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Cytotoxic effect of Carvacrol on human cervical cancer cells

[#]Mehdi SJ[1], [#]Ahmad A[2], Irshad M[1], Manzoor N[2], *Rizvi MMA[1]

[1] Genome Biology Lab, Department of Biosciences, Jamia Millia Islamia, New Delhi-25, India.
[2] Enzyme Kinetics Lab, Department of Biosciences, Jamia Millia Islamia, New Delhi-25, India.

[#] Both the authors have equal contribution

***Corresponding Author:** rizvijmi@gmail.com

Abstract

Carvacrol is a component of numerous aromatic plants which has been evaluated for substantial pharmacological properties. Although the carvacrol induced cytotoxicity has already been reported, but no such study have been made on human cervical cancer, HeLa and SiHa cells. Therefore, in the present study an attempt has been made to investigate the cytotoxic effect of carvacrol on cervical cancer cells. Cytotoxicity induced by carvacrol was determined by different assays like MTT assay and LDH assay. Apoptosis was measured by DNA fragmentation assay. The study clearly showed the dose dependent cytotoxic effect of carvacrol in HeLa and SiHa cells at an IC_{50} of 50 mg L^{-1} by both the cytotoxic assays respectively. The dying cells showed characteristics of apoptosis such as, DNA fragmentation. The data in the present study clearly demonstrated cytotoxic effects of carvacrol on human cervical cancer cells. Carvacrol could have a potential therapeutic significance in treating cancer.

Keywords: Carvacrol; Cervical cancer; HeLa cells; SiHa cells; Apoptosis.

Introduction

In recent years, the interest in medicinal plants and their biologically active derivatives has increased, in relation to the possible development of novel potential drugs for several pathologies of relevant social impact (Hedberg, 1993; Heinrich and Gibbons, 2001). It is well known that natural products from the extracts of medicinal plants are used in the treatment of skin, respiratory, neuromuscular and mental health disorders and also in obstetrics and gynecology (Abo *et al*., 2000; Ahmad *et al*., 1998; Ankli *et al*., 2002; Dutta *et al*., 1998; Pinn, 2001).The anti-tumor activity and the possible applications of medicines from medicinal plants for cancer prevention have been recently described (Chiu and Wu, 2002; Cragg and Newman, 1999; Katsube *et al*., 2003; Mukherjee *et al*., 2001; Richardson, 2001; Tatman and Mo, 2002; Wargovich *et al*., 2001; Popov *et al*., 2001; Ruffa *et al*., 2001).

Essential oils and their components have been timely honored for their pharmaceutical properties. Plant-derived substances have recently become of great interest owing to their versatile applications (Baris *et al*., 2006; Khan *et al*., 2010). The development of pharmaceuticals begins with identification of active principles, detailed biological assays and dosage formulations, followed by clinical studies to establish safety, efficacy and pharmacokinetic profile of new drug (Iwu *et al*., 1999). Carvacrol (5-isopropyl-2-methylphenol) (Figure I) is a natural isopropyl cresol and is credited with a series of pharmacological properties including antimicrobial and antifungal effects (Pina-Vaz *et al*., 2004; Braga *et al*., 2007; Chami *et al*., 2005). Carvacrol is generally recognized as a safe food additive and used as a flavoring agent in baked foods, sweets, beverages and chewing gum (Fenaroli, 2002). It is also well known that essential oils, which are rich in carvacrol, possess strong antioxidant properties equivalent to those of ascorbic acid, butyl hydroxyl toluene (BHT) and vitamin E (Aeschbach *et al*., 1994; Mastelic *et al*., 2008). Since many antioxidants exert anticarcinogenic effects (Kallistratos *et al*., 1994; Evangelou *et al*., 1997; Liasko *et al*., 1998; Karkabounas *et al*., 2002), it is possible that carvacrol functions in a similar way and the anti-proliferative properties of carvacrol on non-small cell lung cancer cells, A549, chronic myeloid leukemia cells, K562, murine B16 melanoma cells and human metastatic breast cancer cell line, MDA-MB 231 have been shown (He *et al*., 1997; Horvathova *et al*., 2007; Karkabounas *et al*., 2006; Koparal and Zeytinoglu, 2003; Lampronti *et al*., 2006; Arunasree, 2010).

Based on this hypothesis and reported data, the present study is aimed to evaluate the cytotoxic effects of carvacrol on human cervical cancer HeLa cells and SiHa cells. Quercitin at

an IC_{50} value of 50 µM was used as positive control to assess the efficacy of carvacrol (Wei *et al*., 2007).

