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RESEARCH ARTICLE

A High-throughput Quantitative Expression Analysis of Cancer-related Genes in Human HepG2 Cells in Response to Limonene, a Potential Anti-cancer Agent

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Abstract: ***Background*:** Citrus bioactive compounds, as active anticancer agents, have been under focus by several studies worldwide. However, the underlying genes responsible for the anticancer potential have not been sufficiently highlighted.

***Objectives*:** The current study investigated the gene expression profile of hepatocellular carcinoma, HepG2, cells after treatment with Limonene.

***Methods*:** The concentration that killed 50% of HepG2 cells was used to elucidate the genetic mechanisms of limonene anticancer activity. The apoptotic induction was detected by flow cytometry and confocal fluorescence microscope. Two of the pro-apoptotic events, caspase-3 activation and phosphatidylserine translocation were manifested by confocal fluorescence microscopy. High-throughput real-time PCR was used to profile 1023 cancer-related genes in 16 different gene families related to the cancer development.

***Results*:** In comparison to untreated cells, limonene increased the percentage of apoptotic cells up to 89.61%, by flow cytometry, and 48.2% by fluorescence microscopy. There was a significant limonene-driven differential gene expression of HepG2 cells in 15 different gene families. Limonene was shown to significantly (>2log) up-regulate and down-regulate 14 and 59 genes, respectively. The affected gene families, from the most to the least affected, were apoptosis induction, signal transduction, cancer genes augmentation, alteration in kinases expression, inflammation, DNA damage repair, and cell cycle proteins.

***Conclusion*:** The current study reveals that limonene could be a promising, cheap, and effective anticancer compound. The broad spectrum of limonene anticancer activity is interesting for anticancer drug development. Further research is needed to confirm the current findings and to examine the anticancer potential of limonene along with underlying mechanisms on different cell lines.

Keywords: Anticancer, cancer-related genes, flavonoids, HepG2, high-throughput PCR, limonene, tumor suppressor.

1. INTRODUCTION

Antioxidants are natural plant products that inhibit the adverse effects of the reactive oxygen species produced in plants so they enable plants to survive. Moreover, antioxidant components of the natural products constitute the major source of human health promotion and maintenance [1].

With the increasing level of the carcinogenic and mutagenic substances in the environment and emergence of chemotherapy resistant cancer cells and microbes, the research to explore new anticancer and antimicrobial compounds has become crucial day after day [2]. This necessitates more efforts to find effective and inexpensive anticancer and antimicrobial agents from natural products which lack the common side effects of the commercially available agents [2, 3].

Limonene is a natural cyclic monoterpene (C10) which is present in nature as two enantiomers, (+) and (-) with potential chemopreventive and antitumor activities [4]. The monoterpenes limonene has been shown to induce apoptosis in various cancer cell lines, but its mechanism of action is yet to be completely clarified [5].

Many studies provided evidence that limonene from citrus fruits can interfere with several cell-signaling pathways attributing to its anticancer activity [6, 7]. However, the molecular and genetic mechanisms of the anticancer activity of limonene are still not fully studied and explored. Accordingly, this study was designed as an attempt to elucidate the anticancer mechanisms of limonene at genetic level by using

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high-throughput quantitative PCR. With a chip panel covering 1023 genes of 16 cancer-related families, the differentially expressed genes between limonene- treated and untreated cancer cells were investigated. It is believed that the results of this study could open the gate wide for better understanding of the anticancer activity of limonene and help in the development of new anticancer agents.

2. MATERIALS AND METHODS

2.1. Preparation of the Compound

The monoterpene, (R)-(+)-Limonene, ≥ 93%, with density 0.84 gm/ml,W26 330-3-K, CAS number 5989-27-5 (Sigma, Germany) was prepared by dissolving in absolute dimethyl sulfoxide (DMSO), (BIO BASIC INC., USA) and was incubated at -20^{0}C as stock solution at a concentration of 64mg/ml. The stock solution was then diluted in 10% Roswell Park Memorial Institute-1640 (RPMI-1640) medium for preparing the working concentrations (20, 40, 80, 160, 320, and 640µg/ml) before each test.

2.2. Cell Culture

The cancer cell line, hepatocellular carcinoma cells (HepG2; ATCC HB-8065), was used to evaluate the cytotoxic and apoptotic effects of limonene along with scrutinizing HepG2 gene expression affected by limonene. HepG2 ells were cultivated in monolayer at humidified 5% CO_2 atmosphere and at 37°C in RPMI-1640 culture medium w/L-glutamine (biowest, USA) supplemented with 10% fetal bovine serum, FBS (Sigma, Germany), 50 U/ml penicillin-streptomycin (Biowest, USA), and 2.5 µg/ml amphotericin B (Biowest, USA).

