Evaluation of *in vitro* Anticancer Activity of Vulpinic Acid and its Apoptotic Potential Using Gene Expression and Protein Analysis

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ABSTRACT

Lichens and their secondary metabolite are still among the many unexamined natural sources in the drug industry. This study was designed to evaluate the cytotoxic effects of vulpinic acid lichen secondary metabolite and 6.25, 12.5, 25, 50, 100, 200 and 400 μM concentrations that treat cancer cell lines (CaCo2, HepG2, Hep2C, RD Wehi) and normal cells (Vero and L929) by MTT assay. The aim of this study was to determine the apoptotic effect of vulpinic acid on a molecular level. The determination of apoptotic molecular patterns of vulpinic acid was performed on western blot and quantitative real-time PCR (qRT-PCR) analysis. In our study, transcriptome changes on both gene and protein levels showed similar results. The determination of, mRNA levels of *Bax*, *Bcl-2* and *P53* genes were showed by qRT-PCR in cancer and normal cell lines. The results of the study showed that IC_{50} value of vulpinic acid altered the mRNA levels of *Bax, Bcl-2* and *P53* genes in all examined cancer cells when compared to the untreated cells. When the mRNA levels of the examined genes were compared, it was observed that *Bax* gene showed more expression increase in all cell lines when compared to *Bcl-2* and *P53* genes. This is the first evaluation of the apoptotic effect of vulpinic acid secondary metabolite on mRNA levels. The current study highlights some points regarding vulpinic acid and its use for cancer treatment.

Key words: Vulpinic acid, Apoptotic effect, qRT-PCR, Western blot.

INTRODUCTION

Cancer, caused by uncontrolled division of cells in the body, is a serious public health problem and a very common disease in the world. Cancer disease is significantly influenced by genetic, physical and socio-economic conditions.[1] In recent years, cancer has steadily increasedas a health problem throughout the world sincemore effective treatments have not still been found.[2,3] Treatments like surgery, chemotherapy and radiotherapy are frequently used methods for cancer therapy. Many laboratories and studies focus on the search for an effective solution of this serious health problem. Natural products derived from different organisms have proven to be promising sources of anticancer drugs. Therefore, in recent years, especially synthetic molecules and biological compounds isolated from fungus, microorganisms and plants have demonstrated therapeutic effect against various types of cancer cell lines. Lichens are the sources for important anticancer active compounds in the drug industry. Lichens are described morphologically and physiologically and they form a symbiotic association with fungi and algae.[4] Researchers

have identified approximately 1000 differentsecondary metabolites of the lichen species. Lichens and their secondary metabolite are still among the many unstudied natural sources in the pharmaceutical sector.[5,6,7] One of the secondary metabolites obtained from lichen is vulpinic acid and it was first isolated in 1831 by Bebert.[8] In 1925, vulpinic acid was isolated by Mazza from pulvinic acid derivative.[9] With bright yellow appearance, vulpinic acid has toxic effects on reindeer if it consumes very high concentrations of *Letharia vulpina*.[8] Vulpinic acid is referred to by IUPACas 3-hydroxy-5-oxo-4-phenyl-3-2-(2H)-furan, from which phenylacetate is formed and it has a molecular weight of 322.32 g.mol^{-1} and a chemical structure of $C_{19}H_{14}O_5$.[8]

