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Original Article

Cytotoxicity and pro-apoptotic activity of carvacrol on human breast cancer cell line MCF-7

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ABSTRACT

The aim of this study was to evaluate anticancer activity of carvacrol against human breast cancer cell line (MCF-7). Cytotoxic of carvacrol was determined by MTT and LDH assays and induction of apoptotic was analysed by expressional analysis of anti- and pro-apoptotic regulatory genes by reverse transcriptase PCR and DNA fragmentation assays. The result showed that the carvacrol cytotoxicity against MCF-7 cancer cells was in dose dependent manner at 24 and 48 h time points ($p < 0.05$). IC_{50} of carvacrol at 48 h time point was 244.7 ± 0.71 µM. Carvacrol treated MCF-7 cells showed induction of apoptosis through p53 dependent and Bcl-2/Bax pathway. Also, carvacrol treatment induces caspase-3, -9, and -6 enzymes gene expression and genomic DNA fragmentations. Our study confirmed that the carvacrol inhibited growth and induced apoptosis regulatory genes in MCF-7 cells.

Keyword: Carvacrol, cytotoxicity, apoptosis, caspases, DNA fragmentation

INTRODUCTION

Carvacrol (2-methyl-5-(1-methylethyl)-phenol) is a natural monoterpenic phenolic compoundpresent mostly in oregano (*Origanum vulgare*), thyme (*Thymus vulgaris*), and savory (*Satureja hortensis*) essential oils [1]. Carvacrol containing origanum essential oil reported to have antiseptic, antibacterial, antiviral and antifungal activities [2-4]. Similarly, carvacrol containing thyme essential oil has been reported for antioxidant, antimicrobial, antitussive, expectorant, antispasmodic, and antibacterial activities [5-7]. During recent year, carvacrol compound has been extensively examined for its biological properties and reported to have antioxidant, antitumor, antimutagenic, antigenotoxic, antimicrobial, analgesic, antispasmodic, anti-inflammatory, angiogenic, antiparasitic, antiplatelet andhepato protective activities [8-11]. Also, carvacrol demonstrated anxiolytic-like effect and antidepressant-like properties [12]. It has been reported to beneficial in the prevention of renal injury, diabetes and parkinson's disease [13-15].

Cancer is a one of the most dreadful disease for humankind and breast cancer is a second leading cause of death in woman, and estimated 5,22,000 death in 2012 [16]. Since 2008, breast cancer incident has increased by more than 20%, while mortality has increased by 14% [16,17]. However, no such concrete chemotherapeutic modality is available to prevent or cure breast cancer or other cancer disease. In recent years major research has been focused on chemical compounds isolated from plants and herbs for the prevention and treatment of cancer which have been considered as nontoxic or very less toxic [18,19]. Also, natural compounds possess strong antioxidant properties have been considered as a rational strategy for dietary approaches to cancer prevention [20,21]. Carvacrol reported to have strong antioxidant properties which were equivalent to the ascorbic acid, butyl hydroxyl toluene (BHT) and vitamin E [22,23]. Anti-proliferative properties of carvacrol against non-small cell lung cancer cells (A549), chronic myeloid leukemia cells (K562), murine melanoma cells (B16) and cervical cancer cell lines (SiHa and HeLa) have been reported [24-28]. Also, carvacrol treated cells reported to induce the formation of apoptotic bodies and increase DNA fragmentation in human metastatic breast cancer cells (MDA-MB231); activation of caspase-3, cleavage of PARP and decrease of Bcl-2gene expression in hepatocellular carcinoma cell line (HepG-2) [29,30]. Given the various actions of

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carvacrol in different models, the effect of carvacrol on apoptosis regulatory gene and caspase gene in human breast cancer cell line (MCF-7) has not been studied. We, first time evaluated the anti-proliferative activity carvacrol against human breast cancer cells (MCF-7) and examined of expressional level of anti-apoptotic and pro-apototic regulatory genes (Bcl-2, p53 and Bax genes) and caspase-3, 6- and -9 enzymes genes.

MATERIALS AND METHODS

Reagent and chemicals: Carvacrol compound was purchased from (Sigma-Aldrich, St. Louis, MO, USA). Stock solution of carvacrol was prepared in dimethyl sulfoxide (DMSO) and diluted to final concentration in the culture medium. Final concentration of DMSO employed as vehicle never exceeded 0.03% and had no discernible effects on MCF-7 cells in comparison with the untreated control. Tissue culture medium constituents purchased from HiMedia (Mumbai, India). All chemicals and solvents were analytical grade purchased from Merck (Mumbai, India).

Cell culture: Human breast cancer (MCF-7) cell line was obtained from National Centre for Cell Sciences (NCCS), Pune, India. Cells were grown as monolayer cultures in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and antibiotics (100 U / mL penicillin and 100 mg/L streptomycin) in a humidified atmosphere of 5% CO_2 at 37°C in T-25 flasks and sub cultured twice a week [31].

