.

- HT29

The highest changes affect mitochondrial membrane potential in HT29 cells were seen after 72 hours exposure to paclitaxel and NEPP while the lowest potential was in NOB–treated cells. Exposure of human colon cancer HT29 cells with EPP, NEPP, NOB, TEC, PERS and paclitaxel (at IC50 concentrations as calculated from MTT assay) significantly ($P < 0.05$) decreased the percentage of cells negative for rhodamine-123 from 99.38% in untreated cells to 63.89%, 52.52%, 76.52%, 60.75%, 63.24% and 46.99%, respectively (Figure 18).

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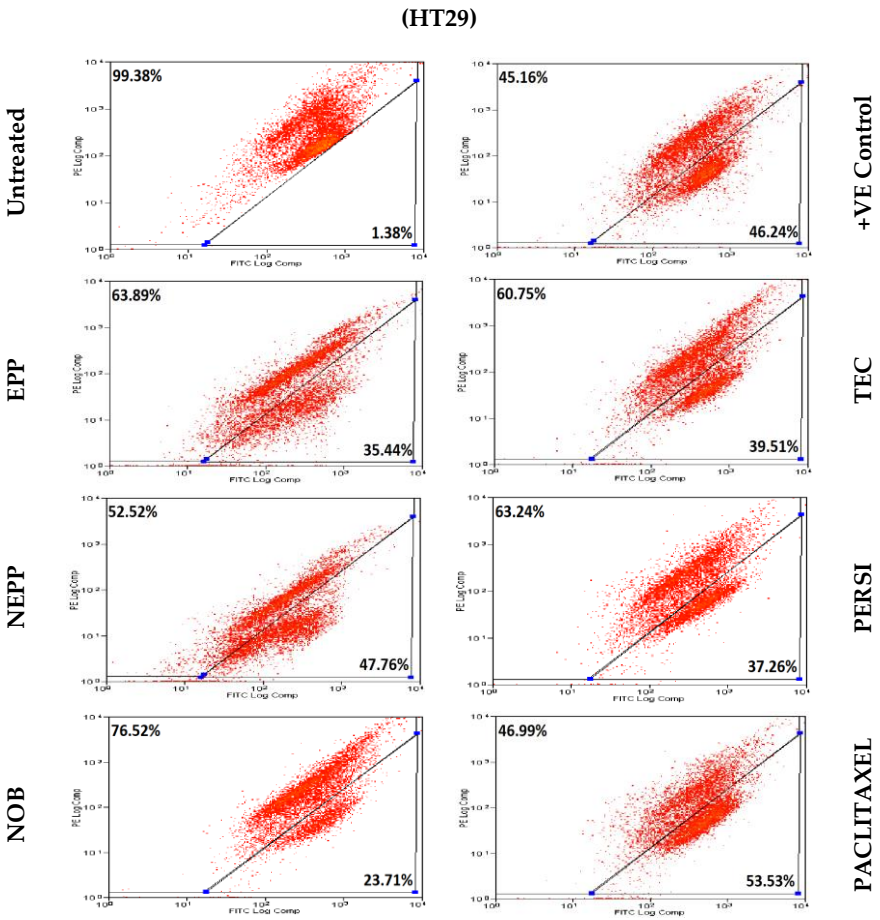

Figure 17. Effect of Extractable polyphenols (EPP); Non-Extractable polyphenols (NEPP); Nobiletin (NOB) and Persicogenin (PERS) from Barhi date kernels (BDPK) extract, and chemotherapeutic drug paclitaxel (TAXO) on the integrity of mitochondrial membrane of HT29 cells. HT29 cells were treated at the respective IC50 concentration of EPP, NEPP, NOB, TEC, PERS, and TAXO, and with the positive control (50 µM CCCP) and incubated for 72h and analysed by flow cytometry. Data are presented the fluorescence intensity.

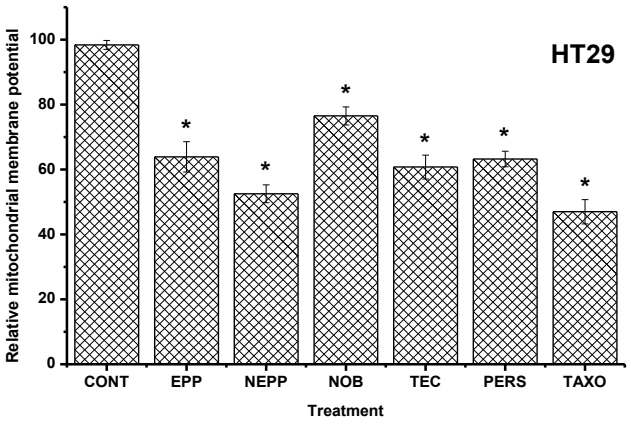

Figure 18. Extractable polyphenols (EPP); Non-Extractable polyphenols (NEPP); Nobiletin (NOB) and Persicogenin (PERS) from Barhi date kernels (BDPK) extract, and chemotherapeutic drug paclitaxel (TAXO) induced dissipation of mitochondrial membrane potential in HT29 cells using JC-1 fluorescence dye. HT29 cells were treated at the respective IC50 concentration of EPP, NEPP, NOB, TEC, PERS, and TAXO, incubated for 72h and analysed by flow cytometry. Data are presented the fluorescence intensity. * represents significant results ($p<0.05$) compared with untreated/controlled (CONT) HT29 cells.