2.3. Cytotoxicity Assay

The MTS (3-(4,5dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2(4-sulfophenyl)-2H-tetrazolium) colorimetric method (Cell Titer 96 Aqueous One Solution Cell Proliferation Assay, Promega, USA) was used. Briefly, triplicates of 1 ×10^5 HepG2 cells/well were treated with 200 µl/well of the limonene working solution prepared in RPMI-1640 maintenance medium (2% FBS). The negative control wells contained DMSO in RPMI-1640 maintenance medium with final volume of 200 µl/well. All the plates were incubated in humidified 5% CO_2 at 37°C for 24 h. Later, the wells' contents were removed and were replaced with 200 µl/well of RPMI-1640 maintenance medium. The plates were re-incubated for 48 h at the same conditions. MTS solution (20µl) in RPMI-1640 culture medium (100 µl) was added to each well. Absorbance at 490 nm was measured after 4 h, using a 96-well plate ELISA reader (Sunrise Basic, Austria). The concentration of limonene that killed 50% of the cells, namely IC50, was calculated [8].

2.4. Apoptosis Detection Methods

2.4.1. Flow Cytometry Analysis

The level of apoptosis in HepG2 cells treated with limonene concentration that killed 50% of the cells (IC50) was evaluated. The ice-cold ethanol fixed pellets were re-suspended in 500 µl of DNA staining solution containing 25 µl of propidium iodide (PI) at the final concentration of 1 mg/ml (MP Biomedicals, LLC, IIIKrick, France), and 50 µl Ribonuclease A from bovine pancrease (1 mg/ml), (Sigma, Germany) in the PBS (Siddik, 2010). The assay was measured in duplicate for each sample. The propidium iodide fluorescence of individual nuclei was measured using CyAn ADP apparatus (BECKMAN COULTER, USA). The software Summit (V4.3) was used to analyze the flow cytometry results.

2.4.2. Dual Apoptosis Assay

A kit of dual apoptosis assay (Biotium Inc, USA) was used along with NucView 488 caspase-3 substrate and sulforhodamine 101 (Texas Red) Annexin V. This kit detects two important apoptosis events: caspase-3 activation and phosphatidylserine (PS) translocation. The procedure was executed according to the manufacturer's instructions. After 24 h of incubation, HepG2 cells with a final cell concentration 1 ×10^6 cell/chamber were re-incubated with limonene IC50 (389 µg/ml) for additional 24 h. The control negative slides contained solvent in the medium. The treated cells were re-incubated with maintenance medium for further 48 hours. Confocal laser scanning microscope (FV 1000, FLOUVIEW, OLYMPUS, Japan) was used to examine HepG2 cells. Image processing was done by using FV 1000, FLOUVIEW, Version 2.0 (OLYMPUS, Japan). The stained positive apoptotic cells were counted. The apoptotic index was calculated as the number of apoptotic cells relative to the total number of cells. The assay was entirely carried out in triplicates.

2.4.3. SmartChip Real-Time PCR System

SmartChip Human Oncology Gene Panel Version 1.5.1 from (WaferGen, BIOSYSTEMS, USA) enables gene expression profiling of 4,128 reaction wells on a single sample using the SmartChip Nanodispenser. SmartChip panels were preloaded with pre-optimized PCR primers. These primers were used to investigate, in quadruplicates, 1023 genes belonging to 16 functional groups (Table **1**). Nine endogenous controls (GAPDH-2, TAF-10, GUSB, TAF11, HPRT, PPIA, HMBS, ACTB, and B2M) and 4 exogenous controls (WGBS-YCF1-4) were used. The SmartChip Human Oncology Gene Panel was used to measure the differential gene expression from 0.5 µg of RNA of limonene-treated HepG2 cells vs. untreated cells. Only the significantly up- and down-regulated genes, 2 fold change, are displayed in this study.

2.4.4. RNA Extraction

Triplicates of HepG2 cells (1 ×10^5 cell/ well) in RPMI-1640 maintenance medium with or without limonene were incubated for 72 h. The limonene IC50 (389 µg/ml) was used. Total RNA from the harvested cells was isolated using GF-1 kit (Vivantis Technologies, Malaysia). RNA extraction from HepG2 cells (1 ×10^7 cell/ well) was done according to the manufacturer's protocol. The RNA concentration used in the downstream experiments was 514ng/µl. Total RNA quality was checked by Agilent 2100 Bioanalyzer (Agilent, USA); RNA integrity number was measured as well. The extracted RNA was stored at −80°C.

Table 1. The gene families and the number of genes in each family which covered by SmartChip Real-Time PCR System from WaferGen using SmartChip human oncology gene panel.