Cytotoxicity assay: Cellular metabolic activity was evaluated by MTT assay as described by Mehdi et al.[28]. Briefly, 2x 10^4 cells transferred into 96 flat well plate and incubated at 37°C in a humidified air atmosphere enriched with 5% (v / v) CO_2 for 24 h in order to let the cells attach to the bottom of each well. Various concentrations of carvacrol (140 - 450 µM) in triplicate were treated for 24 and 48 h respectively. At the end of the treatment duration, the culture medium was replaced with fresh medium and added 20 µL of MTT (5 mg / mL in PBS) for 4 h. Formazan crystals were formed by mitochondrial reduction of MTT by lives cell only, were solubilised in DMSO (200 µL / well) and the absorbance was read at 570 nm after 10 min incubation on the *i*Mark Microplate Reader (Bio-Rad, USA).

$$\% \text{ Cell survival} = \frac{\text{Expremental } (OD_{570})}{\text{Control } (OD_{570})} \times 100$$

The mean percentage (± standard deviation (SD)) cell survival was plotted against the corresponding carvacrol concentration and the best fit line was used to derive the estimated IC_{50} value from the concentration that could provide a 50% cell survival [32].

LDH enzyme assay: Lactate dehydrogenase enzyme (LDH) is a stable cytosolic enzyme, released into the medium after disruption of cell membrane [31,32]. Various concentrations of carvacrol (140 - 450 µM) in triplicate were treated for 24 and 48 h respectively. Treated cells were centrifuge at 3000 rpm for 5 min at 4°C and cell free medium was used for quantification of LDH enzyme according to the commercially available Cytoscan™-LDH assay Kit (G-Biosciences, USA). The reaction mixture was read at 490 nm using *i*Mark Microplate Reader (Bio-Rad, USA). Percentage cytotoxicity was calculated from the equation:

$$\% \text{ Cytotoxicity} = \frac{(\text{Control } OD_{490} - \text{Soptaneous } OD_{490})}{\text{Control } OD_{490}} \times 100$$

Reverse transcriptase-polymerase chain reaction (RT-PCR): The mRNA expression of apoptosis related gene was determined after the treatment of various concentration of carvacrol for 48 h. The treated and untreated MCF-7 cells were harvested and washed with phosphate buffer saline (PBS) using centrifugation at 5000 rpm for 5 min at 4ºC. Total RNA was extracted using TRIZOL reagent (Invitrogen) according to the manufacturer's instruction. RNA preparations were carefully checked by gel electrophoresis and found to be free of DNA contamination. 1µg of total RNA was used for cDNA synthesis using RevertAidTM fisrt stranded cDNA synthesis Kit (Fermentas Life Science, USA) with random hexamers. cDNA was used for the detection of mRNA expression of p53, Bcl-2, Bax, Caspase-3, -6 and -9 gene using specific oligonucleotide primers (Table 1)[33]. GADH gene was used as an internal control. A 25µL PCR mixture containing 2 µL of cDNA, 1U taq DNA polymerase, 1.5 mM MgCl2, 0.2 mMdNTP and 20 pmole specific oligonucleotide primers. A cycle profile consisted of 30 sec at 94°C for denaturation, 30 seconds (at 52.7°C, 56.3°C, 65.4°C, 54.6°C, 49.3°C, 51.6°C, and 55.2°C for GapDH, p35, Bcl-2, Bax, caspase-3, -6 and -9 respectively) for annealing, and 30 sec at 72°C for final extension in amplification reactions (Eppendorf , Norwalk). The amplified product was observed on 2% agarose gel electrophoresis and finally photograph was taken on Gel Doc system (Bio-Rad, USA).

DNA fragmentation assay: To analyze the hallmark of apoptosis, genomic DNA fragmentation assay was performed [31,33]. Carvacrol (IC_{50} concentration for 48 h) treated MCF-7 cells were harvested and centrifuged at

5000 rpm for 5 min at 4ºC. The cell pellets were washed with PBS and fragmented genomic DNA was isolated by DNA ladder assay kit (G-Biosciences, USA). Fragmented DNA was separated by electrophoresis in 1.8% (w / v) agarose gel. It was visualized under UV light and photograph was taken under the Gel Doc system (Bio-Rad, USA).

Statistical analysis: The mean value ± standard error of mean (SEM) was determined from triplicate samples for each experimental group. Statistical significance was examined with ANOVA test.

