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Original article

THE EFFECTS OF CARVACROL ON APOPTOSIS OF H-*RAS* AND N-*RAS* TRANSFORMED CELL LINES

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Abstract

Carvacrol is a phenolic monoterpen found in Thyme plant which is used as a source of flavour in food. The aim of this study was to investigate apoptotic effects of carvacrol on H-ras transformed 5RP7 and N-ras transformed CO25 cell lines and possibilities of carvacrol as being a chemoterapeutic drug. The data was shown that carvacrol has cytotoxic effects on both cell lines upon time- and concentrations. IC_{50} values were determined 0.04 mg/mL for 5RP7 and 0.1 mg/mL for CO25 cell lines. Depending on the IC_{50} values, although carvacrol induced morphological changes on both cell types, mobility of phosphatidilserine localisation analysed by flow cytometry was only detected on 5RP7 cells. Beside, DNA laddering which is the late apoptotic determinant was seen on H-ras transformed cells but not on N-ras transformed cells at concentration IC_{50} value and below. These results indicated that H-ras transformed cells are more sensitive to carvacrol than N-ras tranformed cells. This investigation suggests the possibility that carvacrol may find application in cancer therapy as an antineoplastic drug.

Key words: *Carvacrol, Apoptosis, N-ras, H-ras, Cell culture, Cancer.*

Karvakrol'ün H-ras ve N-ras Transform Hücre Apoptozu Üzerine Etkileri

Fenolik bir monoterpen olan karvakrol kekik bitkisinde bulunmaktadır ve besinlerde baharat olarak kullanılmaktadır. Bu çalışmanın amacı, karvakrol'ün H-ras transform edilmiş 5RP7 ve N-ras transform edilmiş CO25 hücreleri üzerine apoptotik etkilerinin incelenmesi ve karvakrol'ün kemoterapötik ajan olabilme olasılığının değerlendirilmesidir. Elde edilen sonuçlar, karvakrol'ün her iki hücre üzerinde de zamana ve doza bağımlı sitotoksik etkisinin olduğunu göstermiştir. IC_{50} değeri 5RP7 hücreleri için 0.04 mg/mL ve CO25 hücreleri için ise 0.1 mg/mL olarak tespit edilmiştir. Bu değerler doğrultusunda, karvakrol her iki hücre tipinde morfolojik değişime neden olduğu halde akım sitometrisi kullanılarak tespit edilen fosfotidilserin hareketliliği yalnızca 5RP7 hücrelerinde gözlenmiştir. Bunun yanı sıra, geç apoptoz mekanizmasının göstergesi olan DNA'nın merdiven görünümü, IC_{50} değerinde ya da altındaki bir değerde H-ras onkogenine sahip hücrelerde gözlenirken, N-ras onkogenine sahip hücrelerde gözlenmemiştir. Elde edilen bu sonuçlar, H-ras hücrelerinin N-ras hücrelerine oranla karvakrol'e daha hassas olduğunu göstermiştir.

Anahtar kelimeler: *Karvakrol, Apoptoz, N-ras, H-ras, Hücre kültürü, Kanser.*

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INTRODUCTION

Apoptosis is a cellular process by which damaged cells actively facilitate their own demise without damaging their neighbours, thus selectively removing themselves from the cellular population (1). Apoptotic process is charecterized by distinctive morphological changes that include condensation of nuclear chromatin, cell shrinkage, nuclear disintegration, plasma membran blebbing and the formation of membrane-bound apoptotic bodies (2). In addition to these major changes, in many cells biochemical changes occur. These include an early increase in unbound cytosolic Ca^{+2} (3), hydronium ions, ceramide (4) together with dilation and rupture of the endoplasmic reticulum. One of the morhological changes is phosphatidilserine redustribution to the outer face of cell membran which can be detected by annexinV-FITC (5). A molecular hallmark of apoptosis is degradation of nuclear DNA into oligonucleosomal-lenght fragments of 180 bp or multiple breaks as the result of activation of endogenous endonucleases (6) that enzymes are depend on cytosolic calcium. Calcium compartmentalization also influences protein kinase C, tyrosine kinase, proteases, sphyngomyelin, sphingosine and arabinosyl cytosine, which all are important in apoptotic pathways (7).