Gene Family	Number of Genes per Family
ADME*	173
Apoptosis	198
Cancer	325
Cell Cycle/ Proliferation	52
Cardiovascular Disease	226
DNA damage repair	36
Drug Target	43
G-protein coupled receptor	31
Growth factor	12
Homeostasis/ Metabolism	9
Inflammation	143
Kinase	194
Proteinase	11
Signal Transduction	303
Transcription Factor	95
NeuroDisease/ Phosphotase	10

*Drug absorption, distribution, metabolism and elimination genes.

2.4.5. High Throughput Real-Time RT-qPCR

The reverse-transcription of 0.5 μg of the RNA of HepG2 cells was carried out using iScript ™ cDNA Synthesis Kit (BIO-RAD, Canada); the manufacturer's instructions were followed. The synthesized cDNA was stored at −80°C for qRT-PCR reaction.

Real-time qPCR assay using SYBR green I DNA binding dye was used. This assay included a SmartChip array (72 X 72) which was analyzed by following WaferGen protocols. A starting sample of 500 ng was dispensed yielding DNA equivalent of 96 pg of sample per well. The results were reported in the form of C_t (threshold cycles) and T_m (melting temperatures) for amplicon melting analyses.

The up- and down- regulation of the target genes were analyzed by SmartChip Software (WaferGen, BIOSYSTEMS, USA). To keep the precision of readings, standard deviation <0.25 Ct between replicates was used. Thermal cycling of primers was 95°C for 180sec once, then for 40 cycles: 95°C for 60sec, and 60°C for 70sec with amplicon size 80-120 bp. At each run, two negative tissue controls (NTC), chip no. 34565 and 35935, and two positive tissue controls (PTC), chip no. 34576 and 35939, were used.

The calculation of fold change in genes expression used the Comparative Ct Method ($2^{-\Delta\Delta Ct}$ Method) and as follows: $\Delta\Delta Ct = \Delta Ct$ treated – ΔCt untreated, where: ΔCt treated = Ct treated – All Mean treated and ΔCt untreated = ΔCt untreated – All Mean untreated.

2.5. Data Analysis

The data in the current study are shown in mean±SD. The data analysis was conducted by using SPSS software version (12.0.0.2). The effect of the tested compound on the inhibition of cell growth was evaluated by using 95% confidence intervals. IC50 value was calculated using linear regression index equations. Regarding flow cytomteric analysis, The R2 fraction represented the sub-G apoptotic cells, and the percentage of cells at different phases of cell cycle was calculated from the total cells minus apoptotic cells. For the real-time qPCR, SmartChip qPCR software exerted all data analysis. Two log and more of up- and down- regulation of the studied genes was considered a significant differential expression. The only genes with significant differential expression, in response to limonene, were included in the results of this study. By using student *t*-test, P values less than 0.05 were considered significant for the variations among the percentages of apoptotic cells.

3. RESULTS

3.1. The Cytotoxic Effect of Limonene on HepG2 Cells

The limonene concentration that killed 50% of the hepatocellular carcinoma cells was 389μg/ml. The optimal time for cell treatment was 72 h and no cytotoxic effect was detected after 24 h and 48 h by this concentration.

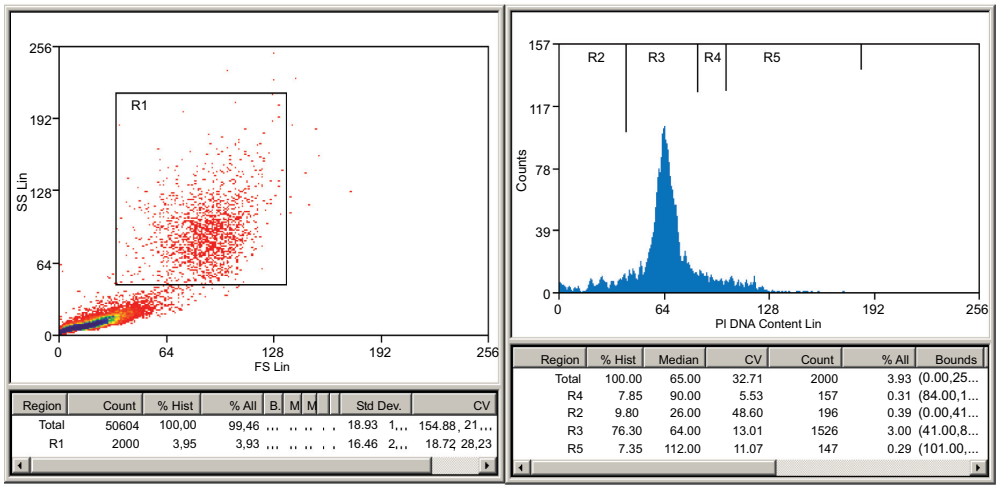
3.2. Detection of Apoptosis by the Flow Cytometry Technique