Table 1: Oligonucleotide primers

p53	F- 5' CCAGCAGCTCCTACACCGGC 3'
	R- 5' GAAACCGTAGCTGCCCTG 3'
Bcl-2	F- 5' GGTCGCCAGGACCTCGCCGC 3'
	R- 5' AGTCGTCGCCGGCCTGGCG 3'
Bax	F- 5' GAGCTGCAGAGGATGATTGC 3
	R- 5'CCGGGAGCGGCTGTTGGGCT 3'
Caspase-3	F- 5' GTACAGATGTCGATGCAGC 3'
	R- 5' CACAATTTCTTCACGTGTA 3'
Caspase-6	F- 5' ATGCTTTAATGATCTTAAAGC 3'
	R- 5' ATAAATGTGATTGCCTTCGC 3'
Caspase-9	F- 5' CCTGCGGCGGTGCCGGCTGC 3'
	R-5' GTGTCCTCTAAGCAGGAGAT 3'
GAPDH	F- 5' GTGATGGGATTTCCATTGAT 3'
	R- 5' GGAGTCAACGGATTTGGT 3'

RESULTS

Cytotoxicity: The MTT assay result showed that dose dependent reduction in the viability of MCF-7 cells at 24 and 48 h time point ($p < 0.005$) (Figure 1). The IC_{50} of carvacrol in MCF-7 cells was determined after 48 h showed 244.7 ± 0.71µM ($p < 0.05$). However, at IC_{50} dose of carvacrol showed 20.8% cytotoxicity at 24 h time point. Also, the cytotoxicity trend of carvacrol examined by MTT assay was significantly correlated with the LDH leakage assay at 24 and 48 h time point ($r > 0.983$).

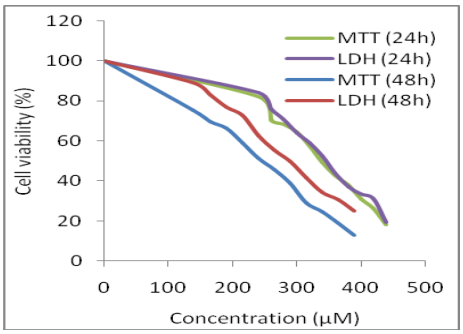

Figure 1: Viability of carvacrol treated MCF-7 cells determined by MTT and LDH assays.

Both MTT and LDH leakage assay, probability work on two different principals. In MTT assay, metabolically active cells reduced MTT salt to purple formazan by mitochondrial succinate dehydrogenase enzyme. However, LDH is a stable cytosolic enzyme which releases into culture medium due to lose of membrane integrity.

Expression analysis of pro- and anti-apoptotic genes: Expressional analysis of apoptosis related (Bcl-2, Bax and p53) genes in MCF-7 cells have been studied by Reverse Transcriptase-PCR. GapDH gene expression was used as internal control to monitor the mRNA expression of apoptosis related genes. Also, densitometry analysis was used for the study of relative band intensity of mRNA on gel. Treatment of MCF-7 cells with carvacrol for 48 h has increased mRNA transcripts of p53 gene dose-dependent manner (Figure 2). Treatment of carvacrol at IC_{50}show increased of p53 mRNA relative band intensity by 6.02 fold in MCF-7 cells in comparison with the untreated control. Also, carvacrol treated MCF-7 cells showed down-regulation of Bcl-2 gene mRNA and up-regulation of Bax gene mRNA in a dose-dependent manner at 48 h time point ($p < 0.05$) (Figure 2).

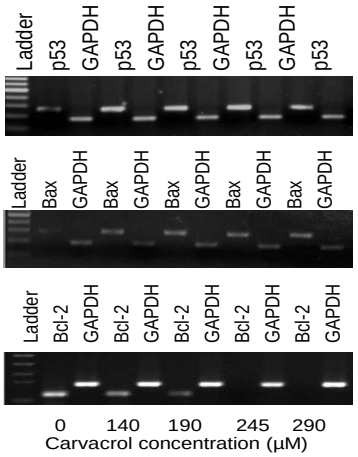

Figure 2: Expressional pattern of p53, Bax and Bcl-2 genes in MCF-7 cell line treated with carvacrol.

Densitometry analysis revealed that, low dose treatment of carvacrol exhibited significant mRNA band intensity of Bcl-2 gene, whereas with the increase of carvacrol concentration, mRNA transcript of Bcl-2 gene reduced and mRNA transcript of Bax gene increased. It was further noted that, with the increase of carvacrol concentrations Bax/Bcl-2 genes expression ratio increased in MCF-7 cell line.

Expression analysis of caspase genes: Caspase family is an important marker in apoptotic

signalling which determined the apoptosis in carvacrol treated MCF-7 cell line. Carvacrol treated MCF-7 cell line exhibited significant mRNA expression of caspase-3, -6 and -9 genes dose dependent manner (Figure 3). At IC_{50} of carvacrol, mRNA transcripts of caspases genes increased many folds in compared with the untreated control ($p < 0.001$-0.017).

