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Caspase 3-mediated cytotoxicity of mealworm larvae (*Tenebrio molitor*) oil extract against human hepatocellular carcinoma and colorectal adenocarcinoma

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ABSTRACT

Ethnopharmacological relevance: Mealworm larvae (MWL) (*Tenebrio molitor*) have been traditionally used in Asian countries for the treatment of liver diseases, including cancer. However, to date, there is marginal information on the mechanisms underlying the anticancer activity of MWL oil.
Aim of the study: This study aims to determine the *in vitro* effect of MWL oil on human hepatocellular carcinoma (HepG2) and colorectal adenocarcinoma (Caco-2) cells growth in order to produce insect-derived chemotherapeutic agents against cancer.
Materials and methods: MWL oil was extracted, and its effects on cancer cells growth were investigated, by the MTT reduction, AO/EB staining, Hoechst 33258 nuclear staining, apoptosis, comet, and caspase activity assays.
Results: MWL oil inhibited HepG2 and Caco-2 growth, with IC_{50} (48 h) values of 0.98% for HepG2 and 0.37% for Caco-2 cells. In addition, flow cytometry analysis demonstrated that 24 h-MWL oil treatment increased early and late apoptosis from 0.04% to 39.77% and from 2.06% to 74.34% on HepG2 and Caco-2 cells, respectively. The mechanism of apoptosis was associated with the death receptor pathway by the activation of caspases -8, -9, and -3, and correlated to its fatty acids action.
Conclusion: Results of this study demonstrated the potential of MWL oil in the development of natural anticancer therapeutic agents.

1. Introduction

Insects represent more than 90% of all animal species on Earth (Bulet et al., 2003; Dai et al., 2013). However, they are an underestimated resource containing a number of bioactive compounds, including anticancer agents. It is known that insects synthesize pheromones, defensins, venoms, and toxins, which might have acquired from plants and transformed for their own use. Some of such molecules possess antitumor potential (Costa-neto, 2005). The amount of bioactive compounds increases during the larval stage (Yoo et al., 2007), because of which insects become resistant to bacterial and viral infections. Recently, there has been an increasing interest on investigating insect bioactive compounds for their therapeutic potential (Nongonierma and Fitzgerald, 2017).

Mealworm beetle (*Tenebrio molitor*) belongs to the order Coleoptera of the family Tenebrionidae. Larvae of this species are known as yellow mealworms or mealworm larvae (MWL) (Han et al., 2014). MWL are a common pest that infests stored food products worldwide (Gao et al., 2018). They are the most widely reared insects for human consumption in many countries because of their high nutritional value and they are currently investigated as source of bioactive compounds (Paul et al., 2017; Seo et al., 2017). Previous studies have shown that MWL may possess anticancer agents (Lee et al., 2015; Liu et al., 2009). MWL have been traditionally used in Asia to treat liver diseases including cancer (Lee et al., 2015). Commonly, the treatment involves consumption of the whole insect body. Given that traditionally people did not use extracts; in our preliminary study, MWL protein and oil were extracted to compare their antiproliferative activity on cancer cells, demonstrating higher activity of the oil, as compared with the protein.

Anticancer compounds exist in natural food compounds (Norikura

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et al., 2011). Recently, there is an increasingly interest of treating cancer in a sustainable manner using natural food compounds (Yan et al., 2014; Yang et al., 2014; Zhang et al., 2019). For decades, natural bioactive compounds have been investigated as potential drugs for use in medicine and food (Ding et al., 2019; Seo et al., 2017). In folk medicine and particularly in Chinese traditional medicine, insect body extracts have been widely used for the treatment of a wide range of diseases, including cancer (Ratcliffe et al., 2011).

Cancer is one of the leading causes of death in the world (Chalamaiah et al., 2018; Sulaiman, 2015). In particular, hepatocellular carcinoma and colorectal cancer represent two of the most diagnosed cancers and leading causes of cancer-related deaths worldwide (Arun et al., 2018; Prashant et al., 2017; Song et al., 2016). Resistance of cancer to anticancer drugs has been observed (Umayaparvathi et al., 2014). Furthermore, traditional chemotherapeutic drugs for the treatment of hepatic and colorectal cancer may cause serious toxicity to normal tissues at high concentrations, because they target both cancer and normal cells (Duan et al., 2016; Huang et al., 2012). Therefore, there is an urgent need for developing natural antitumor drugs which are safe and effective in cancer therapy, particularly hepatic and colorectal cancer (Li et al., 2013).

MWL oil anticancer activity has not yet been studied. The aim of this study was to determine the *in vitro* effect of MWL oil on human hepatocellular carcinoma (HepG2) and colorectal adenocarcinoma (Caco-2) cells growth in order to produce natural insect-derived chemotherapeutic agents against cancer.

2. Materials and methods

2.1. Reagents, culture media, and larvae

Thiazolyl blue tetrazolium bromide (MTT), acridine orange (AO), ethidium bromide (EB), Hoechst 33258, and annexin V-FITC apoptosis detection kit were purchased from Sigma-Aldrich (St. Louis, MO). Streptomycin-penicillin solution, trypsin EDTA, and phosphate buffered saline (PBS) (without Ca and Mg) were purchased from Hyclone Laboratories (Logan, UT). BCA protein assay kit was obtained from ThermoFisher Scientific (Pierce Biotechnology, Rockford, IL). Caspase-3, -8, and -9 assay kits were purchased from KeyGen Biotech (Nanjing, Jiangsu, China). Minimum Essential Medium (MEM), Dulbecco's Modified Essential Medium (DMEM), and MEM non-essential amino acids solution 100X were purchased form Gibco (ThermoFisher Scientific, Gran Island, NY). Fetal bovin serum (FBS) was obtained from Gemini Bio-Products, Inc. (Calabasas, CA). Freeze-dried MWL were purchased from the local market and stored at -20 °C. Larvae were finely ground in a food processor and passed through a 40-mesh sieve prior to processing and analyses.

2.2. MWL oil extraction

MWL total lipid was obtained by Soxhlet extraction. An amount of 4 g of ground freeze-dried larvae were loaded to a Soxhlet apparatus and extracted with 40 mL of petroleum ether (Sinopharm Chemical Reagent, Co Ltd., Shanghai, China) at 50 °C for 6 h. Next, solvent was removed under vacuum at 40 °C in a rotary evaporator (R-210 BUCHI Labortechnik AG, Flawil, Switzerland) at 350 mbar for about 30 min, and further increase up to 60 °C until no solvent was observed. Lipid extracts were stored in amber glass bottles under nitrogen atmosphere at -20 °C for further analysis.

2.3. Fatty acids analysis

Fatty acid composition of MWL oil was determined by gas chromatography (GC) analysis. The first step was methylation of fatty acids to obtain their respective fatty acid methyl esters (FAME). For this, 20 mg of oil were added into a testing tube with cap, plus 1 mL internal standard, 0.7 mL KOH (10M), and 5.3 mL methanol, after which mixture was heated at 55 °C for 1.5 h in a water bath, shaking the tube every 20 min for 5 s. After heating, the tube was taken and cooled to room temperature, following by addition of 0.50 mL 12M H_2SO_4. Then, the tube was heated again at the same conditions as detailed above, and 3 mL of hexane were added to the tube, vortexed for 5 min, and centrifuged at 2500×*g* for 10 min. Next, 1 mL anhydrous Na_2SO_4 was added to the tube and after 5 min, it was vortexed for 10 min. FAMEs-containing supernatant was pipetted, filtered through a 0.22 µm filter, and analyzed by GC. For this, GC-FID was used for the analysis of FAMEs, which was equipped with a DB-FFAP capillary column 30 m × 0.53 mm id, 1 µm (Agilent Technologies Inc., Palo Alto, CA). Column temperature was set at 140 °C for 1 min and programmed to 220 °C at 2 °C/min for 15 min; whereas injector and detector temperatures were set at 250 °C and the flow rate of H_2 (carrier gas) was set at 40 mL/min. FAMEs (1 µL) were injected into the GC equipment at split ratio of 1:10. Fatty acids were then identified by comparison of their retention time with those of standards (FAME C8–C22 standards, Supelco, Bellefonte, PA) (Fogang Mba et al., 2017).

Table 1
Fatty acid profile of MWL oil.

