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ANTIPROLIFERATIVE AND APOPTOTIC EFFECTS OF THE ESSENTIAL OIL OF *ORIGANUM ONITES* AND CARVACROL ON HEP-G2 CELLS

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ABSTRACT

The essential oil *Origanum onites* L. and its phenolic constituent carvacrol were examined for their cytotoxic and apoptotic effects in a human hepatocellular carcinoma cells Hep-G2. WST-1 and neutral red uptake assays were performed to determine the inhibitory effects of the oil and carvacrol on the growth of the cells. Possible induction of apoptosis by *Origanum* oil and carvacrol was further investigated by acridine orange/ethidium bromide (AO/EB) staining. Results showed that the *Origanum* oil and carvacrol was significantly cytotoxic and induced apoptosis in Hep-G2 cells. IC_{50} value of essential oil and carvacrol was found about 0,009% (v/v) and 500 μM, respectively. After incubation of the cells with *Origanum* oil and carvacrol, characteristics of apoptotic morphology such as chromatin condensation, shrinkage of the cells and cytoplasmic blebbing was observed. In conclusion, both essential oil and its major constituent carvacrol significantly exhibited cytotoxic and apoptotic activities in hepatocellular carcinoma cells, indicating its potential for use as an anticancer agent.

Keywords: *Origanum* oil, carvacrol, Apoptosis, Cytotoxicity, Hep-G2.

ORIGANUM ONITES UÇUCU YAĞI VE KARVAKROLÜN HEP-G2 HÜCRELERİ ÜZERİNDEKİ ÇOĞALMAYI ENGELLEYİCİ VE APOPTOTİK ETKİLERİ

ÖZ

Origanum onites L. uçucu yağı ve onun fenolik bileşeni olan karvakrolün insan kanserli karaciğer hücreleri Hep-G2 üzerindeki sitotoksik ve apoptotik etkileri çalışılmıştır. Hücrelerin çoğalması üzerine *origanum* uçucu yağının ve karvakrolün engelleyici etkilerini belirlemek için WST-1 ve nötral kırmızısı alım yöntemleri uygulanmıştır. *Origanum* yağı ve karvakrolün olası apoptotik etkisi akridinoranj/etidyumbromür (AO/EB) boyama yöntemiyle araştırılmıştır. Sonuçlar değerlendirildiğinde, Origanum yağı ve karvakrolün Hep-G2 hücrelerinde anlamlı derecede sitotoksik etkiye sahip olduğu ve bu hücrelerde apoptozu uyardığı gözlenmiştir. Uçucu yağın IC50 değeri % 0.009, karvakrolün 500 mM olarak belirlenmiştir. *Origanum* uçucu yağı ve karvakrol ile inkübasyonun ardından hücrelerin kromatin yoğunlaşması, hücre büzülmesi, sitoplazmik keselerin oluşumu gibi karakteristik apoptoz morfolojisi gösterdiği belirlenmiştir. Sonuç olarak, hem *Origanum* uçucu yağı hem de onun temel bileşeni olan karvakrol, insan kanserli karaciğer hücrelerinde anlamlı bir sitotoksik etki ve apoptotik aktivite sergilemiştir ve bu özelliklerinden dolayı, her ikisi de antikanser ajan olarak kullanılabilme potansiyeline sahiptir.

Anahtar Kelimeler: *Origanum* yağı, Karvakrol, Apoptoz, Sitotoksisite, Hep-G2.