There are various studies related to the determination of anticancer activity of lichen secondary metabolites in the literature.[10-16] As a result of these studies, lichen secondary metabolites may provide important results related withits use as candidate drug molecules. Usnic acid and its enantiomers are the most extensively studied lichen secondary metabolites.[17,18,19] Vulpinic acid has also been examined for malignant mesothelioma cells MM98, vulvar carcinoma cells A431 and Keratinocytes HaCaT cells by Burlando *et al*.[20] According to their study results, vulpinic acid extracted from *Letharia vulpine* shows minimum toxic effect when compared with usnic acid, salazinic, gyrophoric and evernic acid.[20] The potential use of vulpinic acid and gyrophoric acid for cosmetic purposes was evaluated against the harmful effects of UVB radiation, and 2,5 J/cm^2 UVB radiation was applied to human keratinocyte (HaCaT) cell for the determination of the activity.[21] After that, 25, 50, 100, 200, 400 and 800 µM vulpinic acid and gyrophoric acid concentrations were applied on the exposed and unexposed cells to UVB radiation. The effect of UVB radiation was determined by MTT, apoptosis and cytoskeleton alteration assay. When the effect of the two lichen secondary metabolites was compared, vulpinic acid showed more protective effects on HaCaT cells exposed with UVB radiation.[21] Koparal (2015) demonstrated cell viability and antiangiogenic effect of vulpinic acid and usnic acid secondary metabolites against HepG2 hepatocarcinoma cell, NS20Y neuroblastoma cancer cell and HUVEC endothelial cell lines. Koparal reported that concentrations of vulpinic acid determined IC$_{50}$value 168±3.33 on HepG2, 68.83±1.58 on NS20Y, 231,94±25.4 on HUVEC for 48 h.[22] Vulpinic acid, as a biologically significant molecule, represents easy production in large amounts for the drug industry.

The primary aims of this research include *i)* the determination of the cytotoxic effects of vulpinic acid lichen secondary metabolite on cancer and normal cells, which

have never been studied before. For this purpose, vulpinic acid was dissolved in DMSO (14 mM) and diluted in different concentrations (6.25, 12.5, 25, 50, 100, 200 and 400 µM) with DMEM and treated to cancer cell lines (CaCo2, HepG2, Hep2C, RD and Wehi) and normal cells (Vero and L929). Cell viability was determined by the MTT assay; *ii)* the precise mechanisms of cytotoxic effects of vulpinic acid remain unclear at molecular level. Therefore, apoptosis-related proteins and genes were performed to determine expression patterns of transcriptome level in cancer and normal cell lines by using western blot and quantitative real-time PCR (qRT-PCR) analysis. This is the first evaluation of the apoptotic effect of vulpinic acid secondary metabolite on mRNA levels.

MATERIALS AND METHODS

Vulpinic acid

Vulpinic acid was dissolved in 14mM DMSO and stock solutions of vulpinic acid were prepared to 400 µM concentrations. Double serial dilutions of the vulpinic acid (200, 100, 50, 25, 12.5, 6.25 µM) were also prepared in stock solution.

Cell culture

CaCo2 (Human colorectal adenocarcinoma cell line), HepG2 (Human hepatocellular carcinoma cell line), Hep2C (Human HeLa carcinoma cell line), RD (Human rhabdomyosarcoma cell line), Wehi (Mouse fibrosarcoma cell line), L929 (Mouse subcutaneous connective tissue cell line) and Vero (African green monkey kidney cell line) cells were cultured in DMEM (PAA Laboratories, Austria); Wehi in RPMI-1640 containing 10% FBS (Gemini Byproducts, Calabasas, CA), 2 mM L-glutamine and 1% penicillin and streptomycin. Cells were maintained in a humidified incubator at 37°C and 5% CO_2.

Cell viability assay

Cells were seeded in to 96-well plates at a density of 1×10^4 cells in each well. The cells were incubated overnight and were checked microscopically to see if they were suitable for the assay. Vulpinic acid concentrations were diluted with suitable medium to each cell type. Concentration (6.25, 12.5, 25, 50, 100, 200 and 400 µM) and time (24, 48 and 72 h) dependent cytotoxicity were assessed by MTT assay. The absorbance was recorded at 570 nm on microplate reader. All experiments were performed in three independent biological and three technical replicates. Absorbance values were converted to logarithm base for the calculation of IC$_{50}$ values, and then, the calculations were performed using Graphpad Prism 6.