Flow cytometry was used to measure the apoptosis in HepG2 cells treated 72h with limonene versus apoptosis in untreated cells. Apoptosis was confirmed by integrating the results of both flow cytometry and RT-qPCR. The flow cytometric analysis showed remarkable potential of limonene to induce apoptosis in the treated cells in comparison to untreated cells after 72 h, Fig. (**1**). The flow cytometric analysis of HepG2 cells treated with the IC50 of limonene revealed an increase in the percentage of the apoptotic cells. The apoptotic cells increased significantly ($P < 0.05$) in the limonene-treated cells (89.61%) when compared to the untreated cells (9.8%). Hence, limonene significantly induced apoptosis in the treated HepG2 cells.

3.3. Detection of Apoptotic Cells by the Confocal Fluorescence Microscopy

The morphological changes of apoptosis were examined by confocal fluorescence microscopy. Two markers were used NucView 488 caspase-3 substrate and sulforhodamine 101 (Texas Red) Annexin V. The kit provided a convenient tool for simultaneous detection of caspase-3 activity and phosphatidylserine (PS) translocation. NucView™ 488 Caspase-3 substrate was able to detect caspase-3 activity within individual whole cells without inhibiting caspase activity [9]. The NucView™ 488 caspase-3 substrate was bifunctional, allowing detection of caspase-3 activity and visualization of apoptotic nuclear morphology by staining the nucleus bright green. On the other hand, Annexin V binds to the translocated PS macromolecule. PS translocated from the inner to the outer leaflet of the plasma membrane, thus produces red fluorescence border around the cell [9, 10].

(a)



(b)

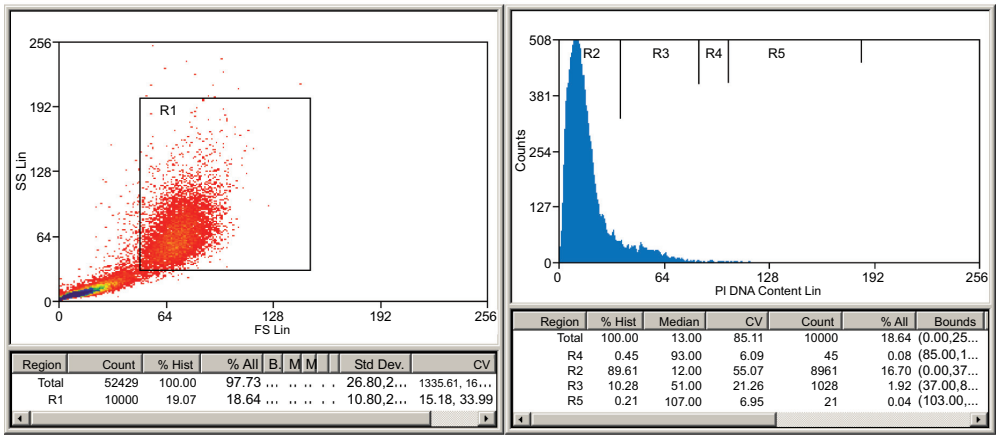


Fig. (1). DNA content frequency histograms representing HepG2 cells after 72 h from (**a**) untreated cultures (**b**) cultures treated with limonene IC50. The treatment affected the cell cycle distribution and induced apoptosis. The cells were stained with PI. Fluorescence of the PI-stained cells was measured using CyAn ADP apparatus and Summit (V4.3) software. The software program provides the estimate of percentage of cells with fractional DNA content (apoptotic cells: R2) and cells in G0/G1 (R3), S (R4), and G2/M (R5) phases of the cycle. Total cell number (R1).

The morphological changes like detachment, rounding of cells, and cell shrinkage were considered as apoptosis-related events [10]. The treated cells were compared with good spread cells in the negative control plates. The limonene IC50 (389 µg/ml) induced apoptosis in 48.2% of HepG2 cells after 72 h of treatment, Fig. (**2**).

3.4. Detection of the Up- and Down-regulation of Gene Expression by SmartChip Real-time PCR

The quantitative analysis by real-time PCR revealed a clear picture on limonene anticancer mechanisms. The treatment of HepG2 cells with limonene for 72 h resulted in up-regulation (>2 log) of 14 different cancer-related genes while limonene down-regulated (>2log) 59 different cancer-related genes. The differential gene expression of limonene treatment was evident in 15 out of 16 gene family groups as shown in Table **2**.