Fatty acid		Content	
		mg/g	%
C8	Caprylic acid	0.14 ± 0.03	0.01 ± 0.00
C10	Capric acid	0.22 ± 0.00	0.01 ± 0.00
C12	Lauric acid	5.17 ± 0.11	0.29 ± 0.00
C13	Tridecanoic acid	0.89 ± 0.00	0.05 ± 0.00
C14	Myristic acid	35.78 ± 0.83	1.98 ± 0.00
C14:1	Myristoleic acid	0.14 ± 0.05	0.01 ± 0.00
C15	Pentadecylic acid	3.60 ± 0.05	0.20 ± 0.00
C16	Palmitic acid	293.91 ± 6.11	16.27 ± 0.06
C16:1	Palmitoleic acid	33.08 ± 1.53	1.83 ± 0.04
C17	Margaric acid	4.03 ± 0.11	0.22 ± 0.01
C18	Stearic acid	12.23 ± 0.41	0.68 ± 0.01
C18:1	Oleic acid	827.60 ± 14.31	45.79 ± 0.11
C18:2	Linoleic acid	505.59 ± 16.88	27.99 ± 0.19
C18:3	α-Linolenic acid	53.78 ± 1.29	2.98 ± 0.00
C22	Docosanoic acid	1.17 ± 0.26	0.06 ± 0.01
C22:1	Erucic acid	29.39 ± 2.44	1.63 ± 0.10
SFA		**357.14 ± 7.69**	**19.77 ± 0.06**
MUFA		**568.20 ± 20.89**	**31.46 ± 0.05**
PUFA		**880.86 ± 15.59**	**48.77 ± 0.11**
UFA		**1449.06 ± 36.49**	**80.23 ± 0.06**
Total FA		**1806.20 ± 44.18**	**100.00**
n-6/n-3		**15.37 ± 0.04**	**15.37 ± 0.04**

SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; UFA: unsaturated fatty acids; FA: fatty acids; n-6/n-3: omega-6/omega-3 ratio.

2.4. Cell culture

Human colorectal adenocarcinoma cell line Caco-2 was cultured in MEM, supplemented with 20% FBS, 1% non-essential amino acids, 100 U/mL streptomycin, and 100 U/mL penicillin. Human liver cancer cell line HepG2, human cervical cancer cell line HeLa, and mouse fibroblast cell line L929 were cultured in DMEM, supplemented with 10% FBS, 100 U/mL streptomycin, and 100 U/mL penicillin. Cells were grown in culture flasks in an incubator at 37 °C with 5% CO_2, in humidified atmosphere. Cells were harvested with trypsin-EDTA and washed with PBS.

2.5. MTT cell viability assay

The effect of MWL oil on tumor cell lines growth was determined by the MTT reduction assay, according to Duan et al. (2016), with some modifications. Caco-2, HepG2, HeLa, and L929 cells were seeded in 96-

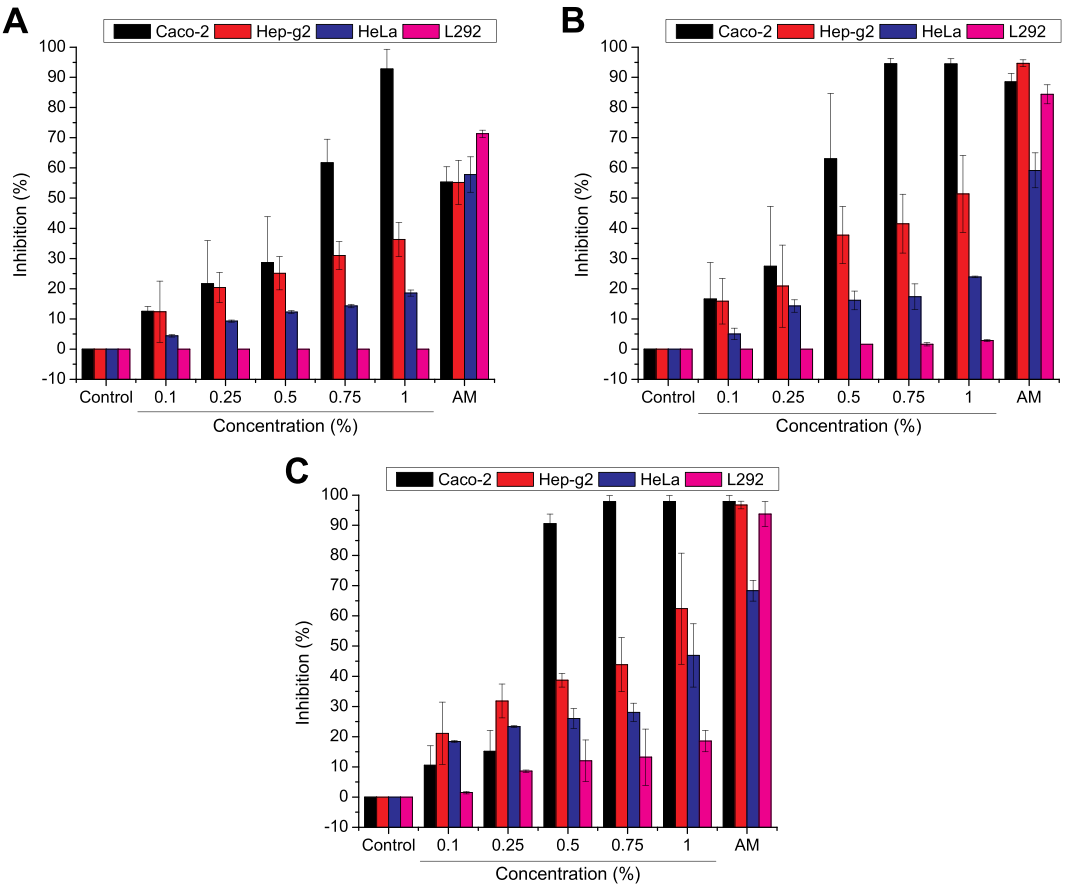

Fig. 1. Percentage of inhibition of Caco-2, HepG2, HeLa, and L929 cells treated with MWL oil for 24 (A), 48 (B), and 72 (C) h. Cell viability was determined by MTT cell viability assay. Control: cells with no test compounds. Acrylamide (AM): 10 mM/L. Data are expressed as mean ± standard deviation from five independent experiments. Treatments were significantly different compared to the control ($P < 0.01$).

well plates at a density of 1×10^4 cells/well and incubated for 24 h. Then the cells were treated with 0, 0.1, 0.2, 0.5, 0.75, and 1% MWL oil for 24, 48, and 72 h, using 10 mM/L acrylamide as a positive control. Next, culture medium was removed and 100 µL of MTT solution (1 mg/mL in PBS) were added and incubated in the dark at 37 °C for 4 h, after which, MTT was removed and formazan crystals were solubilized with 150 µL dimethyl sulfoxide (DMSO) (Sinopharm Chemical Reagent Co Ltd., Shanghai, China); plates were then shaken in an orbital shaker for 15 min. The color developed in each well was measured in a microplate spectrophotometer (BioTek Eon, BioTek Instruments, Inc., Winooski, VT) at a wavelength of 550 nm. Results were expressed as the percentage of inhibition of cell viability. IC_{50} was defined as the concentration of sample that inhibited 50% cell viability.

2.6. Apoptosis morphological changes assay

Apoptotic cells were visualized using acridine orange (AO) and ethidium bromide (EB) double-staining according to Ribble et al. (2005), with some modifications. Caco-2 and HepG2 cells were seeded in 96-well plates at a density of 1×10^4 cells/well and incubated for 24 h. Then 0, 0.5, 0.75, and 1% concentrations of MWL oil were added to the cells for 24, 48, and 72 h. Next, plates were centrifuged at 1000 rpm for 5 min (Allegra X-12R Centrifuge, Beckman Coulter, Brea, CA) and stained with 8 µL AO/EB dye mix (ratio 1:1, containing 100 µg/mL of AO and 100 µg/mL of EB in PBS). Cells were then observed under a fluorescent microscope (Leica DMI 4000B, Leica Microsystems, Germany) under blue filter.

2.7. Hoechst 33258 nuclear staining assay

Nuclear changes and apoptotic body formation on Caco-2 and HepG2 cells were visualized using Hoechst 33258 staining, according to Kasibhatla et al. (2006), with some modifications. In brief, 1×10^4 cells/well were seeded in 96-well plates and incubated for 24 h. Then 0, 0.5, 0.75, and 1% concentrations of MWL oil were added to the cells for 24, 48, and 72 h. Next, plates were centrifuged at 1000 rpm for 5 min and stained with 1 µL of Hoechst 33258 solution (1 mg/mL in PBS) and incubated for 10 min. Cells were then observed under a fluorescent microscope.

2.8. Cell apoptosis assay

Caco-2 and HepG2 cells apoptosis was detected by flow cytometer according to Huang et al. (2012), with some modifications. In brief, 3×10^5 cells were seeded in 6-well plates and incubated for 24 h to attach to the plate. Then, 0, 0.5, 0.75, and 1% MWL oil were added to the cells. After 24 h, cells were harvested and centrifuged at 1000 rpm for 5 min, after which 500 µL of binding buffer were added to the cell pellets and passed through a 200-mesh sieve transferring them to plastic 12 × 75 mm test tubes. Then, 5 µL of annexin V FITC conjugate and 10 µL of propodium iodide solution were added to cell suspensions, after which, tubes were incubated at room temperature in the dark for 10 min, and immediately analyzed by a Gallios flow cytometer (Beckman Coulter, Brea, CA). Data were analyzed using Kaluza Analysis 2.1 software (Beckman Coulter).