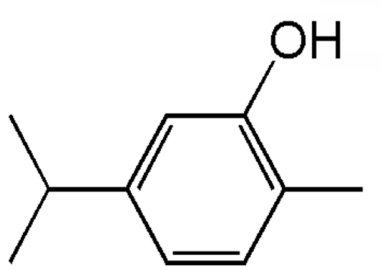

Figure I: Chemical structure of Carvacrol.

Materials and Methods

Chemicals

Carvacrol (98%) and Quercitin (≥95%) (quercitin was used as positive control) were purchased from Sigma–Aldrich (USA). DMEM (Dulbecco's modified Eagle's medium), MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide) and 0.25% trypsin and 0.02% EDTA mixture was purchased from Himedia (India). *Cytoscan™-LDH* Cytotoxicity Assay Kit was purchased from GBiosciences (USA). Fetal bovine serum (FBS) was from Biowest (USA). All the other chemicals and reagents were purchased from local companies and were of molecular biology grade. Carvacrol and Quercitin were dissolved in DMSO and stored at 4°C.

Cell lines and culture conditions

Human cervical cancer cell lines, HeLa and SiHa were obtained from National Centre for Cell Sciences (NCCS) Pune, India. Both the cell lines were grown as monolayer cultures in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and antibiotics (100 units/ml penicillin and 100 mg L^{-1} streptomycin) in a humidified atmosphere of 5% CO_2 at 37°C in T-75 flasks and were sub cultured twice a week.

Cytotoxicity assay

The cytotoxic effect of carvacrol was assessed in human cervical cancer HeLa and SiHa cells by the MTT assay (Chou and Talalay, 1984). Briefly, cells were seeded at a number of 2 X 10^4 per well onto 96-well plates (200µl/well) in triplicates, allowed to attach and grow for 24 h and subsequently exposed to different carvacrol (25 mg L^{-1}-500 mg L^{-1}) concentrations for 48 h.

At the end of the treatment, the medium was removed and cells were incubated with 20µl of MTT (5 mg/ml in PBS) in fresh medium for 4 h at 37°C. After four hours, formazan crystals, formed by mitochondrial reduction of MTT, were solubilized in DMSO (150 µl/well) and the absorbance was read at 570 nm after 10 min incubation on the iMark Microplate Reader (BioRad, USA). Percent inhibition of cytotoxicity was calculated as a fraction of control (without carvacrol) and the cytotoxicity of carvacrol was expressed as IC_{50}.

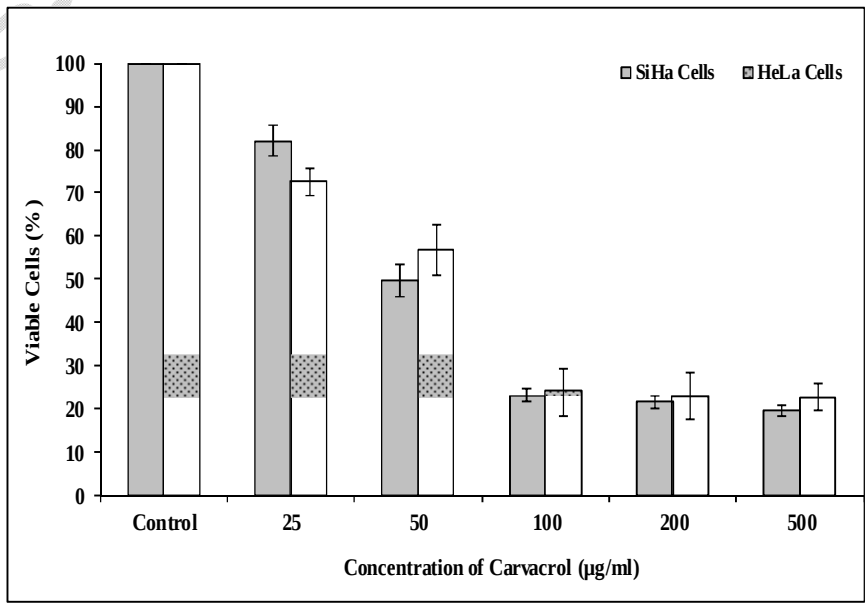

Figure II: Dose-dependent effect of carvacrol on SiHa and HeLa cell proliferation. Both the cells were cultured in 10% FBS medium and treated with 25, 50, 100, 200 and 500 mg L^{-1} carvacrol for 48 h and cell proliferation was monitored by MTT assay. The percent viable cells were calculated in comparison to untreated cells taken as 100%. Values were expressed as mean ± SD and the experiment was performed in triplicate (P<0.05).