Quantitative polymerase chain reaction (qRT-PCR)

All examined cells were treated with IC_{50} values of vulpinic acid for 48 h.Total RNA was extracted from the cultured cells using TRIzol reagent (Genezol) according to the manufacturer's protocol. The purity and concentration of the total RNA were determined by spectrophotometry using the 260 nm/280 nm ratio (Nanodrop 1000, Thermo Scientific) and RNA controlled with 1% agarose gel electrophoresis. The first strand cDNA was synthesized byRosche First Strand cDNA Synthesis Kit (Rosche). cDNA synthesis was performed with 2 μg of RNA, 2.5 μM anchored-oligo $(dT)_{18}$, 1x transcriptor high fidelity reverse transcriptase reaction buffer, 20 U protector RNase inhibitor, 1 mM deoxy-nucleotide mix, and 10 U transcriptor high fidelity reverse transcriptase at final concentration. After that, we continued 10 min at 65°C, 30 min at 55°C and 5 min at 85°C. Sequence of *P53*, *Bcl-2* and *Bax* target genes were designed with Primer3 program and *GAPDH* was used as housekeeping gene. Sequences of primers were as follows; *Bax* (forward, TCCCCCCGAGAG-GTCTTTT; reverse, CGGCCCCAGTTGAAGTTG); *Bcl-2* (forward, CTGCACCTGACGCCCTTCACC; reverse, CACATGACCCCACCGAACTCAAAGA); *P53* (forward, AACGGTACTCCGCCACC; reverse, CGTGTCACCGTCGTGGA); *GAPDH* (forward, AACGGGAAGCTTGTCATCAATGGAAA; reverse, GCATCAGCAGAGGGGGCAGAG). Quantitative Real-time PCR was conducted with Roche Light Cycler 480 Real Time, using SYBR Green I Master. The PCR reaction conditions were as follows: after pre-denaturation, 10 min at 95°C, 40 cycles of 10 s at 95°C, 30 s at 60°C and 8 s at 72°C. All experiments were performed in three independent biological and technical triplicates.

Western Blot Analysis

The aim of western blot analysis was to detect the levels of apoptotic protein. Cancer and normal cells were exposed to IC_{50} value of vulpinic acid for 48 h. Cells were harvested and lysed in 150 μl lysis buffer. Total protein concentrations were qualified using the Bradford protein assay. Forty micrograms of protein of all supernatant were separated by electrophoresis on 10% SDS polyacrylamide gel. The gel was then transferred to PVDF membranes, and then blocked for 2 h with Tris-buffered saline and Tween20 containing 5% nonfat milk. After that, the membranes were incubated with primary antibodies specific to Bcl-2, P53 and Bax (Santa Cruz Biotech) at 4°C overnight. This was followed by incubation at room temperature with the appropriate anti-rabbit, anti-goat and anti-mouse IgGimmunoglob-

ulins conjugated (1:10.000) for 2 h. β-Actin was used as internal control for relative quantitation. The results were analyzed by ELISA systems.

Data analysis

Analyses of MTT, qRT-PCR and western blot assays were performed independently in triplicate. All values of the MTT assay and western blot analyses were calculated as mean ± standard deviation, and then were analyzed by Student's ttest using SPSS 15.0 (SPSS Inc., Chicago, IL, USA).For qRT-PCR assay, the relative mRNA expression level of each targeted gene was normalized against selected reference genes as calculated by the $2^{-\Delta\Delta Ct}$ method. Results obtained from fold change in gene expression were analyzed with one way ANOVA on SPSS 15 (SPSS Inc., Chicago, IL, USA). $p<0.05$ value indicated a confidence level of 95%.