2.9. Comet assay

Alkaline single-cell gel electrophoresis was performed to analyze DNA damage of Caco-2 and HepG2 cells by MWL oil. For this, cells were seeded in microplates at a density of 1×10^5 cells/mL and incubated for 24 h. After incubation, cells were treated with 0, 0.5, 0.75, and 1% MWL oil for 24, 48, and 72 h. A layer of 1% normal melting agarose was placed onto microscope slides; 10 μL of cell suspensions containing 1×10^6 cells were then mixed with 75 μL of 1% low melting agarose maintained at 37 °C, and covered with a coverslip. After agarose solidification, the coverslip was removed and the slides were immersed in a lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH10, 1% Triton X-100, and 10% DMSO) for 2 h at 4 °C. After lysing, slides were gently removed from the lysis solution and placed side by slide on a horizontal gel box near one end. The buffer reservoirs were filled with electrophoresis buffer (pH > 13), until completely covering the slides and kept at 4 °C for 20 min to allow DNA unwinding. After this, slides were subjected to electrophoresis at24 V and 300 mA for 20 min. They were then neutralized with 0.4 M tris buffer (pH 7.5) for 5 min, stained with ethidium bromide (10 μg/mL in PBS) for 5 min, and examined at 400 × by a fluorescence microscopy (Duan et al., 2016). Images were analyzed using Tritek CometScore 2.0 (Sumerduck, VA).

2.10. Caspase activity assay

Caspase-3, -8, and -9 activities on Caco-2 and HepG2 cells were

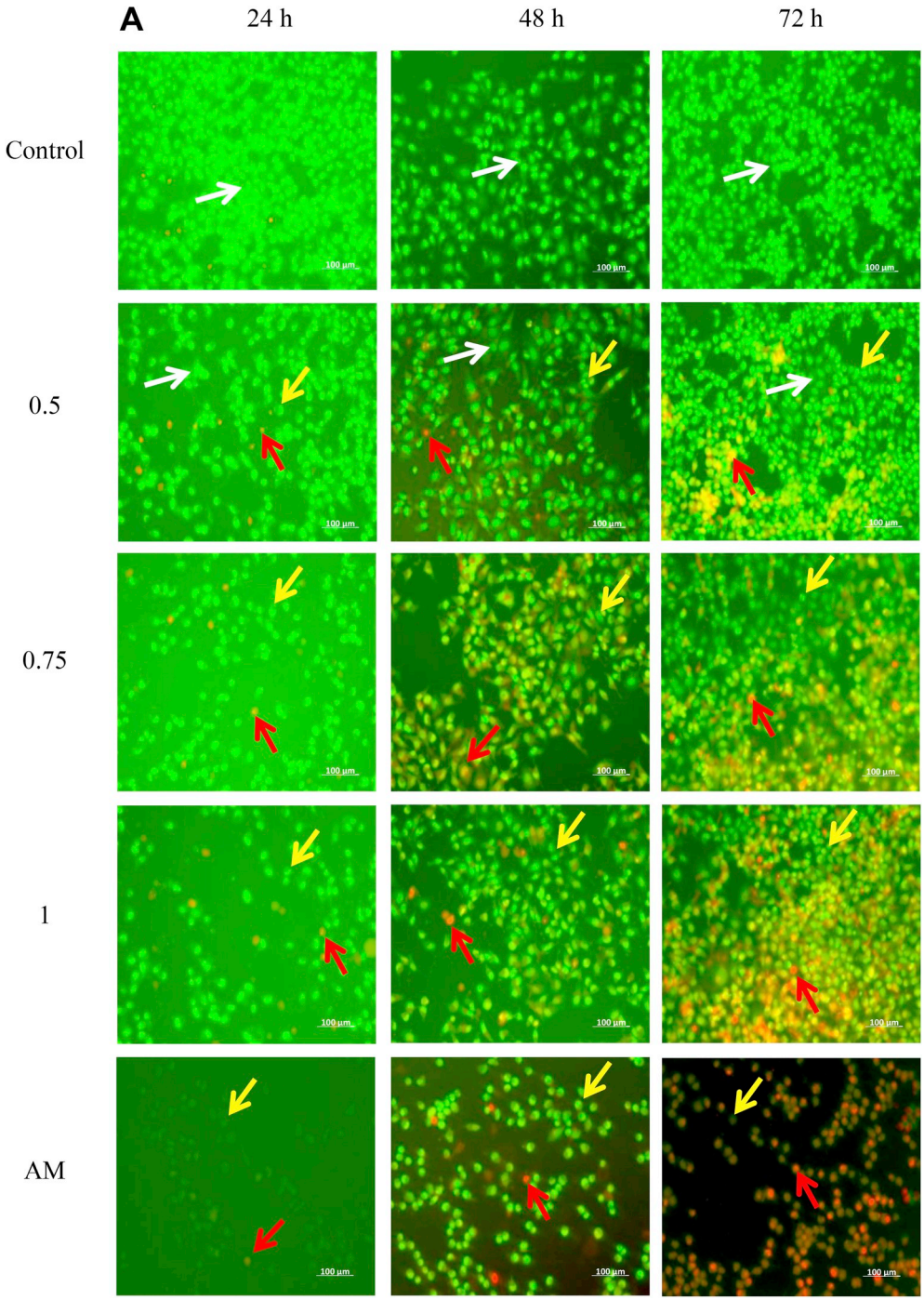

Fig. 2. Morphological changes of HepG2 (A) and Caco-2 (B) cells treated with different concentrations of MWL oil for 24, 48 and 72 h. Cells were stained with AO/EB and seen in an inverted fluorescent microscope (200 ×). Control: cells with no test compounds. MWL treatments: 0.5, 0.75, and 1%. Acrylamide (AM): 10 mM/L. Viable cell Apoptotic cell Necrotic cell.

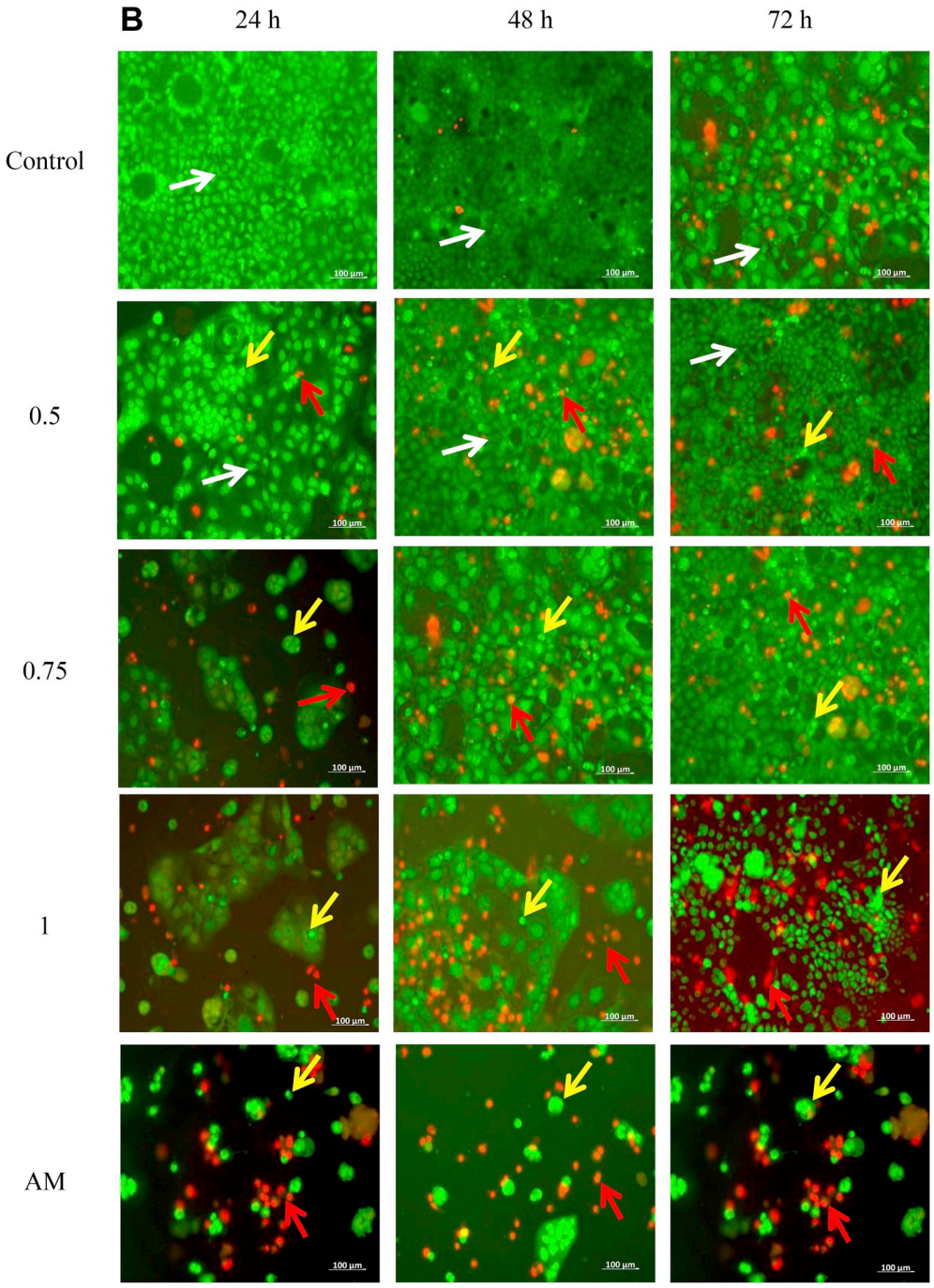

Fig. 2. (*continued*)

measured by a caspase activity assay kit, following manufacturer's procedures and according to Li et al. (2018). with some modifications. In brief, cells were seeded at a density of 3×10^5 cells in 6-well plates and incubated for 24 h to attach to the plate. After incubation, cells were treated with 0, 0.5, 0.75, and 1% of MWL oil and protein or positive control (10 mM/L of acrylamide) for 48 h. Cells were lysed in 100 μL cold lysis buffer and incubated on ice for 15 min. Then, cells were centrifuged at 1000 rpm for 5 min, after which supernatant protein concentrations were determined by the BCA protein assay. Next, 50 μg of protein were mixed with 50 μL of 2 X reaction buffer and 5 μL of caspase substrate in a 96-well microplate and incubated for 4 h at 37 °C. Optical densities were then read at 405 nm. Caspase activation was calculated by the $OD_{inducer}/OD_{control}$ ratio.