LDH assay

Cytotoxicity induced by carvacrol was assessed by lactate dehydrogenase (LDH) leakage into the culture medium. Following exposure to the carvacrol the culture medium was aspirated and centrifuged at 3000 rpm for 5 min in order to obtain a cell free supernatant. The activity of LDH in the medium was determined using a commercially available *Cytoscan™-LDH* Cytotoxicity Assay Kit. Percent inhibition of cytotoxicity was calculated as a fraction of control (without carvacrol) and the cytotoxicity of carvacrol was expressed as IC_{50}.

Detection of apoptotic DNA fragments

DNA fragmentation was detected by agarose gel electrophoresis. $1X10^6$ HeLa and SiHa cells were plated in 30 mm culture plate. When the cells of reached approximately 70% confluency, increasing concentrations of carvacrol (25 mg L^{-1}-500 mg L^{-1}) were added and the cells were incubated for 48 h. After 48 hours, cells were harvested and pelleted by centrifugation (Eppendorf 5804R, Germany). Cellular DNA was extracted by SDS/proteinase K treatment, phenol–chloroform extraction, and ethanol precipitation as described previously (Sambrook and Russell, 2001) and then dissolved and stored in TE buffer and the DNA samples obtained were analyzed by 2% agarose gel electrophoresis. After electrophoresis, the gels were stained with ethidium bromide, and visualized as a DNA ladder with UV.

Statistical analysis

Viable cell ratios were evaluated by Student '*t*' test. The data were presented as arithmetic mean ± standard deviation. A value of $P < 0.05$ was considered to be statistically significant.

Results

Carvacrol inhibited proliferation of HeLa and SiHa cells

Data on the cytotoxic effects of carvacrol using two human tumor cell lines *in vitro* are showed in Figure II and III. Carvacrol has cytotoxic effects *in vitro* at clinical acceptable concentrations (IC_{50} values ≤ 50 mg L^{-1}) by MTT methods and LDH methods respectively. The cytotoxic effect of carvacrol was determined using concentrations ranging 25 mg L^{-1}- 500 mg L^{-1} for 48 h. After 48 h exposure, carvacrol induced concentration-dependent cytotoxic effects in cervical cell lines with IC_{50} of 50±3.89 mg L^{-1} and 50±5.95 mg L^{-1} in SiHa and HeLa cells, respectively using MTT method and 55±0.15 mg L^{-1} and 50±0.05 mg L^{-1} in SiHa and HeLa cells, respectively by LDH method.

Validation of apoptosis measurement by DNA laddering

In our results, the cells were treated with carvacrol, and the DNA was directly extracted and run on agarose gel. DNA hyperfragmentation, if presented, was seen as a stepwise ladder of DNA fragments.

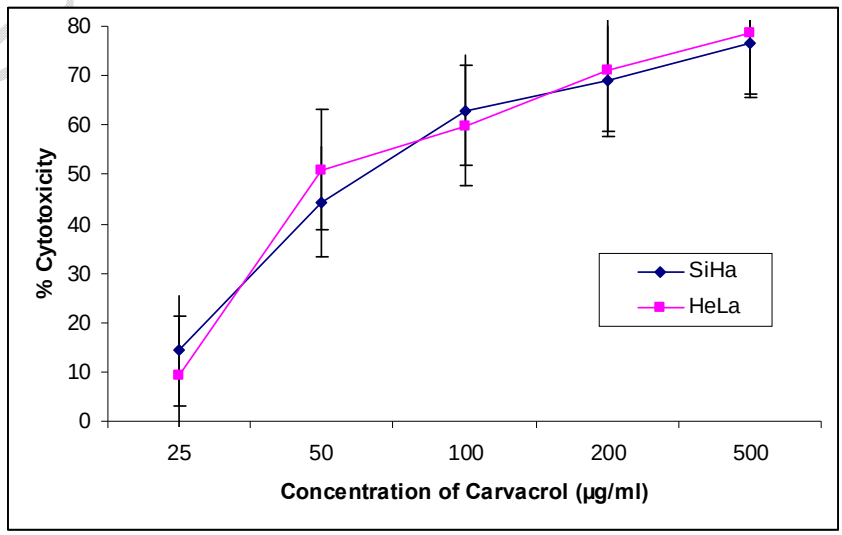

Figure III: Dose-dependent effect of carvacrol on SiHa and HeLa cell proliferation. Both the cells were cultured in 10% FBS medium and treated with 25, 50, 100, 200 and 500 mg L^{-1} carvacrol for 48 h and cytotoxicity was monitored by LDH assay. The percent cytotoxicity was calculated in comparison to untreated cells taken as 100%. Values were expressed as mean ± SD and the experiment was performed in triplicate ($P<0.05$).