RESULTS

## Effects of vulpinic acid on the viability of different cancer cells

Seven vulpinic acid concentrations were used to treat CaCo2, HepG2, Hep2C, RD, Wehi, Vero and L929 cell lines for 24, 48 and 72 h. Compared with time-dependent manner of vulpinic acid effects, vulpinic acid significantly inhibited the growth ofall examined cells in a 48 h timeand dosedependent manner (Figure 1). As shown in Figure 2, vulpinic acid showed the best effects on CaCo2 cancer cell line from all studied cells (Figure 2). The highest rates of cell death were observed in approximately 12.5 μM concentration vulpinic acid for 48 h on CaCo2cancer cell line ranged from 87.4% ($p<0.05$) (Figure 2). The value of the IC_{50} of vulpinic acid for CaCo2, HepG2, Hep2C, RD, Wehicancer cell lines was determined to be 13.7, 23.8, 25.3, 34.4 and 38.6μM, respectively. Vulpinic acid was, for the first time,
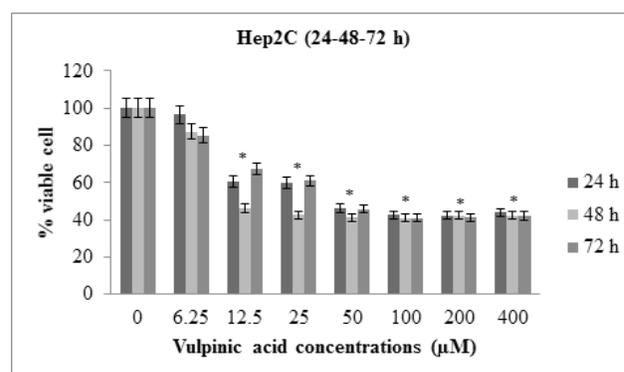
Figure 1: Comparative representation of Hep2C cell line (values are statistically significant at * p < 0.05 respective control).
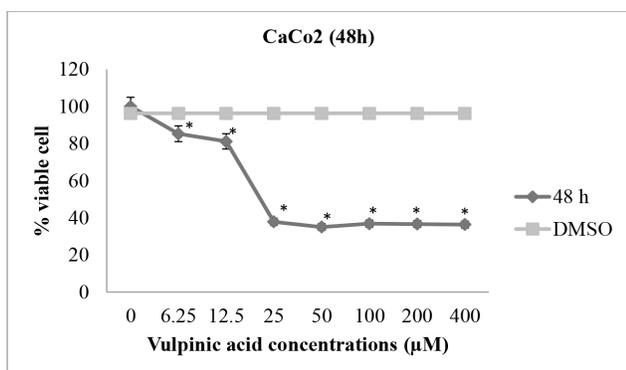
Figure 2: Dose response curves for the effect of vulpinic acid on CaCo2 cell line for 48 h (values are statistically significant at * p < 0.05 respective control).
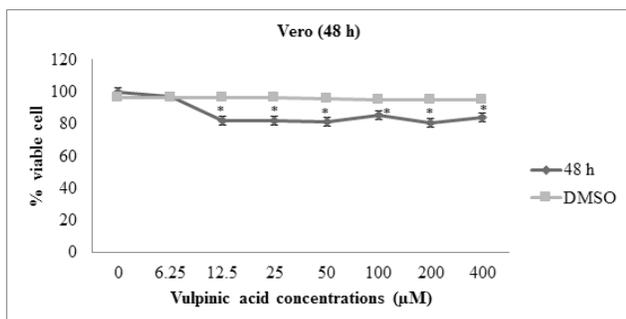
**Figure 3: Dose response curves for the effect of vulpinic acid on Vero cell line for 48 h (values are statistically significant at * p < 0.05 respective control).**

exposed to CaCo2, HepG2, Hep2C, RD, Wehi cancer cells, Vero and L929 normal cell lines in our study. This result suggests that vulpinic acid significantly inhibited the proliferation of cancer cell lines ($p<0.05$).As a more interesting result, the cytotoxic effect of vulpinic acid was on cancer cells and it had little cytotoxic effect on normal cells ($p<0.05$). The application of vulpinic acid did not have a significant decrease in cell viability on L929 and Vero normal cell lines at all increasing concentration/time options ($p<0.05$) (Figure 3). The viability of Vero and L929 normal cells only decreased 12% and 17%, respectively ($p<0.05$) (Figure 3).One of the most valuable results of this study is that vulpinic acid inhibited cancer lines but it had very little inhibiting effect on normal cells.