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1. INTRODUCTION

The Essential oils of *Origanum* species have been used in folk medicine as a remedy against burns and wounds, agriculture, alcoholic beverages, fragrance and flavouring substances of food products (Sivropoulou et al., 1996; Aligiannis et al., 2001; Şahin et al., 2004; Baser, 2008). Due to their rich carvacrol content as monoterpenic phenols, essential oils of *Origanum* genus have been shown scientifically to possess various biological activities such as antioxidant, anti-fungal, antimicrobial, insecticidal, anti-melanogenic, anti-inflammatory and wound healing (Tabanca et al., 2001;Vincenzi et al., 2004; Chorianopoulos et al., 2004; Baser, 2008; Bakkali et al., 2008; Aslim et al., 2008; Kordali et al., 2008; Karpouhtsis et al., 2008; Govaris et al., 2010; Ding et al., 2010; Fuentes et al., 2010; Süntar et al., 2011). Essential oil of *Origanum onites* L. and its major component carvacrol exhibited a strong antimutagenic effect on TA98 and TA100 strains of *S. typhimurium* (Ipek et al. 2005). Furthermore, angiogenic effects of essential oils of *Origanum minutiflorum* were investigated in shell-lesschick embryo culture. This study demonstrated that *O. minutiflorum* provided dilatation in the blood vessels and increase in the number of new vessels (Goze et al., 2010). In specifically, carvacrol affected the cells via interaction with the cytoplasmic membrane by changing its surface and permeability (Ultee et al. 1999; Storia et al., 2011). Recently, an investigation by Genomic profiling revealed a transcriptional response to carvacrol in *S. cerevisiae*. Genes involved in alternate metabolic and energy pathways and stress response were prominently upregulated, whereas repressed genes mediated ribosome biogenesis and RNA metabolism (Rao et al., 2010). Kim et al. (2010) reported the altered expression of 74 genes associated with metabolic pathways in intestinal intraepithelial lymphocytes of carvacrol-fed chickens.

Anti-cancer, anti-proliferative and apoptotic effects of essential oil and several extracts from *Origanum* species on various cell types including leukemic cell, platelets and breast adenocarcinoma cells were also reported by others (Chinou et al.2007; Jelnar et al., 2009; Roula et al., 2010; Al-Kaladeh et al., 2010). Furthermore, carvacrol inhibited tumorigenesis and the growth of murine melanomas, chronic myeloid leukemia cells, N-*ras* transformed mouse myoblasts and human breast cancer cells (He et al. 1997; Stammatia et al. 1999; Zeytinoglu et al., 2003; Karkabounas et al., 2006; Lampronti et al., 2006; Horvathov et al., 2007; Arunasree, 2010).

The present study is aimed to investigate the cytotoxic and apoptotic effects of both essential oil of *Origanum onites* L. (Ipek et al., 2005) and carvacrolin a hepatocarcinoma cell line, HepG2. Cytotoxicity was investigated by WST-1 and neutral red uptake (NR) assays. Apoptotic morphology of the cells was examined and scored by acridine orange/ethidium bromide double staining (AO/EB). We report that *Origanum* essential oil and its major component carvacrol possessa significant cytotoxic and apoptotic activity Hep-G2 cells indicating their potential use as anti-cancer compound at least for hepatocarcinoma.

MATERIAL AND METHODS

Essential oil of *Origanum onites* and Carvacrol

Carvacrol (2-methyl-5-(1-methylethyl)-phenol) was purchased from Sigma-Aldrich as %98 purity (Figure 1). Essential oil of *Origanum onites* L. is kindly provided from Prof.Dr. K.C.H. Baser (Anadolu University, Turkey). Content of the oil has been analysed before as given in the Table 1 (Ipek et al., 2005). Different concentration of essential oil (0.002-0.02% v/v) and carvacrol (50-800 μM) were dissolved in DMSO and used freshly for each experiment.

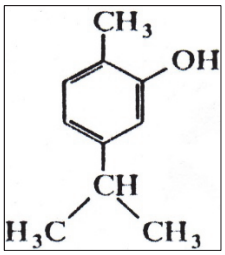

Figure 1. The structural formula of carvacrol cited in the present work.

Table 1. Major constituents of essential oil of *Origanum onites* L. analysed by GC (Ipek et al., 2005).

COMPOUND	%
Carvacrol	74.0
Linalool	7.2
Thymol	4.4
p-cymene	3.0
b-bisabolene	1.4
Caryophyllene oxide	1.3

Cell Culture

A human liver hepatocellular carcinoma cell line HepG2 (DSMZ, Germany) were maintained in Dulbecco Modified Eagle Medium (DMEM) (Sigma) supplemented with 10% (v/v) of foetal bovine serum (FBS) (PAA Lab. GmbH), penicillin/streptomycin at 100 units/ml and 2 mM L-glutamine as adherent monolayers. Cultures were incubated at 37^{o}C under 5% CO_2 / 95% air in a humidified atmosphere. Cells were passaged and harvested by 1% trypsin/EDTA (Sigma).