In contrast to apoptosis, necrosis is an accidental cell death often caused by toxic agents. Necrosis is characterized by swelling and bursting of the cell, leading to release of cytoplasmic materials into the extracellular space, as well as by random cleavage of DNA. Although toxic agents have the potential to cause necrosis, some of them can interfere with intracellular signalling pathways and induce apoptosis instead of necrosis (2).

Carvacrol (5-Isopropyl-2-methylphenol) used in this study, the predominant monoterpenic phenol which occurs in many essential oils of the family Labiatea including Origanum, Satureja, Thymbra, Thymus and Corydothymus species used through the ages as a source of in food. Carvacrol (Figure 1) has antibacterial (8, 9), antifungal (10), insecticidal (11) and antioxidant (12) effects.

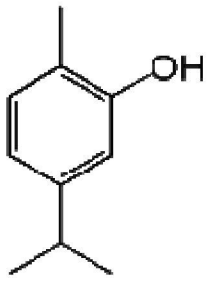

Figure 1. Chemical structure of carvacrol

Treatment of Hep-2 cells derived from a human larynx carcinoma induced the apoptotic phenotype (13). Besides these effects carvacrol seemed to protect lymphocytes from the genotoxic effects of heterocyclic amine IQ and mitomycin C at non-toxic concentrations below 0.05 mM, but at the higher concentration of 0.1 mM carvacrol itself induced DNA damage (14). Previous study showed that carvacrol inhibited DNA synthesis above 60 μg/mL concentration on CO25 cell line (15). Because of that we made further investigations about apoptosis with carvacrol on both N-*ras* and H-*ras* transformed cell lines.

EXPERIMENTAL

Cell culture

Dr. I. Gibson (University of East Anglia, UK) kindly provided the mouse skeletal muscle cell line, CO25. CO25 cells were derived by transfection of the parenteral C2 cells with a plasmid containing a mutational activated human N-*ras* gene (in codon 61) under transcriptional control of the steroid–sensitive promoter of the mouse mammary tumour virus long terminal repeat (MMTV-LTR). Therefore, to induce transformation, 1μM Dexamethasone (Dex) (Sigma, Deisenhofen, Germany) was added to the cells grown in 10 % Horse Serum (HS) (16, 17).
5RP7 cells were derived by transfection of the parenteral F2408 rat embryo fibroblast cells with a pEI plasmid containing c-Ha-*ras* cloned from T24 carcinoma cells. Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Sigma, Deisenhofen, Germany) supplemented with 10 % (v/v) of Foetal Calf Serum (FCS) (Gibco, UK), penicillin/streptomysin at 100 Units/mL as adherent monolayers. Both cell lines were incubated at 37°C under 5 % CO_2 / 95 % air in a humidified atmosphere.

Stock solutions of carvacrol (5-Isopropyl-2-methylphenol) were prepared in dimethyl sulphoxide (DMSO) and latter dilutions were made with fresh culture medium. (The concentration of DMSO in the final culture medium was < 1% which had no effect on the cell viability) (18).

MTT Assay

The level of cellular MTT (3-[4,5-dimethylthiazol-2yl)-2,5-diphenyl-tetrazolium bromide) (Sigma, Deisenhofen, Germany) reduction was quantified as previously described in Mossmann (19) with small modifications. Briefly cells were cultured (2×10^4 cell /mL) in 96-well plates and 0.0002-0.001-0.002-0.01-0.02-0.1 mg/mL carvacrol added and incubated for 24 or 48 h. After that MTT (5 mg/mL) was added to each well, absorbance was read at 540 nm on a microplate reader (Elx808-IU Bio-Tek plate reader). Every concentration was repeated in three wells and control cell viability was accepted % 100.

Trypan blue staining

Cells (1×10^6 cell/mL) were incubated with carvacrol for 24 or 48 h. Trypan blue exclusion method was used to determine cell count (with a hemocytometer) and viability (clear viable vs blue non-viable cells).