Effects of vulpinic acid on Bcl2 family members

Previous studies have indicated that lichen secondary metabolites are induced by apoptosis of cancer cells.[19] Firstly, in this study, the effects of vulpinic acid on molecular mechanisms associated with apoptosis in examined cancer cells and the expression of apoptosis related proteins were analyzed by western blot assay. The value of the IC$_{50}$ of vulpinic acid demonstrated
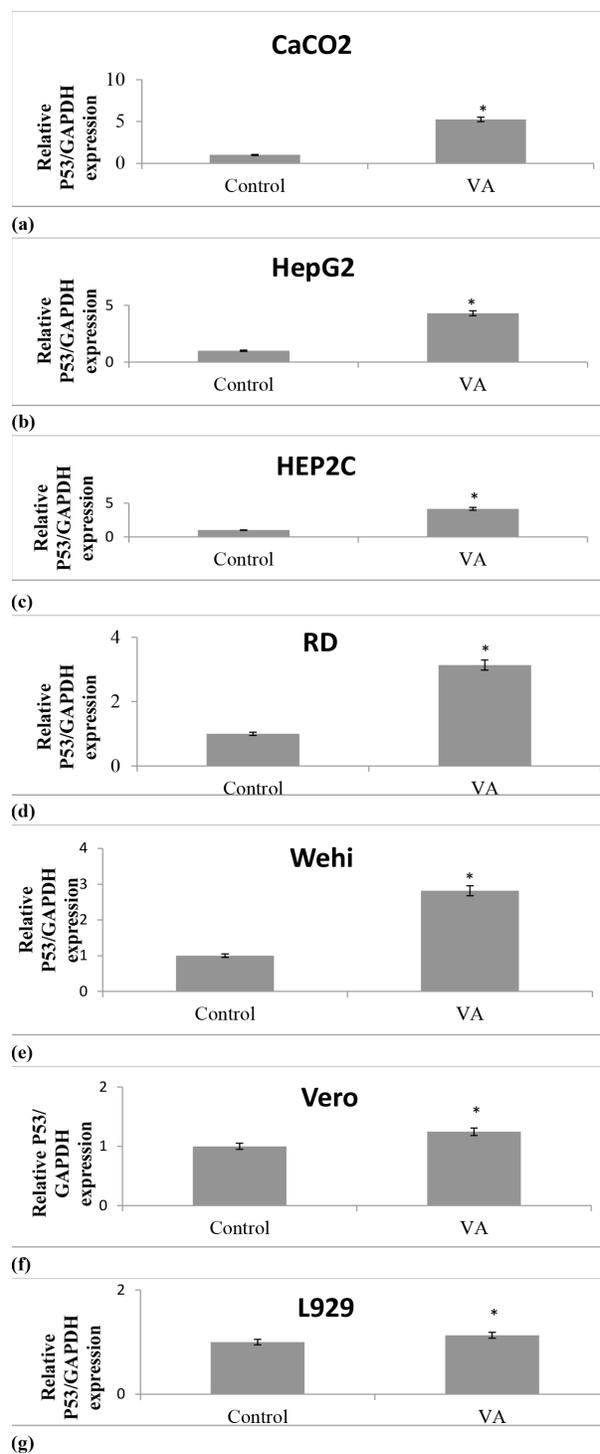
Figure 4: Effects of vulpinic acid on apoptosis-related p53 protein in examined cell.

increasing Bcl2, P53 and Bax protein levels in examined cancer cells in a 48 h concentrationdependent manner ($p<0.05$) (Figure 4). Bax expression was 12 fold higher in CaCO$_2$ cells ($p<0.05$), 10 fold higher in HepG$_2$ ($p<0.05$), 9 fold higher in Hep2C ($p<0.05$), 6 fold higher in RD ($p<0.05$), 5 fold higher in Wehi ($p<0.05$) than in the

corresponding normal cells (Vero and L929) ($p<0.05$). Similarly, p53 expression was 5.2 fold higher in $CaCO_2$ cells ($p<0.05$), 4 fold higher in $HepG_2$ and Hep2C ($p<0.05$), approximately 3 fold higher in RD and Wehi cells (p<0.05) than in the VA non-exposed cells (Vero and L929) ($p<0.05$) (Figure 4). In contrast, Bcl-2 expression was 2.3 fold lower in $CaCO_2$ exposed vulpinic acid ($p<0.05$), approximately from 0.3 fold to 0.5 fold range on lower in examined all cancer cell lines ($p<0.05$).