Limonene treatment revealed significant up-regulation of 4 genes from 173 genes of the ADME gene family ranging from 5.02 to 2.6 log, with mean 3.87 ± 1.14 log. On the other hand, 10 genes in this family were down-regulated because of limonene treatment. The log reduction ranged from -2.1 to -4.9 log, with mean -2.95 ± 0.95 log.

Four out of 198 genes were significantly up-regulated in the Apoptosis gene family. The range was from 3.89 to 2.24 log, at mean 3.13± 0.74 log. On the other hand, 5 out of 198 genes were significantly down-regulated in this family ranging from -2.1 to -3.41 log, at mean -2.53 ± 0.55 log.

Signal Transduction and Cancer gene families showed higher numbers of up- and down- regulated genes. Sixteen genes were significantly up-regulated in these two families from a total of 303 and 325 genes in Signal Transduction and Cancer gene families, respectively. The range of the seven up-regulated genes in Signal Transduction family was 5.02

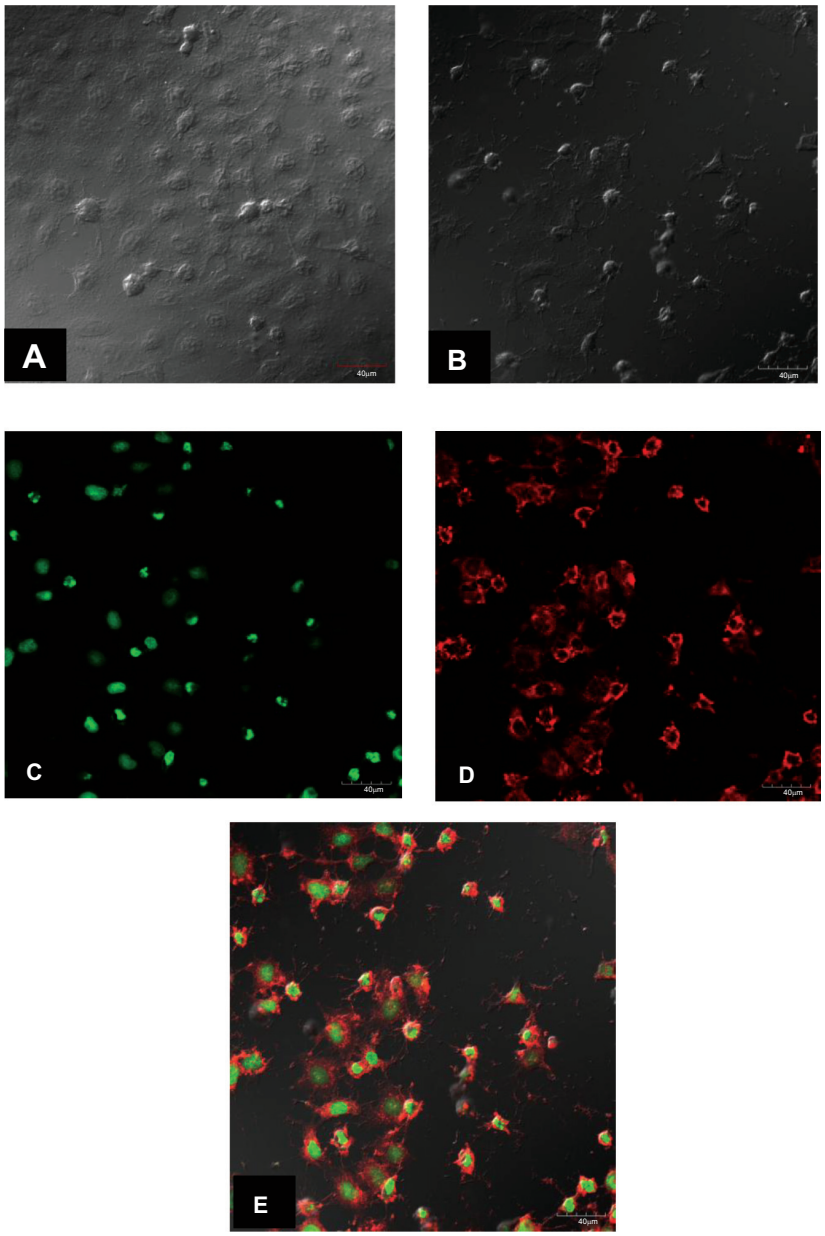


Fig. (2). A) Photomicrograph of HepG2 negative control cell lines. There were no morphological changes in these cells. **B**) Photomicrograph of HepG2 treated cell lines viewed under bright field microscope using confocal laser scanning biological microscope (FV 1000, FLOUVIEW, OLYMPUS, Japan). The morphological changes were clear after 72hs of treatment with limonene IC50. **C**) Photomicrograph of HepG2 treated cell lines stained with caspases-3 substrate. Green nuclei in HepG2 cells indicated caspase-3 activation. **D**) Photomicrograph of HepG2 treated cell lines stained with annexin-V substrate Red border around HepG2 cells indicated phosphatidylserine translocation from inner to the outer leaflet of the cell membrane. **E**) Merged photomicrograph of stained HepG2 treated cell lines. Green nuclei surrounded by red borders indicated the apoptotic HepG2 cells.