Acridine orange / ethidium bromide staining

The percentage of apoptotic cells was measured under the fluorescence microscope (Olympus) by staining the cells with acridine orange and ethidium bromide. Acridine orange (100 μg/mL) was mixed with ethidium bromide (100 μg/mL) in 1× phosphate buffer solution (PBS). Dye mix was incubated 5 min with 5RP7 cells treated with either 0.02 or 0.04 mg/mL carvacrol and CO25 cells treated with either 0.05 or 0.1 mg/mL carvacrol for 8 or 24 h.

Live cells were determined by the uptake of acridine orange (green fluorescence) stain. Live and dead apoptotic cells were identified by the perinuclear condensation of chromation stained by acridine orange or ethidium bromide, respectively and by the formation of apoptotic bodies. Necrotic cells were identified by uniform labelling of the cells with ethidium bromide (20).

Flow cytometric analysis

Following incubation of cells with carvacrol for 24 or 48 h $2\text{-}3 \times 10^6$ cells/mL were washed two times with 1 × PBS and co-stained with Annexin-V and Propidium Iodide (PI) according to the manufacturer's protocol (Roche, Annexin-V-Fluos staining kit). Flow cytometry was performed with a Becton Dickinson FACScan (Becton Dickinson Immunocytometry Systems, Calibur).

Analyses of DNA fragmentation

Following culture of 1 × 10^6 cells/mL in culture medium, cells were treated with carvacrol for 9, 16 or 24 h. After cells were collected and washed with 1 × PBS and DNA was isolated according to the manufacturer's protocol (Roche, Apoptotic DNA-Ladder Kit). The DNA was then loaded on to 1 % agarose gel with ethidium bromide, electrophoresised at 50 V for 2 h and band visualized by UV illumination.

Statistics

The SPSS for windows 11.5 computer programme was used for statistical analyses. Statistical comparison of the results from controls, groups and days parameters were carried out by the one-way analyses of variance (ANOVA) test and post hoc analyses of group differences was performed by the Tukey test. Results were expressed as mean ± S.D.

RESULTS

Cytotoxicity of the carvacrol was determined with a MTT assay as described in Material and Methods. As shown in Figure 2 a, b both cell lines were incubated with various concentrations (0.0002-0.001-0.002-0.01-0.02 mg/mL) of carvacrol.

5RP7 cell line showed cytotoxic effects against carvacrol as a time- and dose-dependent manner. After incubation of cells with 0.1 mg/mL carvacrol for 24 or 48 h, toxicity was found 64 % and 75 %, respectively. However, the lower concentrations (0.04 mg/mL) of carvacrol indicated 50 % toxicity after both 24 and 48 h incubation time. IC_{50} value for 5RP7 cell line was determined as 0.04 mg/mL for this cell line. Carvacrol had not cytotoxic effect on control cell lines (F2408 not contain H-*ras* oncogene) (data not shown).