2.11. Statistical analysis

All experiments were performed three times, unless otherwise specified. Results were expressed as mean ± standard deviation and analyzed using the GLM analysis and Duncan's test for comparison of means ($P < 0.05$) by the SAS 9.4 software (SAS Institute, Cary, NC).

3. Results and discussion

3.1. MWL oil fatty acids profile

A total of 16 fatty acids were identified in MWL oil (Table 1). The most abundant ones were oleic acid (C18:1) (45.79 ± 0.11%), linoleic

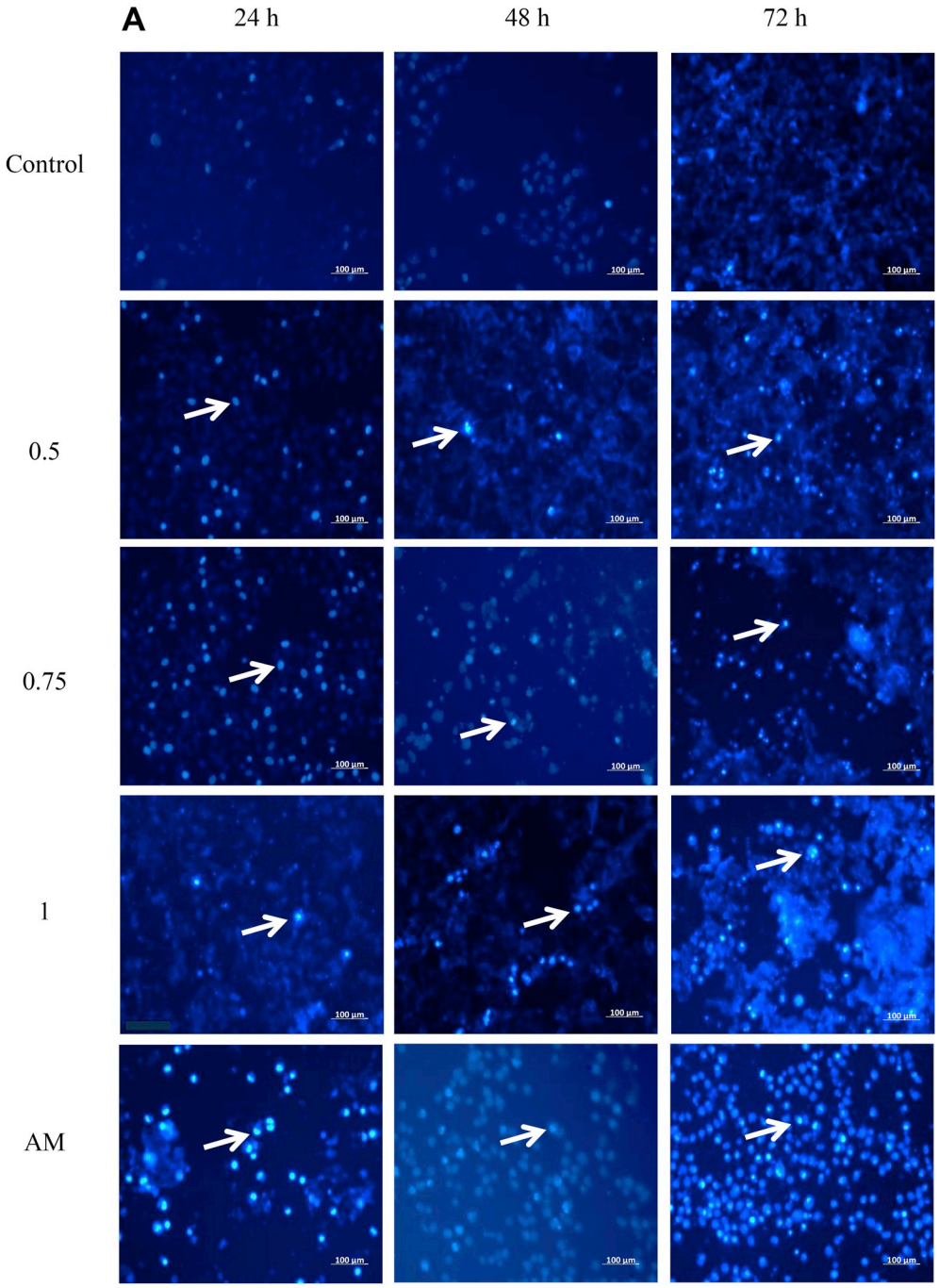

Fig. 3. Nuclear morphological changes of HepG2 (A) and Caco-2 (B) cells treated with different concentrations of MWL oil for 24, 48 and 72 h. Cells were stained with Hoechst 33258 and seen in an inverted fluorescent microscope (200 ×). Control: cells with no test compounds. MWL treatments: 0.5, 0.75, and 1%. Acrylamide (AM): 10 mM/L.
→ Bright chromatin condensation and fragmentation.

acid (C18:2) (27.99 ± 0.19%), palmitic acid (C16) (16.27 ± 0.06%), and α-linolenic acid (C18:3) (2.98 ± 0.00%).

3.2. Effect of MWL oil on HepG2 and Caco-2 cells growth

MWL oil induced significantly ($P < 0.01$) higher HepG2 and Caco-2 tumor cell growth inhibition, compared with that on HeLa cells, in a concentration- and time-dependent fashion, without altering normal L929 cells (Fig. 1). In addition, the maximum inhibition rate of MWL oil on Caco-2 cells was 97.88 ± 2.02% after treatment with 1% for 72 h, whereas treatment with 1% caused 92.74 ± 6.61% inhibition after 24 h. The inhibition rate on HepG2 cells was 62.38 ± 18.39% after treatment with 1% for 72 h, lower compared with that on Caco-2 cells. The inhibitory effect of MWL oil on Caco-2 cells after 24 h of treatment was higher than that of Sepia ink oligopeptide against DU-145, PC-3, and LNCaP prostate cancer cell lines (Huang et al., 2012). After 48 h of treatment, the IC_{50} of MWL oil on Caco-2 and HepG2 was 0.37 and 0.98 % respectively. These IC_{50} were lower than that of a peptide isolated from heated products of half-fin anchovy peptic hydrolysates against prostate cancer PC-3, which was reported to be 11.3 mg/mL (Song et al., 2014). Di Nunzio et al. (2011) reported that polyunsaturated fatty acids (PUFAs), including linoleic acid and α-linolenic acid,