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4. Discussion

There is a growing interest in the cytotoxic effects of various medicinal plants and their application as chemotherapeutic treatments [49,50]. This is due to the fact that plant approaches to cancer treatment are less harsh as compared to those found in current chemotherapeutic drugs [51,52]. These findings suggest that plant extracts are rich in compounds can be a very effective chemotherapeutic treatment of many types of cancer [53].

The changes in cellular morphology, induced by apoptosis, appears similarly in almost all the eukaryotic cells as it affects both the nucleus and the cytoplasm of the cell [54]. However, the time it takes to complete the process of cell death varies from cell to cell based on the stimuli and the pathways that lead to apoptosis [55]. Although MTT and trypan blue exclusion tests are generally non-specific indicators of the toxic effect of the drugs or therapeutic compounds, positive cytotoxicity tests would suggest that physiological or morphological changes have occurred in the treated cells [56].

The results from early MTT and TBE assays of treated HT29 and A549 cells which suggested that our extracts and compounds treatment caused a dramatic decrease in cell viability, were supported by the observations of cellular morphology indicative of apoptotic death.

Cell cycle analysis studies the DNA content found in pre-fixed cells by means of flow cytometry. The technique aims to identify the stage of DNA development within the pre-mitotic phases of the cell cycle [42,43]. As most of the cell underwent apoptosis, the cell cycle arrest effect of NOB, TEC and paclitaxel on HT29 cell were not consistent with that seen in treated A549 cells. Nevertheless, it could state that the main mechanism of the antiproliferative effect of all BDPK extracts and purified compounds on both cancer cells was apoptosis. Another similar study investigated the suppressant effects of ethyl acetate fraction of *Phoenix dactylifera* on human prostate cancer cell line (PC3) line. The result indicated that this plant extract suppressed prostate cancer cell line proliferation by cell cycle progression inhibition at S phase [57].

The results of flow cytometry using Annexin V-FITC and PI were presented as a percentage of Annexin V+/PI- or Annexin V+/PI+ to indicate the status of apoptosis and necrosis induced by the test compounds. Flow cytometry analysis was performed to determine the degree of apoptosis induced in human colon cancer HT29 cells by NOB, TEC, PERS, paclitaxel and the extracts of BDPK EPP and NEPP and to validate the Annexin V-FITC/PI assay.

Here, we can notice that majority of the dead cells in EPP-treated HT29 cells were at the early stage of apoptosis while the NEPP-treated HT29 cells, NEPP-treated A549 cells, and EPP-treated A549 cells were at the late stage of apoptosis. The majority of the dead cells in NOB-treated HT29 cells were at the late stage of apoptosis and necrosis while the dead NOB-treated A549 cells were at the late stage of apoptosis with no necrotic cells.

Furthermore, TEC has resulted in a considerable percentage of necrotic cells among treated A549 cell, while the necrosis was not the favorable death pathway with TEC- treated HT29 cells. The dead PERSI-treated HT29 cells were concentrated at late apoptosis more than that seen with dead PERSI-treated A549 cells.

The mitochondrion is the main source of production of reactive oxygen [58,59]. Reactive oxygen species production is increased when the mitochondrial membrane potential is affected [59,60]. Decreases in mitochondrial membrane potential ($\Delta\psi$) were obtained in A549 and HT29 cancer cells after treatment with all extracts and compounds. Recently published article by [61], indicated that the treatment of breast MCF-7 cancer cell line with *Phoenix dactylifera* L. extract led to Bax protein mobilization into mitochondria organelles to breakdown mitochondrial membrane potentials [61].

Tectorigenin also reduced mitochondrial membrane potential through the mutual activation between Bax and Bcl-2 and induced mitochondrial apoptosis-induced channel (MAC), and release cytochrome c into the cytosol [62]. In isolated cancer cells, flavonoids have been shown to induce lipid peroxidation, increase reactive oxygen species formation, deplete glutathione (GSH) levels, and collapse the mitochondrial membrane potential before causing cytotoxicity [63].

5. Conclusions

The findings indicate that the both Barhi Date Palm Kernels (BDPK) extracts and purified phytochemicals do exert induced cell death on A549 and HT29 cells. The results observed from MTT-assay and trypan blue exclusion indicate that the cytotoxic effects of both the plant extracts; extractable polyphenols (EPP) and non-extractable polyphenols (NEPP) are dose-dependent with higher cell death after 72 hours treatment. Furthermore, the compound activity of the extracts took significant effects ($p < 0.05$) at the expected time. The results also indicated that the extracts induced late stages of apoptosis as there was evidence of the DNA degradation and large percent of the cells population being in the sub-G_1 phase, which is indicative of apoptosis. The extracts of BDPK showed that the cytotoxic effect can be compared to that of paclitaxel as there is indication of DNA degradation and most of the cells that were treated with extracts populated at the upper right quadrant, which is an indication that those cells had undergone late stages of apoptosis. Both extracts; EPP and NEPP exhibited dependent mitochondrial signalling pathway as seen with caspase-9 and induced receptor-mediated (extrinsic) apoptotic pathway as seen with caspase-8. In summary, the present study has revealed the anticancer potentials of BDPK extracts and the three new cytotoxic isolated compounds could be new promising therapeutic candidates in treating lung and colon cancers with no side effects.

Author Contributions: Conceptualization, I.M. and M.M.; methodology, I.M., M.M, M.A, B.Y., N.I.; validation, M.M.; investigation, I.M. and M.M.; resources, M.M., F.Y., M.A., A.R., I.M., M.S., N.K., Y.H., A.H., P.A.; data curation, I.M.; writing—original draft preparation, I.M.; writing—review and editing, M.M.; supervision, M.M, F.Y. and M.A.

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