Cell Viability Assay

The Cell Proliferation Reagent WST-1 (Roche) measures the metabolic activity depends on the mitochondrial enzyme reduction of tetrazolium dye to determine cell viability (Mosman, 1983). HepG2 cells were seeded on to 96-well culture plates at a density of $1X10^4$ cells/well. After 24 h incubation, cells were treated with a series of concentrations ofthe oil or carvacrol for 24, 48 h and 72 h. Then 10 ml of WST-1 solution was added to each well and incubated 3 hours. The relative cell viability was quantified by a microplate reader (ELX 808 IU) at 490 nm. Absorbance values for wells containing medium alone were subtracted from the results obtained with the test wells. The results were compared to control cells treated with DMSO not exceeding 0.1%.

Neutral Red Incorporation Assay

Viability of HepG2 cells was further assessed using neutral red (NR) incorporation assay. HepG2 cells were seeded on to 96-well culture plates at a density of $1X10^4$ cells/well one day prior to the oil or carvacrol treatment. After 24, 48 h and 72 h incubation of cells with the test compounds, cells were treated with 1%NR solution further 3 hours. The incorporated NR crystals was then dissolved with 100 µl of DESORB (50% ethanol + 1% glacial acetic acid) and quantified at 540 nm using Elisa-type spectrophotometer (EL_x800).

Morphological Observation by Acridin Orange/Ethidium Bromide

Morphology of HepG2 cells was initially observed under inverted microscope before performing all experiments. In order to investigate whether the cell death is a cause of apoptosis, acridine orange/ethidium bromide (AO/EB) double staining was performed according to Unlu et al. (2010) to provide the morphological evidence. For this purpose, cells were seeded as $3X10^5$ cells per well on to a six-well plate and incubated with or without different concentration of the oil or carvacrol after 24 h adherence. At the end of the 48 h incubation, cells were harvested and resuspended in culture medium, thenstained with 0.1 mg/mL AO and 0.1 mg/mL EB (Sigma) in PBS. Cells were immediately examined under a fluorescence photomicroscope (Olympus BX50). Apoptotic cells were expressed as a percentage of controls by scored total approximately 1500 cells from two different experiments using the following formula (Duke, 2004);

of % apoptotic cells = LA + DA /LN+LA +DN+ DA x 100

Live cells with bright green normal nuclei (LN)
Live cells with bright green apopotic nuclei (LA)
Dead cells with bright orange apoptotic nuclei (DA)
Dead cells with bright orange normal nuclei (DN)

Statistics

For most experiments, mean values were compared with solvent controls by Dunnett's in ANOVA, SPSS to evaluate statistical differences

RESULTS AND DISCUSSION

Essential Oil

Analysis of essential oil prepared from *Origanum onites* L. by GC was reported as seen in Fig.1 (Ipek et al., 2005). At least six compounds were determined and carvacrol was the major constituent as 74%.

Effects of *Origanum* Essential Oil And Carvacrolon Cell Viability

The inhibitory effects of origanum essential oil on the growth of HepG2 cell were measured using WST-1and NR uptake assays. Results were expressed in terms of relative absorbance of *Origanum* essential oil- or carvacrol-treated cells, in comparison to control cells as seen in the Figure 2. The oil showed reduction in the viability of HepG2 cells from doses of around 0.008% ($p<0.05$ against control cells) and IC_{50} values was determined about 0,009% ($p<0.001$ against control cells) after 24 and 48 h of exposure as shown in Fig. 2A and B. It was our main observation that growth of cells was inhibited in a concentration-dependent manner for 24, 48 and 72 h exposure tothe oil in both WST-1 and NR assays.