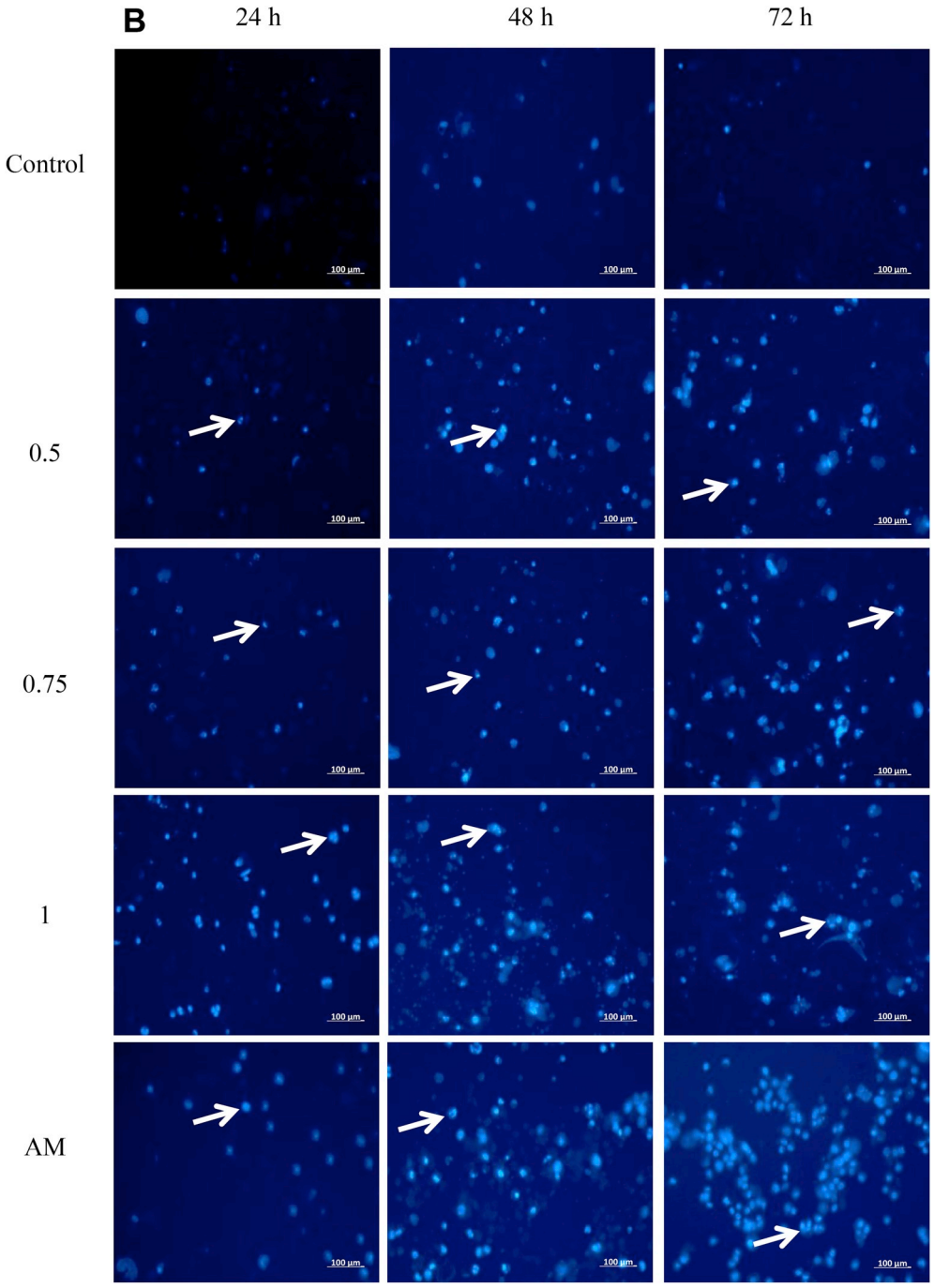

Fig. 3. (*continued*)

inhibited growth on HepG2 cells. Particularly, cell viability was severely more affected in cells treated with n-6 PUFA (i.e. linoleic acid) than with n-3 PUFA (i.e. α-linolenic acid). This correlates the effect of MWL oil against HepG2 cells, in which content of linoleic acid, was higher compared to α-linolenic acid. Another author (Yoo et al., 2007), reported that oleic acid, palmitic acid, and stearic acid isolated from *Protaetia brevitarsis* larvae have shown inhibitory effects against colon tumor cells. These findings demonstrated the anticancer effects of MWL oil which may be associated with different mechanisms against HepG2 and Caco-2 cells. This is the first time that the anticancer activity of MWL oil against HepG2 and Caco-2 cells is reported.

Insects are a promising source of anticancer compounds which can be isolated and used for the production of anticancer drugs. A study carried out in the 1970s, indicated that about 4% of extracts from many species of arthropods possess anticancer activity (Costa-neto, 2005). Other species of insects have displayed anticancer effects against a number of tumor cells. For example, fatty acids from *Eupolyphaga steleophaga* have growth inhibition against HepG2 tumor cells (Wang and Ji, 2009). Blister beetle has shown cytotoxic effects against 41 tumor cell lines (Kadioglu et al., 2014). *Musca domestica* crude extracts have antitumor potential (Cao et al., 2010; Hou et al., 2007). Recent studies have reported the cytotoxic effects of MWL crude extracts on hepatic cancer cells (Lee et al., 2015). With the increasing frequency of colon and hepatic cancer worldwide, the chemotherapy side effects, and the increasing interest of natural compounds with therapeutic effects, insects have become a research priority in the search for bioactive compounds, specifically agents with anticancer activity. MWL seems to be a promising source of bioactive compounds because it is one of the most

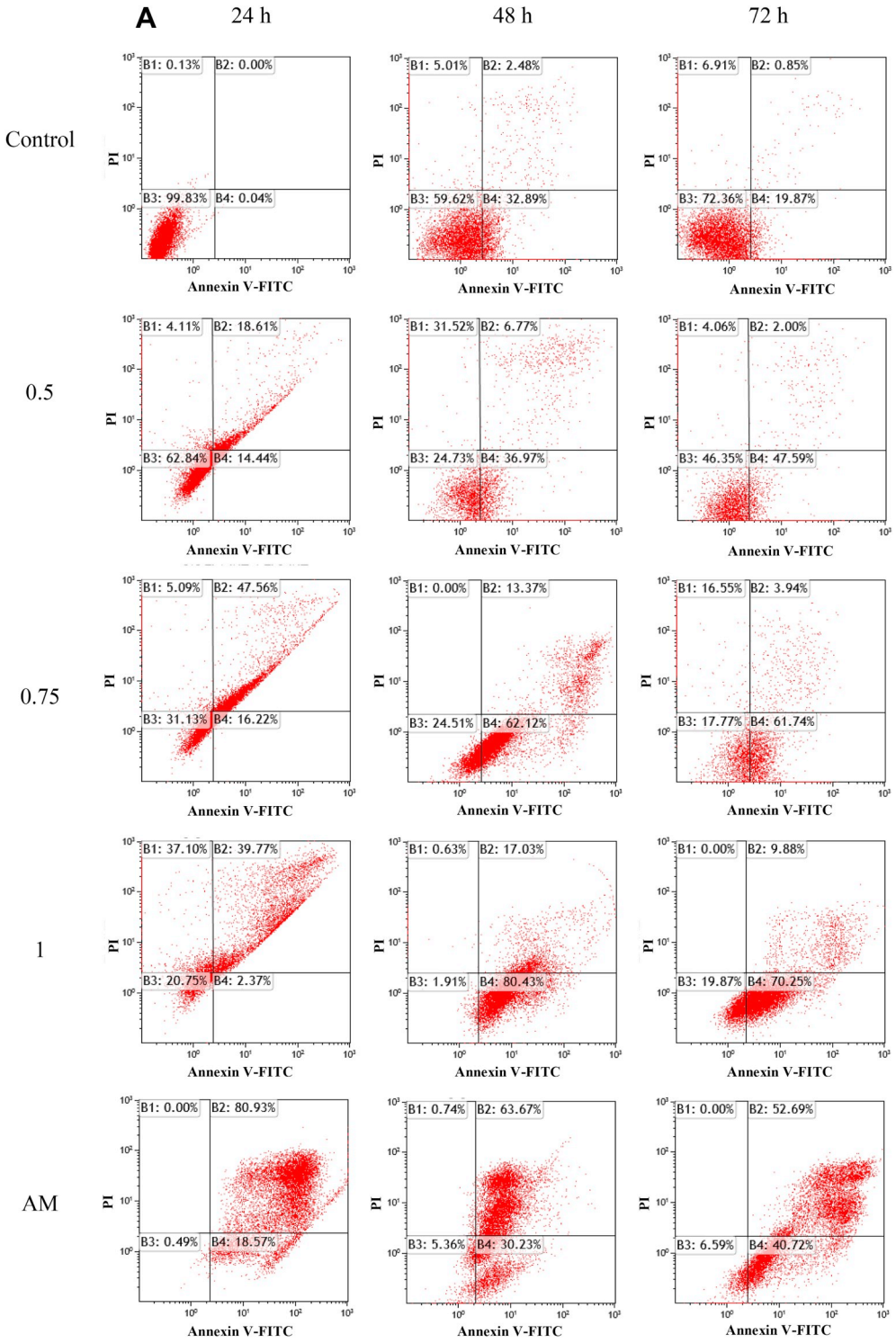

Fig. 4. Flow cytometer analysis of HepG2 (A) and Caco-2 (B) cells treated with different concentrations of MWL oil (0.5, 0.75, and 1%) for 24, 48 and 72 h. For this analysis, cells were stained with Annexin V-FITC/PI double staining. Quadrants: lower left – live cells; lower right – early apoptotic cells; upper right – late apoptotic cells; upper left – necrotic cells. Control cells (cells with no test compounds). AM: cells treated with 10 mM/L acrylamide (positive control).

consumed species of insect in the world and has a lower cost compared with other species of insects. As MWL showed higher inhibitory effects against HepG2 and Caco-2 compared with HeLa; HepG2 and Caco-2 cells were selected for further analyses.