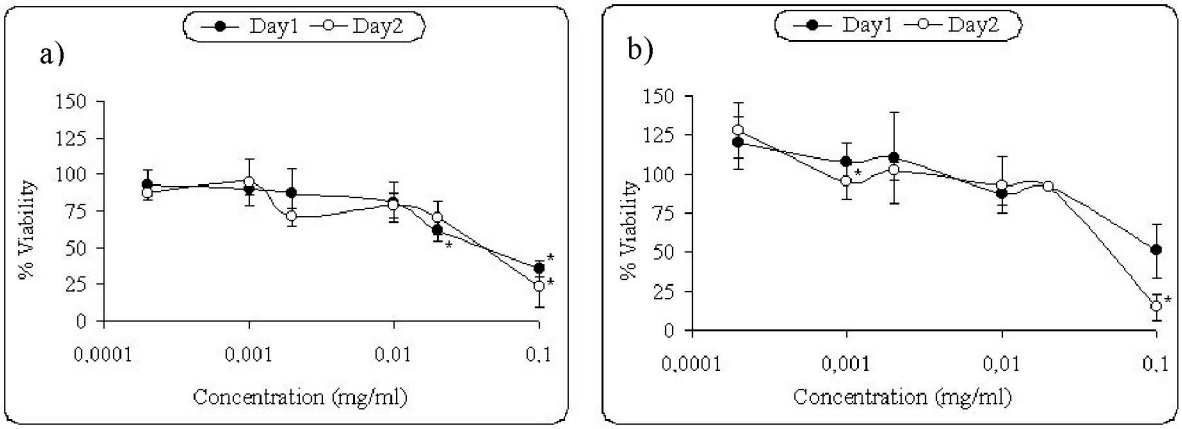

Figure 2. Effects of carvacrol on proliferation of (a) 5RP7 and (b) CO25. Cells were incubated with increasing concentrations (0.0002, 0.001, 0.002, 0.01, 0.02, 0.1 mg/mL) of carvacrol in culture medium for 24 h or 48 h. The proliferative response was then assessed by MTT assay. Results are expressed as the mean % of MTT absorbanse (ratio of absorbanse in carvacrol-treated and control cells) ± SD of 3 independent wells.

CO25 cell line also had a time- and dose-dependent cytotoxic effects. After incubating the cells with carvacrol (0.1 mg/mL) for 24 or 48 h, toxicity was found 50 % and 85.4 %, respectively. The lower concentrations (0.01 mg/mL and 0.05 mg/mL) of carvacrol was also showed 50 % toxicity on CO25 cells. 0.05-0.1 mg/mL concentration was significantly toxic for CO25 cell line in 48 h and IC_{50} value for CO25 cell line was determined as 0.1 mg/mL for 24 h (Table 1). Without Dex, carvacrol did not show any cytotoxic effects on this cell line (data not shown).

Table 1. IC_{50} values for 5RP7 and CO25 cells

	Cell Lines			
	5RP7		CO25	
	Day1	Day2	Day1	Day2
Carvacrol	0.04 mg/mL	0.04 mg/mL	0.1 mg/mL	0.05 mg/mL

IC_{50} values for 5RP7 and CO25 cells treated with carvacrol (0.0002, 0.001, 0.002, 0.01, 0.02, 0.1mg/mL).

All these concentrations of carvacrol were used for Tripan Blue exclusion method and results were consistent with MTT assay (Table 2).

Table 2. % viability of 5RP7 and CO25 cells treated with various concentrations of carvacrol

Cell lines						
	5RP7			**CO25**		
Dose (mg/mL)	0.0002	0.02	0.04	0.0002	0.05	0.1
Time (h)						
8	91	90	80	98	96	80
24	87	84	51	75	82	49
48	75	50	20	74	54	51
72	60	46	19	70	52	53