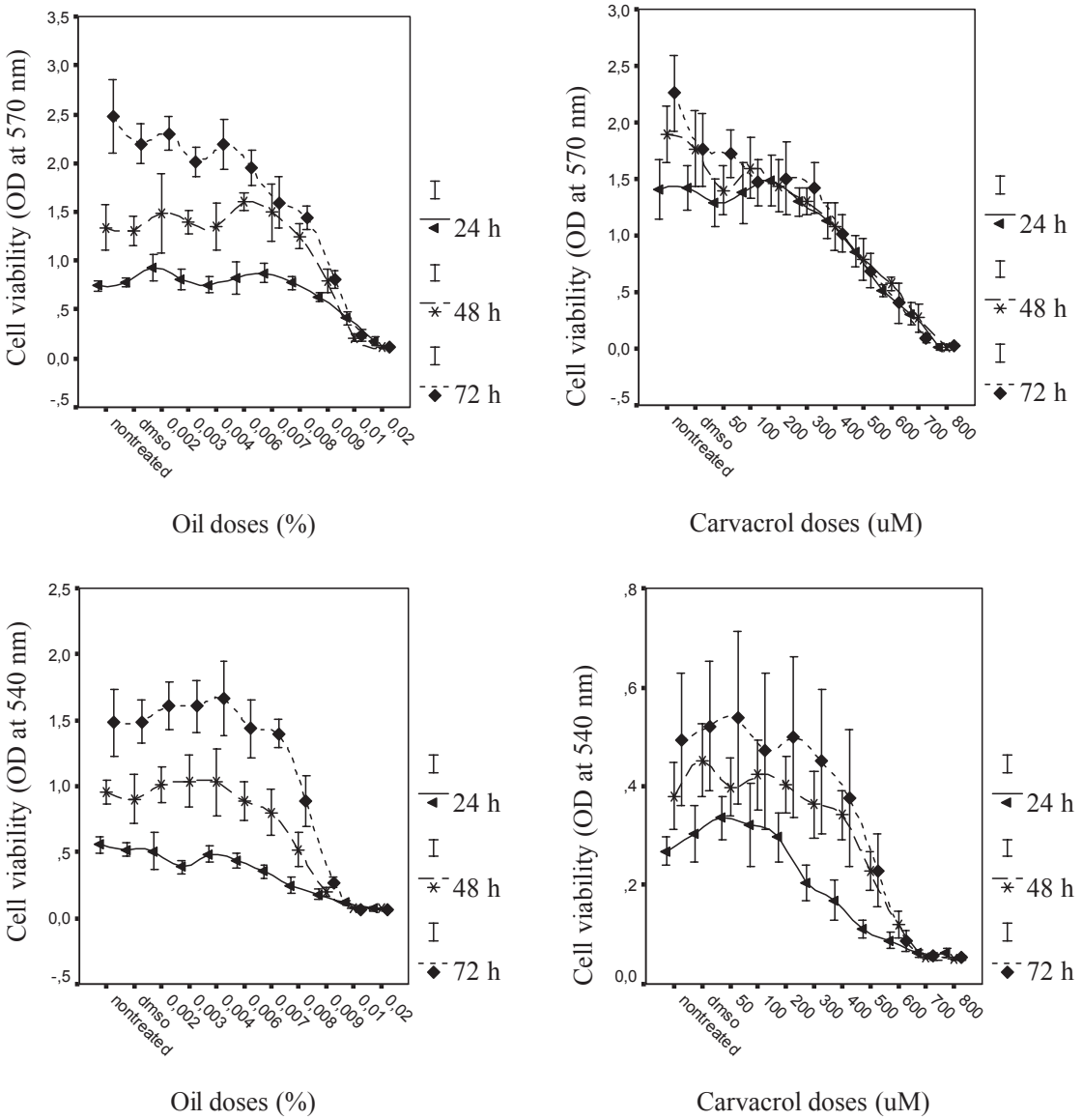

Figure 2. Inhibitory effects of origanum oil (A, B) and carvacrol (D, E) on the growth of HepG2 cells evaluated by MTT (A, D) and neutral red uptake (B, E) assays. Each value is the mean ±S.D. of at least two separate experiments performed in quadruplicate.

Inhibition of the growth was slightly time-dependent since reduction in cell viability from 0.006% doses and IC50 was around 0.008% after 24 h exposure.