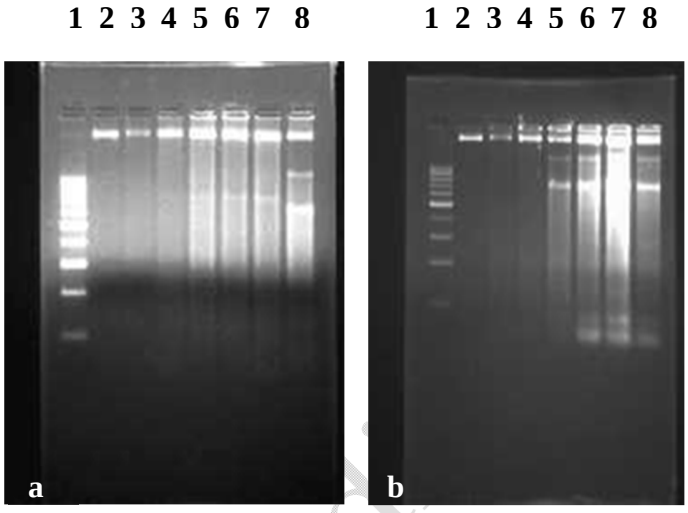

Figure IV: Analysis of DNA fragmentation in human cervical cancer cells treated with carvacrol. **a.** HeLa cells treated with carvacrol. Lane 1: 100 bp DNA ladder; lane 2: Control cells; lane 3: Cells treated with 25 mg L^{-1} carvacrol; lane 4: Cells treated with 50 mg L^{-1} carvacrol; lane 5: Cells treated with 100 mg L^{-1} carvacrol; lane 6: Cells treated with 200 mg L^{-1} carvacrol; lane 7:Cells treated with 500 mg L^{-1} carvacrol; lane 8: Cells treated with 50µM quercitin. **b**. SiHa cells treated with carvacrol. Lane 1: 100 bp DNA ladder; lane 2: Control cells; lane 3: Cells treated with 25 mg L^{-1} carvacrol; lane 4:Cells treated with 50 mg L^{-1} carvacrol; lane 5: Cells treated with 100 mg L^{-1} carvacrol; lane 6: Cells treated with 200 mg L^{-1} carvacrol; lane 7: Cells treated with 500 mg L^{-1} carvacrol; lane 8: Cells treated with 50µM quercitin.

The data (Figure IV) shows that DNA laddering is pronounced for carvacrol (50 mg L^{-1}) in HeLa cells and (50 mg L^{-1}) in SiHa cells respectively. These results confirm that carvacrol can induce apoptosis of HeLa and SiHa cells.

Discussion

Research on biologically active compounds from essential oils has proved them to be potent anti-bacterial, antifungal and anti-oxidant agents (Lampronti *et al*., 2006; Albuquerque *et al*., 2007; Ao *et al*., 2008; Baik *et al*., 2008; Bakkali *et al*., 2008). Carvacrol (5-isopropyl-2-methylphenol) is one of main substances of essential oil possess antimicrobial and antifungal effects (Pina-Vaz *et al*., 2004; Braga, 2007; Chami *et al*., 2005).

Cellular proliferation depends on the rates of cell division and death and, thus, many anticancer drugs have been used to prevent cancer cell division in order to inhibit cancer cell proliferation. In vitro cytotoxicity assays can be used to predict human toxicity and for the general screening of chemicals (Clemedson and Ekwall, 1999; Scheers *et al*., 2001). It has been previously reported that different cytotoxicity assays can give different results depending on the test agent used and the cytotoxicity assay employed (Weyermann *et al*., 2005). The results obtained from the cytotoxicity assays indicate that inhibition of HeLa and SiHa cells was gradually increased by the addition of carvacrol in MTT and LDH assays. It reveals that HeLa and SiHa cell line is susceptible to carvacrol. Although, the LDH leakage assay is based on the release of the enzyme into the culture medium after cell membrane damage whereas the MTT assay is mainly based on the enzymatic conversion of MTT in the mitochondria, both the cytotoxicity assays, employed to assess carvacrol toxicity *in vitro*, showed the similar results.

Apoptosis is a physiological process of cell elimination, and DNA fragmentation is one of the hallmarks of cell apoptosis. The results showed the apoptosis proportion of cells was increased by treatment of carvacrol in both the cervical cancer cell lines.

Conclusion

In summary, the present study demonstrated that carvacrol is a potent anti-cancer compound with an IC_{50} of 50 mg L^{-1}at 48 h inducing growth inhibition in both the human cervical cancer cells. Further research based on animal models may resolve *in vivo* efficacy of carvacrol.

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