% viability was determined by Tripan Blue exclusion method

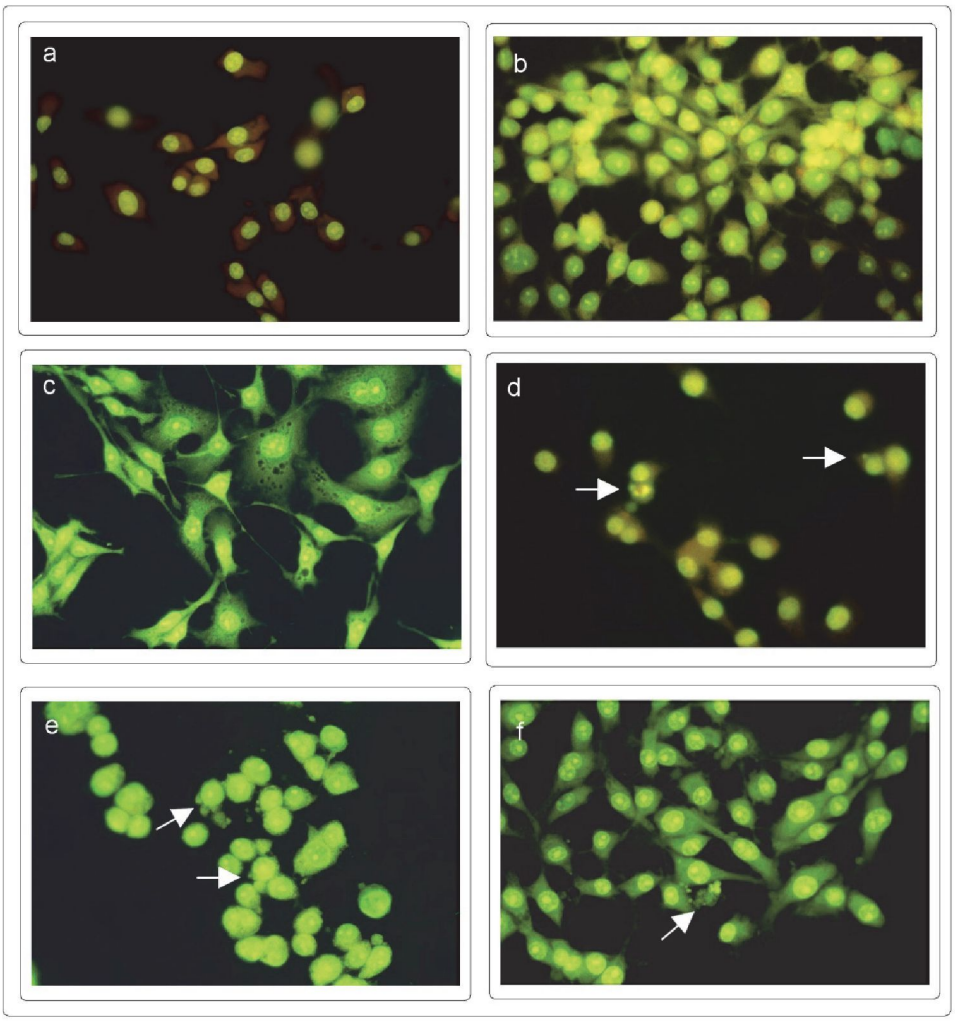

Figure 3. Carvacrol induced morphological changes on 5RP7 cells. Cells were treated with carvacrol 8 h and 24 h. Cellular morhological changes were observed using a fluorescence microscope (Olympus) at the magnitude of 200 ×. Representative photomicrographs of 5RP7 are 8 h conrol (a), 24 h control (b), 0.02 mg/mL carvacrol incubated cells after 8 h (c), 0.04 mg/mL incubated cells after 8 h (d), 0.02 mg/mL carvacrol incubated cells after 24 h (e), 0.04 mg/mL incubated cells after 24 h (f).