The viability of HepG2 cell treated with carvacrol was also evaluated usingWST-1and NR uptake assays. Results were expressed in terms of relative absorbance of carvacrol-treated cells, in comparison to control cells as seen in the Figure 2C and D. The carvacrolexhibited reduction in the viability of HepG2 cells from doses of around 100 μM ($p<0.05$ against control cells) and IC_{50} values was determined about 500 μM ($p<0.001$ against control cells) after 48 and 72 h of exposure. The growth of cells was inhibited in a concentration-dependent manner for 24, 48 and 72 h exposure tothe oil in both WST-1 and NR assays. Inhibition of the growth was slightly time-dependent since reduction in cell viability from 300 μM and IC_{50} was around 400 μM after 24 h exposure determined by NR assay. However, this IC_{50} value is not the same as reported by a previous study (Stammatia et al. 1999), it might be due to cell type and purity of carvacrol.

Here we tested the growth inhibitory effects of the oil and carvacrol using two different assays to detect the viability of cells and compare sensitivity of the assays. WST-1and NR assays are the most common used for the detection of cytotoxicity or cell viability following treatment to toxic compounds (Stammati et al., 1999; Fotakis and Timbrell, 2006 Xiao et al., 2007; Al-Kalaldeh et al., 2010). The WST-1 assay is based on the enzymatic conversion of a water soluble tetrazolium salt to an insoluble purple formazan by succinate dehydrogenase within the mitochondria. On the other hand, NR uptake assay is based on the uptake of the neutral red which is concentrated within the functional lysosomes (Fotakis and Timbrell, 2006). In order to avoid getting positive or negative estimation of the toxicity of a compound, more than one assay should be used to determine cytotoxicity in in vitro studies. Here, we found IC_{50} values of the compounds obtained by WST-1 and NR assay quite similar, indicating the effects of the oil and carvacrol on both mitochondrial and lysosomal pathway in cells.

Effects of The Oil On Cell Morphology

Morphological observation of HepG2 cells was initially performed under an inverted microscope and then florescence microscope after staining AO/EB. AO/EB staining method is based on the differential uptake of fluorescent dyes which bind DNA in viable, apoptotic or necrotic cells (Xiao et al., 2007). Cells incubated with 0,008%and 0,009%(v/v) of the oil (Fig.3B and C) and 300 and 500 μM carvacrolfor 48 h showed obvious changes in their morphology such as the loss of adhesion and rounded in shape compared to control cells (Fig.3D and E). As seen in the microphotographs, treatment of the oil (Fig.3G and H) and carvacrol(Fig.3I and K) caused significant apoptotic cell death.Early apoptotic live cells were observed with bright green areas of condensed/fragmented chromatin (LA), late apoptotic dead cells were with bright orange condensed chromatin (DA) and necrotic or dead cells were with bright orange uniform nucleus (DN). Under control conditions, cell showed round and homogeneous bright green nuclear morphology (LN).

Apoptotic cells were expressed as a percentage of controls by scored total approximately 1500 cells from two different experiment using the following formula (Duke, 2004);

% apoptotic cells = LA + DA /LN+LA +DN+ DA x 100.

Effects of *origanum* essential oil and carvacrolon the morphology and the number of apoptotic cells were significant in a dose-dependent manner as given in Fig.4A and B, respectively. The highest percentage of apoptotic cells was about 65% in cells treated with 0,009% essential oil and 95% treated with 500 μM carvacrol for 48 h. These morphological observations were consistent with the results of the cytotoxicity and apoptosis experiments. High concentrations of the compounds also caused immediate cell death with the morphological features of cell lysis (data not shown).

Carvacrol as a major constituent of essential oils of various medicinal plant exerted quite strong apoptotic effect on cells. In the essential oil, the cytotoxic and apoptotic effect might be related to the presence of some major components such as carvacrol, thymol and thymoquinone (Baser et al., 1993). Previously, Crowell (1999) reviewed prevention and therapy of cancers by monoterpenes. The inhibition of cell growth in melanoma by isoprenoids including carvacrol and its metabolites has reported by several grubs (Crowell et al. 1994; He et al. 1997). Both essential oils from *Origanum* species and carvacrol have been reported to have cytotoxic and apoptotic effects on various cell types (Stammatia et al. 1999; Zeytinoglu et al., 2003; Karkabounas et al., 2006; Horvathov et al., 2007; Chinou et al. 2007; Jelnar et al., 2009**;** Roula et al., 2010; Al-Kaladeh et al., 2010; Arunasree, 2010).