3.3. Effect of MWL oil on morphological changes in HepG2 and Caco-2 cells

To confirm that apoptosis was induced in HepG2 and Caco-2 cells by MWL oil at concentrations of 0.5, 0.75, and 1%, AO/EB dual staining assay was performed. Cells treated with these concentrations for 24, 48, and 72 h showed early apoptosis, late apoptosis, and necrosis in their morphology (Fig. 2). Live cells have a normal green nucleus, early apoptotic cells have bright green nucleus, late apoptotic cells display condensed orange, and necrotic cells normal orange nucleus (Ribble et al., 2005). Most cells in control group exhibited normal green nuclei, whereas bright green and orange nuclei were shown in cells treated

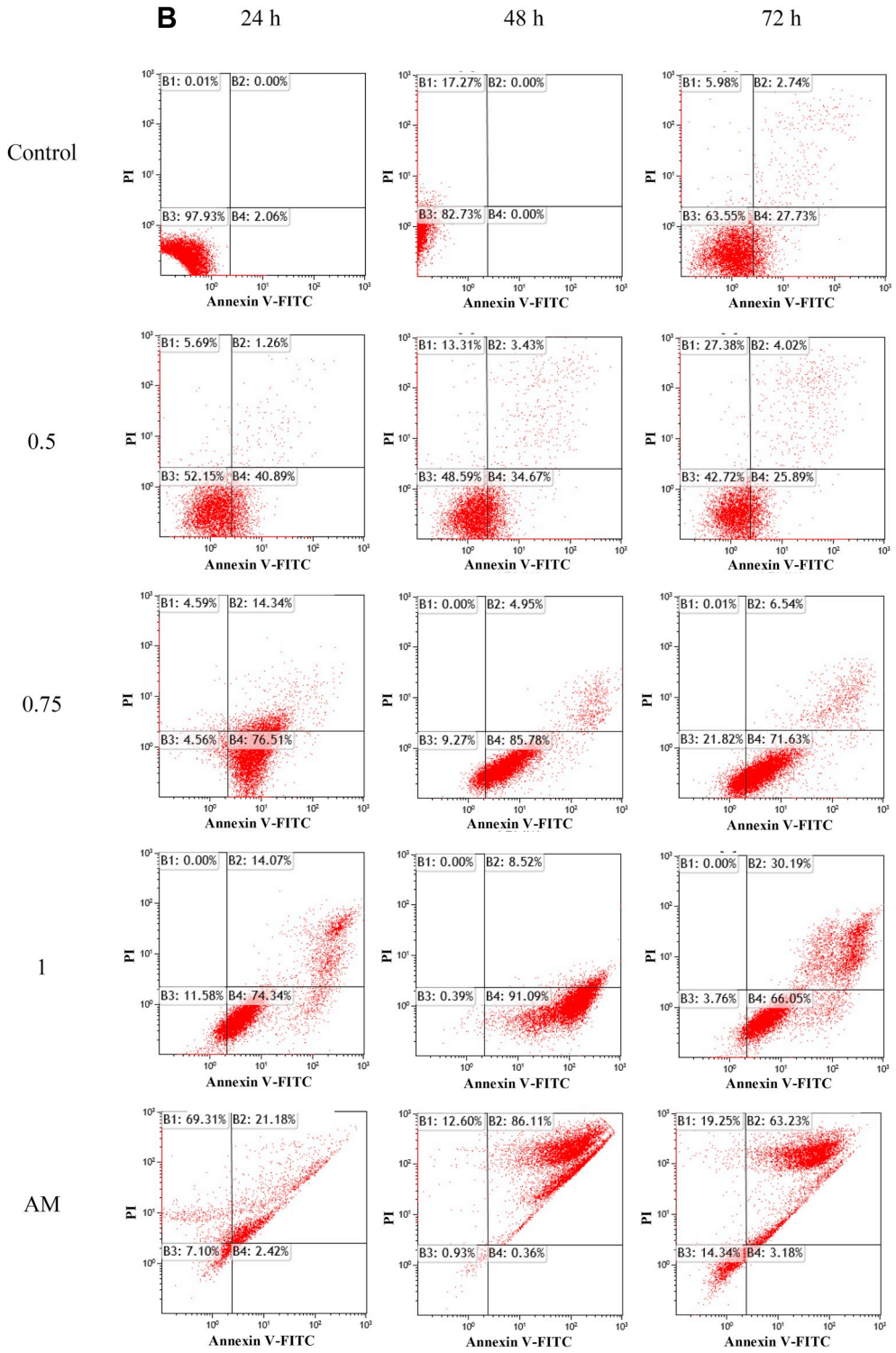

Fig. 4. (*continued*)

with MWL oil. The bright and orange-colored cells were increased with increasing concentrations. These results demonstrated that MWL oil induces apoptosis and necrosis at high concentrations in a concentration- and time-dependent manner.

In addition, nuclear morphological changes of HepG2 and Caco-2 were evaluated by staining the cells with Hoechst 33258. Untreated control HepG2 and Caco-2 cells showed normal chromatin staining. On the other hand, cells treated with MWL oil for 24, 48, and 72 presented bright chromatin condensation and fragmentation which are typical characteristics of apoptotic cells (Fig. 3). Healthy cell nuclei are spherical and DNA is evenly distributed (Crowley et al., 2019). However, cells nuclei presenting chromatin condensation, brightly stained, and marginalization are considered apoptotic (Pang et al., 2019). This morphology is considered one of the main characteristics of cell apoptosis (Umayaparvathi et al., 2014), which can be detected by Hoechst 33258 (Plesca et al., 2008). These results confirmed that MWL oil induces apoptosis against HepG2 and Caco-2 cells.

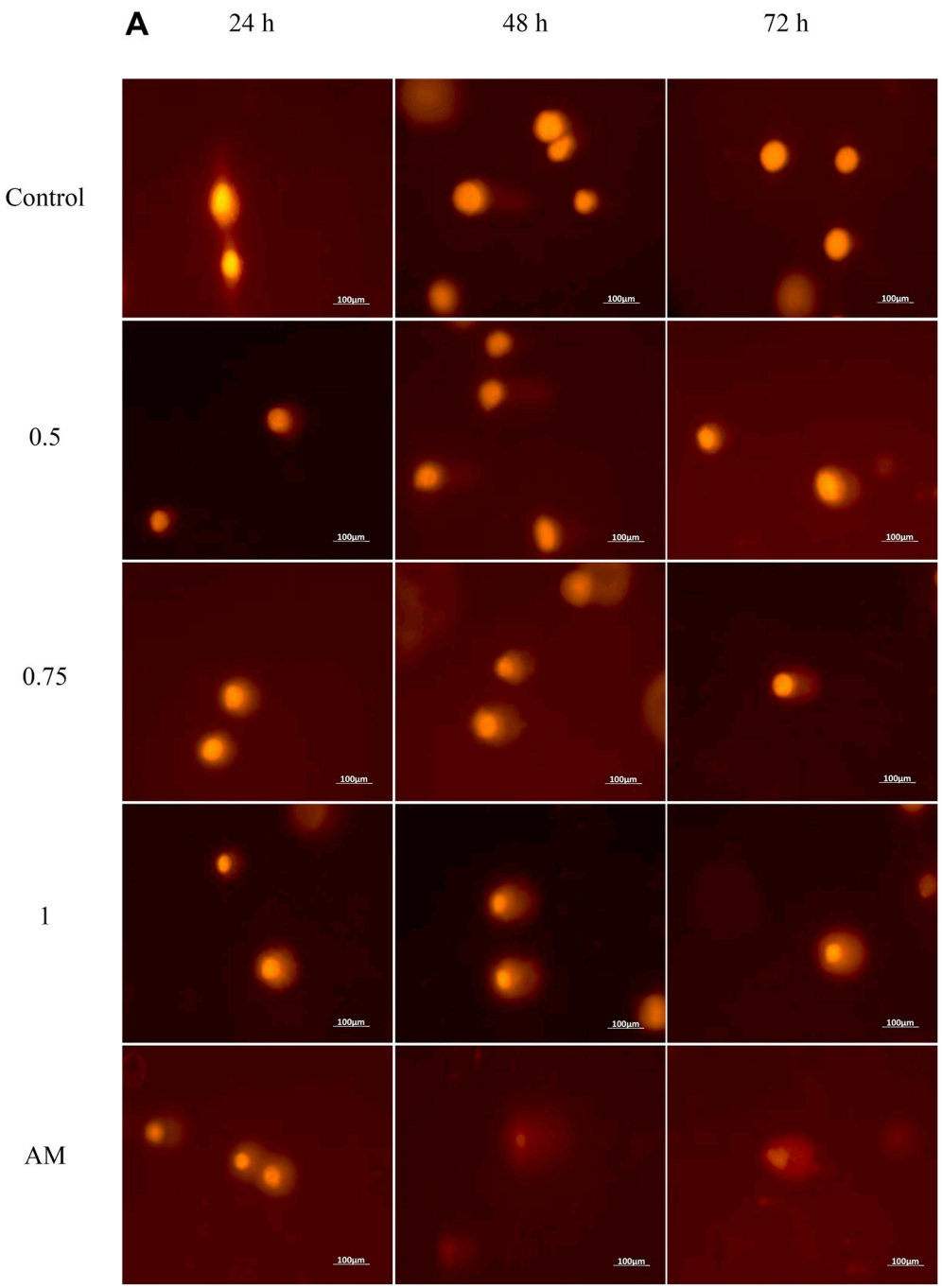

Fig. 5. Alkaline comet assay. HepG2 (A) and Caco-2 (B) cells treated with different concentrations of MWL oil were subjected to electrophoresis, stained with EB and seen through an inverted fluorescent microscope (400 ×) to determine the DNA damage. Percentage of tail DNA (C) and OTM (D) in Caco-2 and HepG2 cells treated with MWL oil for 24 (-1), 48 (-2), and 72 h (-3) (mean ± standard deviation). Control: cells with no test compounds. MWL treatments: 0.5, 0.75, and 1%. Acrylamide (AM): 10 mM/L. Treatments were significantly different compared to the control ($P < 0.01$).