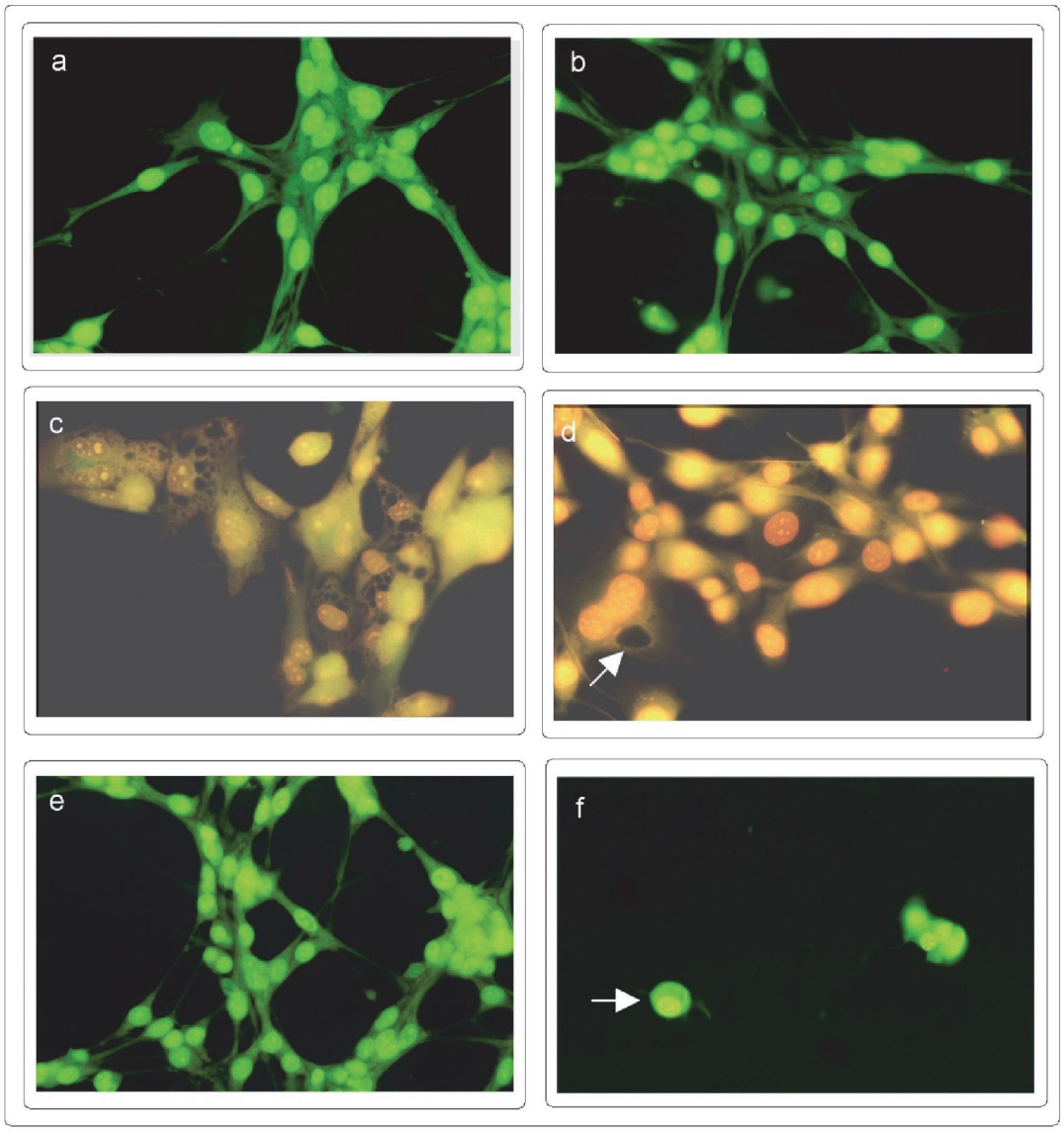

Figure 4. Carvacrol induced morhological changes on CO25 cells. Cells were treated with carvacrol 8 h and 24 h. Cellular morhological changes were observed using a fluorescence microscope (Olympus) at the magnitude of 200 ×. Representative photomicrographs of 5RP7 are 8 h control (a), 24 h control (b), 0.05 mg/mL carvacrol incubated cells after 8 h (c), 0.1 mg/mL incubated cells after 8 h (d), 0.05 mg/mL carvacrol incubated cells after 24 h (e), 0.1 mg/mL incubated cells after 24 h (f).

The change in location of phosphatidylserine in the cell membrane during apoptosis can be detected with Annexin-V (5). Co-staining with Annexin-V and PI allow differantiation of the apoptotic cells (Annexin-V-positive, PI-negative) from the viable (Annexin-V-negative, PI-negative) and necrotic cells (Annexin-V-positive, PI-positive) by flow cytometry analysis. Table 3 displays the percent of both 5RP7 and CO25 apoptotic cells after 24 h incubation with 0.02-0.04 mg/mL and 0.025-0.05 mg/mL carvacrol, respectively. As a control, cells were also

incubated with or without 25 μM Etoposide. The lower concentration (0.02 mg/mL) of carvacrol didn't make any significant change on phosphatidylserine localisation of 5RP7 cells. However, there was a 2.1 fold increase in 0.04 mg/mL carvacrol treated cells and 2.2 fold increase in 25 μM Etoposide treated cells.

On the other hand, even high concentration of carvacrol had no effect on localisation of phosphatidylserine in the CO25 cell membrane.