In conclusion, these data indicate the antiproliferative and apoptotic effects of essential oil of *Origanum onites* L. andits major component carvacrol in a hepatocellular carcinoma cells HepG2 at very low concentration. Induction of apoptotic morphology at these doses is also supported the cell viability data. Therefore, origanum oil and mainly carvacrol may have potential and may find application as an anticancer agent. These results call for further investigations of the mechanism of apoptosis in cancerous cells treated with carvacrolor other constituents of origanum oil such as thymolat molecular level such as related gene regulation and protein activation.

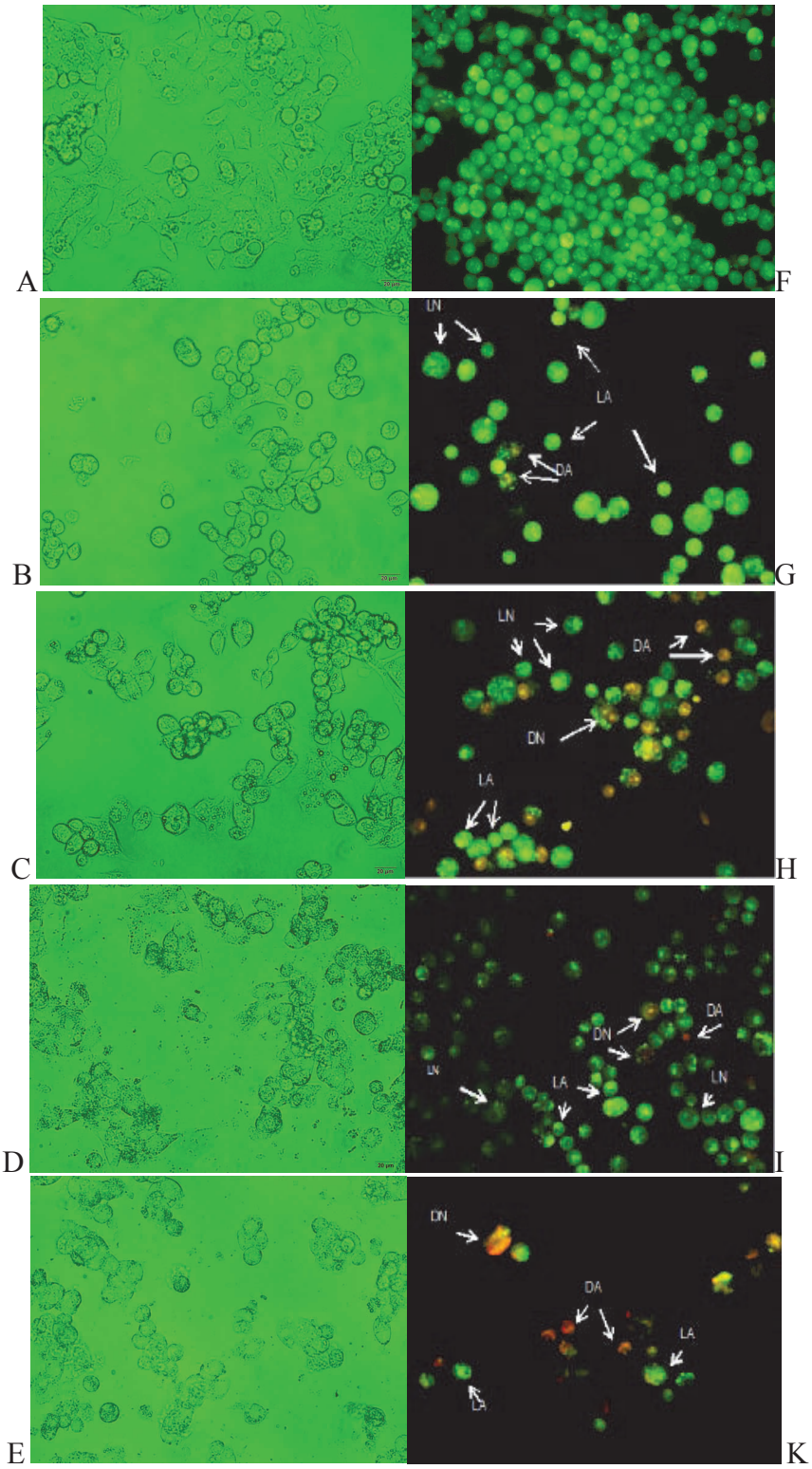