3.4. MWL oil-induced apoptosis in HepG2 and Caco-2 cells assessed by flow cytometer analysis

Apoptosis of HepG2 and Caco-2 cells was analyzed by flow cytometer using annexin V-FITC/PI after treatment with MWL oil at 0.5, 0.75, and 1% for 24, 48, and, 72 h (Fig. 4). After 24 h, treatments with 0.5, 0.75, and 1% of MWL oil revealed that MWL oil treatment on HepG2 and Caco-2 increased early and late apoptosis from 0.04% to 39.77% and from 2.06% to 74.34%, respectively. After 48 h and 72 h of treatment, late apoptosis and necrosis cells increased in all treatments in a concentration- and time-dependent fashion.

Apoptosis is a homeostatic mechanism that balances cell division and death, maintaining the appropriate cell number in the body (Huang et al., 2012). MWL ethanol extracts have shown anticancer effects against human prostate, cervix, liver, colon, lung, breast, and ovary cancer cells. The mechanisms included apoptosis, necrosis, and autophagy (Lee et al., 2015). Additionally, fatty acids from *Protaetia brevitarsis* larva have also shown anticancer effects against colon cancer cells mediated by apoptosis (Yoo et al., 2007). After 24 h of treatment, 0.5, 0.75, and 1% of MWL oil showed higher apoptosis rates against HepG2 and Caco-2 cells compared with 5 mg/mL, 10 mg/mL, and 15 mg/mL of *Sepia* ink oligopeptide against prostate cancer cell lines (Huang et al., 2012).

Previous data have demonstrated the preventive effects of omega-6

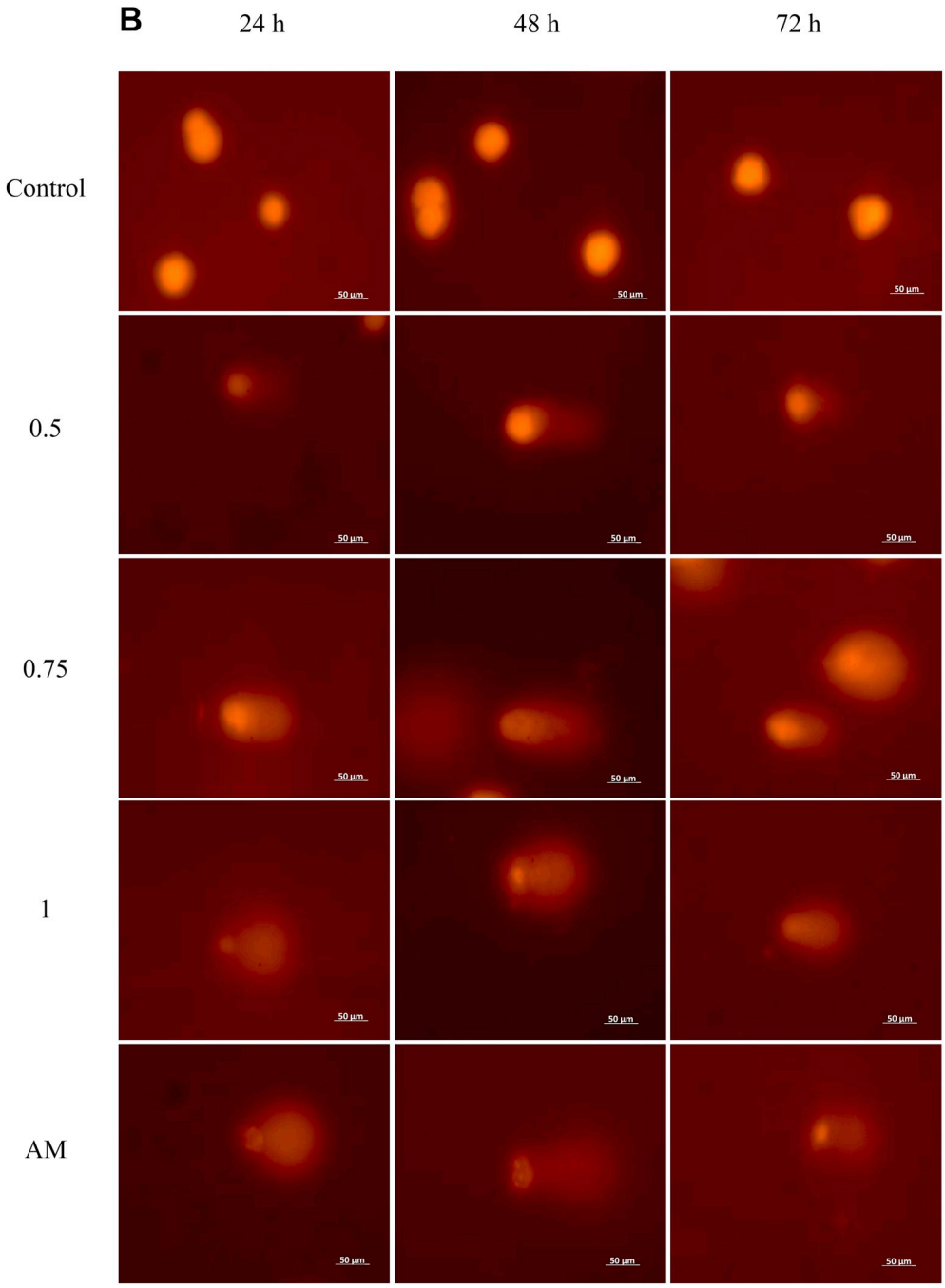

Fig. 5. (*continued*)

and omega-3 PUFAs (i.e. linoleic acid and α-linolenic acid) against HepG2 and colorectal cancer cells (Di Nunzio et al., 2011; Ortea et al., 2018). These PUFAs (linoleic acid and α-linolenic acid) are present in MWL oil, the amounts of which are 27.99 ± 0.19% and 2.98 ± 0.00% respectively. As can be seen, the amount of these fatty acids together is more than 30% of the total fatty acids in MWL oil. Yoo et al. (2007) reported that oleic acid, palmitic acid, and stearic acid isolated from *Protaetia brevitarsis* larvae showed high apoptotic-inducing activity mediated by caspase-3 activation in colon 26 tumor cells. MWL oil contains high relative amounts of oleic (45.79 ± 0.11%) and palmitic (16.27 ± 0.06%) acids and lower relative amounts of stearic acid (0.68 ± 0.01%). These fatty acids may provide MWL oil the anticancer effect against human HepG2 and Caco-2 tumor cells.

3.5. MWL oil induces DNA damage in HepG2 and Caco-2 cells

In order to investigate whether MWL oil induced DNA damage in HepG2 and Caco-2 cells, comet assay was performed (Gagné et al., 2014). MWL oil induced DNA damage which can be quantified by % of DNA in tail and tail moment (Fig. 5). After exposure of MWL oil and protein for 24, 48, and 72 h, DNA damage was induced in HepG2 and Caco-2 cells. Control cell showed undamaged DNA. The percentages of DNA in tail of cells treated with MWL oil were significantly different ($P < 0.01$) compared with that of control. Olive tail moment (OTM) of cells treated with MWL oil also showed significant differences ($P < 0.01$) compared with control cells. Different concentrations of treatments induced different grades of DNA damage as indicated by the presence of DNA in the tail. DNA damage response may lead to death of