to 2.61 log, with mean 3.41 ± 0.99 log, while the range of nine up-regulated genes in Cancer family was 5.02 to 2.24 log, with mean 3.53 ± 0.93 log. The Cancer gene family showed the highest number of the significantly down-regulated genes, 21 genes, among all the tested gene families followed by Signal Transduction family, 19 genes. The range of down-regulation in the Signal Transduction gene family was -2.0 to -4 log, with mean 2.63 ± 0.54 log, while in the Cancer gene family, the magnitude of down-regulation ranged from -2.04 to -4.39 log, with mean -2.75 ± 0.69 log.

The gene family revealed the second highest number of significantly down-regulated genes was the Kinase gene family, 13 out of 194 genes. The range of down-regulation in these genes was from -2.01 to -3.96 log, with mean -2.66 ± 0.59 log. Nevertheless, only two genes in this family group were significantly up-regulated ranging from 2.15 to 1.82 log, with mean 1.98 ± 0.23 log.

For the cardiovascular gene family, 11 genes were significantly down-regulated from total of 226 genes. The down-regulation ranged from -2.51 to -3.8 log, with mean -2.8 ± 0.46 log. Only 3 significantly up-regulated genes were recorded in this family ranging from 4.87 to 2.15 log, with mean 3.15 ± 1.49 log.

Table 2. The up- and down-regulated genes in different gene families after 72 h treatment of HepG2 cells with limonene versus untreated HepG2 cells.

Gene Family	Differentially Expressed Genes [log change]	
ADME	Upregulated	JUN[5.02], FOS-2[4.65], FOS-1[3.23], RARA[2.6]
	Downregulated	BRCA2-2[-2.1], EPHX1[-2.23], CCNC[-2.45], IGF2R[-2.53], XRCC6[-2.56], PPIA[-2.62], ITGB1[-2.71], RB1[-2.91], MGST3[-4.5], CALR[-4.9]
Apoptosis	Upregulated	BAG3[3.89], BTG2[3.58], MAPK8IP2[2.83], BBC3-2[2.24]
	Downregulated	MADD[-2.01], BRCA2-2[-2.1], KRAS2[-2.51], PPIA[-2.62], CDC2[-3.41]
Signal Transduction	Upregulated	JUN[5.02], FOS-2[4.65], FOS-1[3.23], DUSP1[2.86], MAPK8IP2[2.83], ID2[2.71], RARA[2.61]
	Downregulated	FLI[-2.0], CCNA2-2[-2.07], MAPK9[-2.12], REL-1[-2.16], TBK1[-2.24], FN1[-2.25], TGFBI[-2.44], TGFBR1[-2.47], KRAS2[-2.51], IGF2R[-2.51], PPIA[-2.53], PAK1IP1-2[-2.62], CCNA2-1[-2.63], ENG[-2.69], RB1[-2.83], CCNB1-1[-2.91], COL3A1[-3.29], SITPEC[-3.8], HMMR[-4.0]
Cancer	Upregulated	JUN[5.02], FOS-2[4.65], BAG3[3.89], MYCL1[3.85], BTG2[3.58], FOS-1[3.23], ID2[2.71], RARA[2.61], BBC3-2[2.24]
	Downregulated	RRAS[-2.04], BRCA2-2[-2.1], REL-1[-2.24], CRKL[-2.38], FN1[-2.44], TGFBI[-2.47], RB1[-2.91], KRAS2[-2.51], TFDP2[-2.52], IGF2R[-2.53], XRCC6[-2.56], MKI67-2[-2.59], PPIA[-2.62], TSG101[-2.89], MMP2[-3.0], ECT2[-3.08], MCM2-3[-3.13], CCNB1-1[-3.29], BUB1[-3.96], HMMR[-4.12], CTTN[-4.39]
Kinase	Upregulated	MAPK8IP2[2.83], EFNB2[2.15]
	Downregulated	MADD[-2.01], MAPK9[-2.16], CDK2AP1-1[-2.24], TBK1[-2.25], EFNA1[-2.27], CCNH[-2.39], CDC2L1[-2.46], TGFBR1[-2.51], DDR1[-2.6], PAK1IP1-2[-2.63], SMG1[-3.31], CDC2[-3.41], BUB1[-3.96]
Cardiovascular	Upregulated	HBEGF[4.87], CXCL2[2.45], EFNB2[2.15]
	Downregulated	TGFBR1[-2.51], EFNA1[-2.27], FN1[-2.44], TGFBI[-2.47] ,PPIA[-2.62], ITGB1[-2.71], COL5A1[-2.75], ENG[-2.83], MMP2[-3.0], TIMP2[-3.5], COL3A1[-3.8]
Transcription Factor	Upregulated	JUN[5.02], FOS-2[4.65], BTG2[3.58], FOS-1[3.23], RARA[2.61]
	Downregulated	REL-1[-2.24], TFDP2[-2.52], PPIA[-2.62], RB1[-2.91], SMAD6[-3.23]
Inflammation	Upregulated	JUN[5.02], FOS-2[4.65], FOS-1[3.23], CXCL2[2.45]
	Downregulated	FN1[-2.44], TGFBR1[-2.52], PPIA[-2.61]
DNA Damage Repair	Upregulated	
	Downregulated	ASCIZ[-2.06], ANKRD17[-2.09], ATRX[-3.35], PMS2L3[-4.44]
Cell Cycle/ Proliferation	Upregulated	
	Downregulated	ANAPC4[-2.15], RUVBL1[-2.24], KNTC1[-2.51], CDKAL1[-2.79], CDC16[-2.95]
G-protein Coupled Receptor	Upregulated	CXCL2[2.45]
	Downregulated	PPIA[-2.62]
Drug Target	Upregulated	DUSP1[2.86]
	Downregulated	PPIA[-2.62]
Phosphatase	Upregulated	
	Downregulated	DYM[-3.16]
Homeostasis/ Metabolism	Upregulated	
	Downregulated	MOSC1[-3.7]
Proteinase	Upregulated	
	Downregulated	ADAMTS5[-2.6]