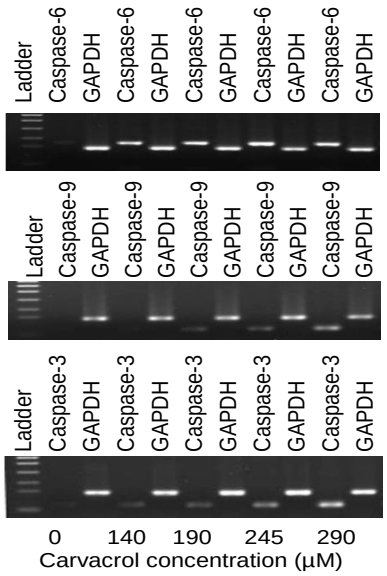

Figure 3: Up-regulation of caspase-3, 6 and -9 genes in MCF-7 cell line treated with carvacrol.

DNA fragmentation analysis: DNA fragmentation assay was performed in carvacrol treated and untreated MCF-7 cells in order to analyse the hallmark of apoptosis. The result showed ladder like appearance in the gel (Figure 4). It is a characteristic feature of apoptosis process in which the genomic DNA is cleaved into fragments by the endogenous endonucleases.

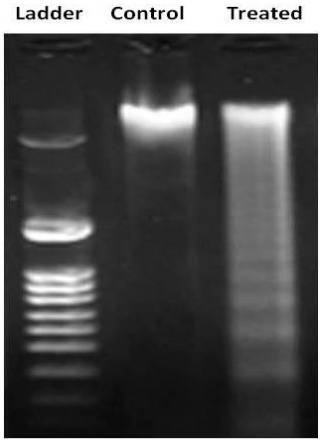

Figure 4: Genomic DNA fragmentation of MCF-7 cell line treated with carvacrol.

DISCUSSION

In vitro toxicity of carvacrol has been reported in several studies; however the toxicity mechanism has not been completely elucidated so far. Interestingly, the IC_{50} of carvacrol found in the present study was lower than the concentration found against human hepatocellular carcinoma cells HepG-2 (IC_{50}, 0.4mM), porcine small intestine cells IPEC-1 (IC_{50}, 525±83 µM) and intestinal cancer cells Caco-2 (IC_{50}, 343±7.4µM) [30,34,35]. However, low cytotoxicity concentration of carvacrol was reported against human metastatic breast cancer cell line MDA-MB 231 (IC_{50}, 100µM) [29]. In previous reports, carvacrol treated cells showed characteristic apoptosis features of prominent morphological changes and flip-flop movement of phosphatidyl serine from inner membrane to the outer membrane of the plasma membrane [26,29]. Also, few study reported that the carvacrol treated cells showed permeabilization of mitochondrial membrane, resulting in a pro-oxidant status and induction of apoptosis [1,30,36]. In fact, alteration of membrane stability leading to release of mitochondrial apoptosis initiation factors (AIFs), cytochrome-*c* and the apoptosis protease-activating factor (Apaf-1) into the cytosol which in turn activates caspase-9,-3 and -7 enzymes [37,38]. In the present study, up-regulation of caspase-3, -6 and -9 genes was observed in carvacrol treated MCF-7 cells in compared to the untreated controls (Figure3). Translational product of caspase-9 gene initiates the cascade of apoptosis whereas caspase-3 is the downstream caspase that plays a pivotal role in the terminal phase of apoptosis [39,40]. As a result genomic DNA fragmentation was detected (Figure 4). The transcriptional product of p53 gene, orchestrates a global transcriptional response that either control cell proliferation or induces apoptosis [41]. p53 gene regulates the apoptosis by interacting with the Bcl-2 family and up-regulation of Bax gene expression through direct transcriptional activation of the Bax promoter with concomitant down-regulation of Bcl-2 gene [42]. Also, apoptotic signalling through mitochondrial events regulated by Bcl-2 family member gene and lower ratio of Bcl-2/ Bax decided the fate of the cell death [43]. In the present study, carvacrol treated MCF-7 cells showed down regulation of Bcl-2 gene and dose dependent up-regulation of Bax gene (Figure 2). Similar pattern of result was observed in carvacrol treated mouse melanomas cells B16 [24]. Hence lowered ratio of Bcl2/Bax, signalling the cells towards apoptotic death [32,44]. The result therefore, indicated that carvacrol induced apoptosis possibly through p53 and mitochondrial pathway. The data presented in this study suggested that carvacrol induced apoptosis is

mediated through death receptor as demonstrated by increased expression levels of p53, bax, caspase-9, -6 and -3 genes.

CONCLUSIONS

This is the first study determined the effect of carvacrol on the regulation of pro-apoptosis genes in breast cancer cells and up-regulation of pro-apoptotic genes confirmed that the carvacrol induced cytotoxicity and apoptosis in MCF-7 cells. This finding suggested that the carvacrol may be a potential chemotherapeutic agent against cancer, however further research warranted for the therapeutic claims.

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