Table 3. Percentage of Annexin V-positive, propidium iodide-negative (apoptotic) cells as determined by flow cytometry

24 hours incubation time		
cell lines		
	5RP7	**CO25**
Control[a]	5.58	2.36
Eto[b]	12.28	3.43
Car[c]	1.74	3.68
Car[d]	11.73	2.01

[a] Control cells were incubated without drugs
[b] 25 μM Etoposide
[c] 0.02 mg/mL carvacrol for 5RP7 cell and 0.025 mg/mL carvacrol for CO25 cell
[d] 0.04 mg/mL carvacrol for 5RP7 cell and 0.05 mg/mL carvacrol for CO25 cell

DNA gel electrophoresis is a classic method for detecting apoptosis through the presence of oligonucleosomal banding or DNA laddering. Cells were incubated with carvacrol for 9, 16 or 24 h, followed by extraction and electrophoretic fractionation of genomic DNA. Fig 5 a, b shows carvacrol induced DNA fragmentation in 5RP7 but not in CO25 cells.

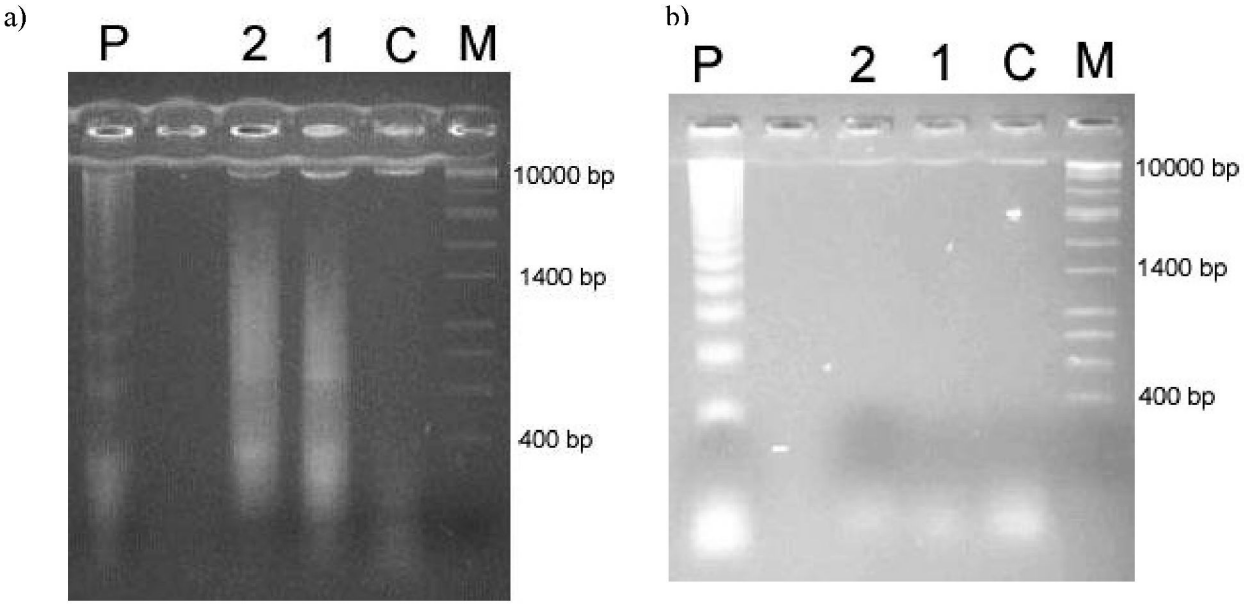

Figure 5. Carvacrol induced DNA fragmentation in 5RP7 and CO25 cells. DNA gel electrophoresis was performed in 5RP7 cells (a) with 0.02 (1), 0.04 (2) mg/mL and in CO25 cells (b) with 0.005 (1), 0.1 (2) mg/mL carvacrol after 24 h. The DNA isolation was also prepared from untreated 5RP7 and CO25 cells (Control, C). U937 cells were induced with 4 μg/mL Camptothecin for 3 h (Positive control, P). M; Marker.