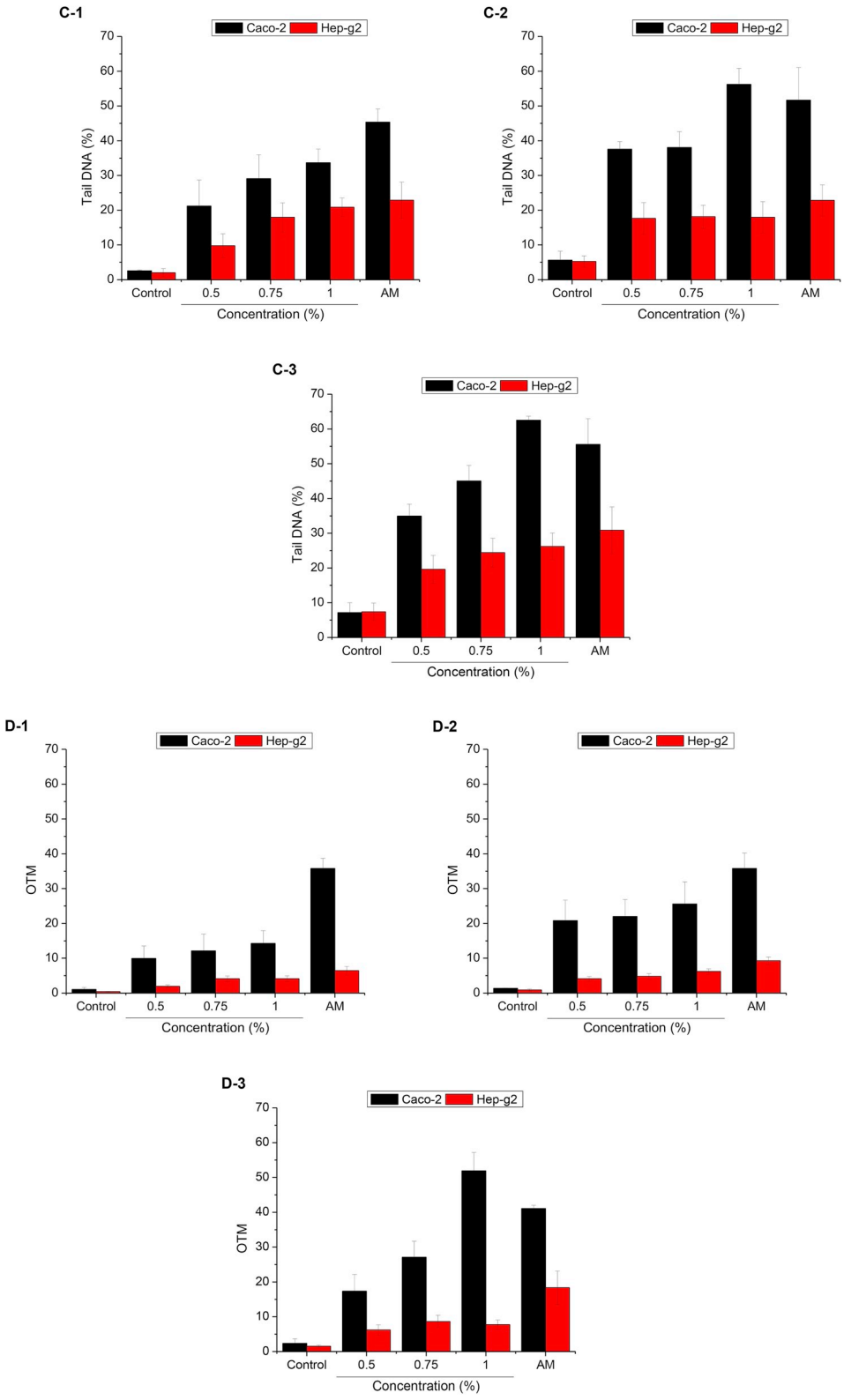

Fig. 5. (*continued*)

cells through apoptosis (Plesca et al., 2008). Induction of growth arrest and apoptosis on HepG2 and Caco-2 cells by MWL oil might be mediated by activation of DNA damage signaling.

3.6. Effect of MWL oil on caspase activities in HepG2 and Caco-2 cells

To uncover the mechanisms underlying apoptosis in HepG2 and Caco-2 cells, caspase-3, -8, and -9 activation was evaluated after treatment with MWL oil. The results show that MWL oil induced apoptosis through the death receptor pathway by the activation of caspases (Fig. 6). The activity levels of caspases increased in a dose-dependent manner ($p < 0.05$). Caspases were significantly activated after 24, 48, and 72 of treatment. Activated caspase -8 and -9 may lead to the activation of caspase-3. MWL oil modulated the extrinsic

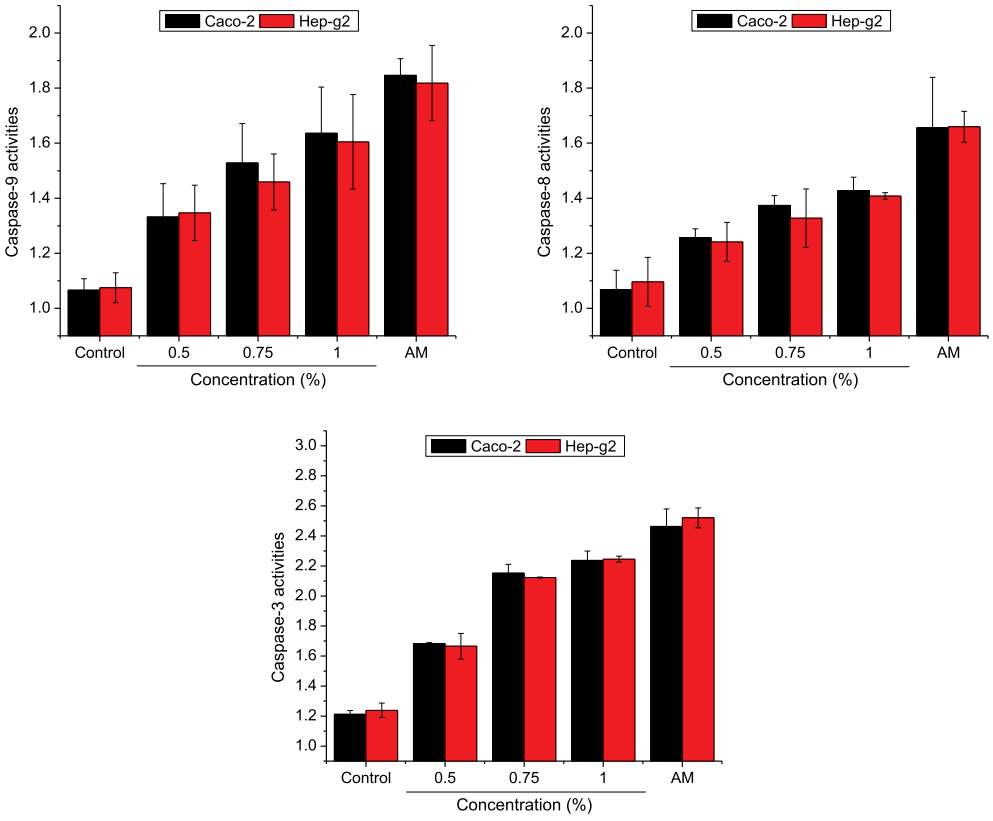

Fig. 6. Caspase activities of HepG2 and Caco-2 cells treated with MWL oil for 48 h. Determination of the activities of caspase-9, caspase-8, and caspase-3 were performed using synthetic caspase substrates. Control: cells with no test compounds. Acrylamide (AM): 10 mM/L. Data are expressed as mean ± standard deviation from three independent experiments. Treatments were significantly different ($P < 0.05$) compared to the control.

pathway of apoptosis in HepG2 and Caco-2 cells through the activation of caspase -8, -9 and -3. This result confirms the results of the comet assay. DNA damage in HepG2 and Caco-2 cells was mediated by the activation of caspase-3. These results were similar to those of *Protaetia brevitarsis* larva. Fatty acids from this larva induced apoptosis mediated by caspase-3 activation in tumor cells (Yoo et al., 2007). Fatty acids from the ground beetle *Eupolyphaga steleophaga* also induced apoptosis against HepG2 tumor cells (Wang and Ji, 2009). Blister beetle has shown cytotoxicity against a wide range of tumor cells (Kadioglu et al., 2014). Insects may provide potential chemotherapeutic agents to treat cancer. In chemotherapeutic agents, apoptosis is a common mode of action. Anticancer agents are successful because they induce apoptosis in cancer cells (Fang et al., 2012). Caspase-3 is one of the key executioners of apoptosis (Mishra et al., 2018). Therefore, these results suggest that MWL oil may induce apoptosis in HepG2 and Caco-2 cells via extrinsic pathway mediated by caspase -9 and -8, with caspase-3 which mediates DNA damage and cell death.

4. Conclusions

MWL oil showed antiproliferative effects against HepG2 and Caco-2 cells. Apoptosis was mediated via death receptor pathway mediated by caspase -8 and -9 followed by the activation of caspase-3; the latter may have induced DNA damage and cell death. The high presence of omega-3 fatty acids, oleic acid, and palmitic acid in MWL oil may be the responsible compounds for their antiproliferative effects. The results of this study suggest that MWL oil might be used in the development of natural cancer therapeutics. Further *in vitro* and pre-clinical studies should be developed to determine the compounds responsible for the anticancer activities.

Author contributions

Qingzhi Ding and Ricardo A. Wu conceptualized and designed the research. Ricardo A. Wu wrote the first draft. Ronghai He, Haile Ma, and Ricardo A. Wu analyzed the data. Ricardo A. Wu, Huijia Lu, Nianzhen Sun, Zhikun Li, Haochen Tang, and Kai Wang performed the experiments. Lin Luo contributed to the discussion and edition of the manuscript. All authors revised and approved the manuscript.

Declaration of competing interest

None.

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