*Drug absorption, distribution, metabolism and elimination genes
*GeneCards®: The Human Gene Database, http://www.genecards.org/

Out of 95 genes in the Transcription Factor gene family, 5 up- and 5 down- regulated genes were found. The range of the upregulated genes was from 5.02 to 2.61 log, with mean 3.82 ± 0.74 log and the range of the downregulated genes was from -2.24 to -3.23 log, with mean -2.7 ± 0.38 log.

Out of 143 genes in the Inflammation gene family, 4 up- and 3 down- regulated genes were discovered. The range of the change was from 5.02 to 2.45 log, with mean 3.83 ± 1.2 log and -2.44 to -2.61 log, with mean -2.52 ± 0.08 log.

Two gene families, the DNA-Damage Repair (36 genes) and the Cell Cycle/Proliferation (52 genes), showed 4 and 5 down-regulated genes, respectively. Interestingly, no up-regulated genes were found in these two gene families. The significantly down regulated genes in the DNA-Damage Repair family were of range -2.06 to -4.44 log, with mean -2.99 ± 1.14 log. On the other hand the significantly down regulated genes in the Cell Cycle/Proliferation were of range -2.15 to -2.95 log, with mean -2.15 ± 0.34 log.

The changes in G-protein Coupled Receptor, Drug Target, Phosphatase, Hemostatsis/Metabolism, and Proteinase gene families were minor in that very few genes were affected after treatment with limonene.

4. DISCUSSION

Previous studies provided evidence on the anti-tumorigenic effect of limonene with high cytotoxic selectivity to cancerous cells. This cytotoxicity was found to be associated with the ability of limonene to affect multiple cellular targets at varying levels [11, 12]. However, the anticancer pathways targeted by limonene have not been fully studied. The ability of limonene to induce apoptosis was investigated in this study by different methods. After limonene treatment, there was a clear up-regulation of Dual specificity protein phosphatase 1 (DUSP1) gene in two apoptotic related families, Signal Transduction and Drug Target. This gene is considered as an important mediator in the human cellular response to environmental stress as well as in the negative regulation of cellular proliferation [13]. These results are in harmony with the increased incidence of apoptotic-positive cells (48.2%) manifested by the confocal fluorescence microscopy. The morphological changes of HepG2 cells in response to limonene treatment exhibited a proof for the ability of limonene to induce apoptosis in comparison to untreated cells. In addition, the flow cytometry analysis revealed an increase in the percentage of apoptotic cells (89.61%) when compared to untreated cells (9.8%).

By using the SmartChip Human Oncology Panel, it was quite feasible to use Real-time qPCR assay to explore the anticancer mechanisms of limonene at the level of gene expression. In the current study, after 72 h of HepG2 cells treatment with limonene, 15 out of 16 gene family groups were shown to have clear changes in the expression of their individual genes. The Growth Factor gene family was the only gene family that limonene did not have any effect on the expression of its genes.