DNA laddering was clearly visible in the lane representing cells incubated with 0.02 mg/mL and 0.04 mg/mL carvacrol for 24 h with 5RP7 cells. After 9 or 16 h incubation time with carvacrol DNA laddering was not seen in 5RP7 cells (data not shown). Also DNA laddering in CO25 cells incubated with different concentrations of carvacrol was not seen. Carvacrol treatment did not cause apoptotic effects on both control cell lines (data not shown).

DISCUSSION

During the last decade, interest in apoptosis has increased significantly due to the fact that its deregulation may have important implications on immun, nervous system and carcinogenesis. Especially naturel product's effects on carcinoma cells are being researched due to less toxic effects on normal cells. Crowell (21) reviewed prevention and therapy of cancer by monoterpens. Before the inhibition of cell growth in melanoma by isopranoids including carvacrol, d-limonen, its metabolites and farnesol has reported by several groups (21, 22, 23). Because of that we investigated the apoptotic role of natural occuring carvacrol -in Thymi plant- on H-*ras* transformed 5RP7 and N-*ras* transformed CO25 cell lines. This study showed that carvacrol induced apoptotic morphological changes both 5RP7 and CO25 cells but internucleosomal DNA fragmentation and early apoptotic determinants were seen only on H-*ras* transformed 5RP7 cell line. Although previous studies showed that carvacrol had inhibited cancer cell proliferation time- and dose-dependent manner (13, 15), there is no evidence about the stimulation of carvacrol to apoptotic pathways of cancer cell lines.

Zeytinoğlu (15) showed that carvacrol showed 50 % toxicity at 60 µg/mL concentration on CO25 cell line and inhibited DNA proliferation at 10 µg/mL concentration, but in our study IC_{50} value was 100 µg/mL for 24 h and 50 µg/mL for 48 h incubation time. The other study showed that carvacrol had 50 % toxicity on Hep-2 cells of 0.02 mM concentration. This differences might be due to purity of carvacrol or cell type.

We carried out the further investigation of carvacrol induced cell death -necrotic or apoptotic- as looking into morphological and biochemical changes *in vitro*. Stammati (13) showed that treatment of Hep-2 cells with 0.6 mM carvacrol caused morphological changes. In our study morphological changes were seen between 0.02-0.04 mg/mL concentrations of carvacrol for 5RP7 cells and 0.05-0.1 mg/mL concentrations of carvacrol for CO25 cells.

The effects of carvacrol on initiation of early apoptosis in these cell lines were investigated by flow cytometric analysis. We found that the apoptotic cell number was increased about 2.1 fold after inducing apoptosis on 5RP7 with carvacrol that increase was approximately similar to effects of control compound, 25µM Etoposide. H-*ras* transformed 5RP7 cell lines might be more sensitive to carvacrol than N-*ras* transformed CO25 cell lines (Table 3).

Some studies showed that carvacrol has anti-mutagenic and anti-tumoregenic activities *in vitro* and *in* vivo systems (23, 24). Besides that was thought that carvacrol could be natural antioxidant as well (25,12). Carvacrol seemed to protect lymphocytes from the genotoxic effects of heterocyclic amine IQ and mitomycin C at non-toxic concentrations below 0.05 mM, but at the higher concentration of carvacrol (0.1 mM) itself induced DNA damage (14). In our study, DNA laddering which is determinant of late apoptosis was seen in H-*ras* transformed 5RP7 cell lines after treatment with 0.02-0.04 mg/mL carvacrol for 24 h incubation time but not in CO25 cell line. This result showed that carvacrol made DNA damage on H-*ras* transformed cells at IC_{50} or $IC_{50}/2$ concentration after 24 h incubation.

CONCLUSION

The results have shown that H-*ras* transformed 5RP7 cells are more sensitive to carvacrol than N-*ras* transformed CO25 cells. Carvacrol is a natural product might be used for cancer therapy because of its apoptotic effects. Preliminary studies were performed on the possible use of the carvacrol mentioned above in antineoplastic therapy. It is thought that the results obtained will enlighten the antineoplastic activity investigations

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