Figure 3. Apoptotic morphologyof HepG2 cells induced by origanum oil (0.008% B,G and 0.009% C,H) and carvacrol (300 µM D,I and 500 µM E,K) after 48 hwas observed under inverted microscope (B,C,D,E) and fluorescence microscope (G,H,I,K) stainingby AO/EtBr. Control cells treated with DMSO under inverted (A) and fluorescence microscope (F). Early apoptotic live cells were observed with bright green areas of condensed/fragmented chromatin [LA], late apoptotic dead cells were with bright orange condensed chromatin [DA] and necrotic or dead cells were with bright orange uniform nucleus [DN]. Under control conditions, cell showed round and homogeneous bright green nuclear morphology [LN]. Mag. 400X.

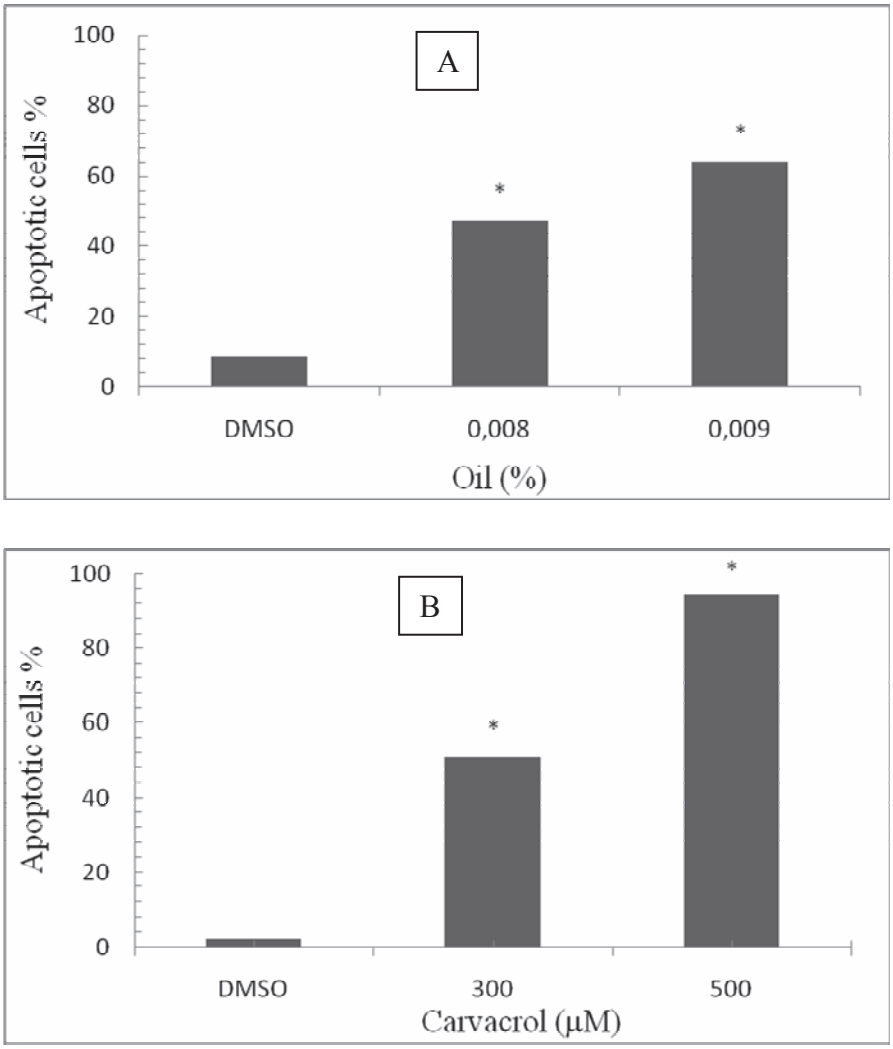

Figure 4. Number of apoptotic cells trated with the oil (A) and carvacrol (B) for 48 h scored and expressed as a percentage of controls.

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