Treatment of cells of hepatocellular carcinoma with limonene brought interesting results about the role of some tumor related genes in the cancer development. One of these genes is JUN, encodes a c-Jun protein in human [14]. The current study showed that JUN gene was differentially over-expressed in HepG2 cells in response to 72h of limonene treatment. This finding is in agreement with the findings of some previous studies which reached to conclusion that JUN expression promotes anticancer effect [15]. Another study done by Kollmann *et al*., found that c-JUN is involved in protecting the promoter region of the tumor suppressor p16 (INK4a), which is consistently methylated over time in c-JUN deficient cells [16]. Similarly, a study found that c-JUN overexpression in conjunction with tylophorine, a phenanthroindolizidine alkaloid, a major medicinal constituent of herb, significantly increase the number of carcinoma cells that were arrested at the G1 phase [17]. Another study revealed that hesperetin, a dietary flavonoid found abundantly in citrus fruits, stimulates alterations in the expression level of JNK (c-JUN NH2-terminal Kinase) thereby induces apoptosis in skin cancer cells [18]. Interestingly, in this study, the up-regulation of JUN gene after limonene treatment was clearly evident in multiple gene families and as follows: ADME, Signal Transduction, Transcription Factor, Cancer, and Inflammation. This might highlight a significant role of JUN up-regulation in the observed anticancer activity of limonene. Moreover, exploiting more than one pathway and gene family, JUN overexpression can be used in treating tumor cells by deploying multiple and different anti-cancer mechanisms. Actually, this point deserves dedicated research to disclose the potential of JUN-activating limonene in the treatment of cancerous cells.

Another overexpressed gene in this study is FOS. The expression of FOS gene is reported by a previous study [8] to weakly induce tumors; however, FOS gene was described by other studies as a potent tumor suppressor [19, 20]. The contradicting findings on the effect of FOS gene on carcinogenesis can be somehow explained. This gene was shown to induce the pro-apoptotic pathways in cancerous cells while it was shown to act as a weak proto-oncogene in normal cells; in consequence, FOS drives cancerous cells to the cycle arrest and suicide [19]. Anyhow, FOS paradoxical effect on cancer needs further investigation.

The homeostasis/metabolism gene family in the treated HepG2 cells demonstrated a down-regulation in only one gene. This gene is the mitochondrial amidoxime-reducing component 1 (MOSC1) gene which encodes a recently discovered enzyme in mammalians. This enzyme plays a major role in drug metabolism, especially in the activation of so-called "amidoxime-prodrugs", though its physiological relevance is largely unknown [21-23].

Finally, this study revealed other limonene-driven up- or down- regulated genes but these genes did not have a clear cancer-related effect. These genes may be drug targets to other diseases and further studies are needed in this endeavor. For example, the gene Dymeclin (DYM), it encodes a protein which is necessary for the normal skeletal development and brain function. Mutations in this gene are associated with two types of recessive osteochondrodysplasia, Dyggve-Melchior-Clausen (DMC) dysplasia and Smith-McCort (SMC) dysplasia, which involve both skeletal defects and mental retardation [24]. This gene was significantly down-regulated in Phosphatase gene family after limonene treatment.

Nevertheless, this study pinpointed a possible adverse effect of limonene. This side effect may develop by the overexpression of Chemokine (C-X-C motif) ligand 2 (CXCL2) in G-protein Coupled Receptor, Cardiovascular Disease, and Inflammation gene families. This gene is known to have a tumor-promoting role to different types of cancers [25, 26]. Therefore, overexpression of CXCL2 gene must be studied in different cell lines in order to assess precisely the nature of this gene activity.

Taken together, this study revealed, at genetic level, good potential of limonene to fight cancer development in human cancer cells in general and in HepG2 cells in particular. The main effector anti-cancer mechanisms shown in this study are related to gene families implicated in apoptosis induction, signal transduction, cancer genes augmentation alteration in kinases expression, inflammation, DNA damage repair, and cell cycle proteins. The disclosed genetic anticancer mechanisms open doors wide for further studies evaluating this anticancer activity of limonene more specifically. In, addition, the studied genetic pathways of anticancer activity of limonene can be used as a model for future anticancer therapies on different cancer cell types.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are base of this research.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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Declared none.

AUTHOR CONTRIBUTIONS

Rand R. and Ahmed S. conceived, designed and performed all the experiments, analyzed the data, and wrote the paper. Fatimah Abu Bakar, Saba Z., and Mohammed Q. designed, helped in conducting and analyzing data of the real-time high throughput qPCR experiment.

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