Secondly, the value of the IC_{50} of vulpinic acid was exposed to all examined cells for 48 h and total RNAs were isolated from cell lines. The RNA concentrations were in the range of 137 to 378ng/μL and 230/260 nm ratios were between 0.47 and 0.99. The integrity of the extracted RNAs was also evaluated by gel electrophoresis. The expression of apoptosis related genes (*Bax, Bcl-2* and *P53*) on cancer and normal cell lines was analyzed by qRT-PCR for the first time in this study. There are no available data in the literature on the effect of vulpinic acid on pro-apoptotic and anti-apoptotic gene expression in different cancer and normal cell lines. Therefore, qRT-PCR assay is demonstrated as one of the most important data explaining the exposure of apoptosis related molecular mechanisms to vulpinic acid on cancer and normal cell lines. Figure 5a-5g summarizes gene expression changes of *Bax, Bcl-2* and *P53*. CaCo2, HepG2, Hep2C, RD and Wehi cancer cell lines exposed to vulpinic acid and the expression of *Bax* gene was increased by 17.2, 14.3, 13.2, 9.8 and 7.4 fold, respectively ($p<0.05$) (Figure 5a-5e). The expression levels of *Bax* gene in Vero and L929 normal cell lines treated with vulpinic acid for 48 h were increased by 1.6 and 1.1 fold, respectively ($p<0.05$) (Figure 5f-5g). When cancer cell lines and normal cell lines exposed to vulpinic acid in respect to *Bax* gene expression were compared, an approximately from 7 and 17fold change was determined in *Bax* gene expression value ($p<0.05$) (Figure 5a-5g). The *P53*gene expression levels also increased in examined cell lines treated with vulpinic acid secondary metabolite. It is markedly inreased by 8.7 and 6.8fold on CaCo2 and HepG2 cancer cell line, respectively when compared with vulpinic acid untreated cells($p<0.05$)(Figure 5a-5b). The transcriptome levels of *P53* genes in the CaCo2 (8.7 fold change) were obviously higher than Vero and L929 normal cell lines (1.4 and 1.9 fold change) ($p<0.05$). In all cancer cell lines, few significant differences in the expression profiles of *Bcl-2* were observed between the other apoptosis related genes (*Bax* and *P53* genes). However Bcl-2 anti-apoptotic gene was 7 fold decreased in CaCo2 and HepG2 cell lines ($p<0.05$). *Bcl-2*was more highly expressed in the CaCo2 cancer cell line (7.3 fold change). There was a six and five fold decrease in the

expression of *Bcl-2* gene in Hep2C, RD and Wehi cell lines, respectively ($p<0.05$).

The current study had similar observations to transcriptome changes of genes and protein levels. It is obvious that both mRNA and protein levels significantly increased in *Bax* and *P53* levels and decreased in *Bcl-2* level ($p<0.05$) (Figure 4 and Figure 5a-5g).

DISCUSSION

In this study, vulpinic acid secondary metabolite had more significant cytotoxic effects on CaCo2, HepG2, Hep2C, RD, Wehi cancer cell lines ($p<0.05$). The effects of vulpinic acid on Bcl2 family members, mRNA and protein levels significantly increased in *Bax* and *P53* levels ($p<0.05$). This is the first paper on the expression value of apoptosis related genes (*Bax, Bcl-2* and *P53*) on cancer and normal cell lines exposed to vulpinic acid analyzed by qRT-PCR assay.

Karaosmanoğlu and Sivas (2015) investigated the inhibitory effect of vulpinic acid on the proliferation of HepG2 hepatocellular carcinoma cell line and F2408 mouse embryonic fibroblasts cell line by MTT assay. Although IC_{50} value of vulpinic acid was determined from 450 to 500 μM on F2408 normal cell line, the value of the IC_{50} of vulpinic acid showed a range of 300-400 μM on HepG2 cancer cell line for 48 h.[23] In our study, we observed 23.8 μM value of the IC_{50} of vulpinic acid on HepG2 cancer cell line for 48 h. When the results of both studies were compared, we obtained lower amounts of the IC_{50} value in our study. As a result of the study, vulpinic acid will be used as a novel drug source in the pharmaceutical industry. Furthermore, Karaosmanoğlu and Sivas (2015) evaluated the apoptotic effect of vulpinic acid using AO/EB dye with fluorescence microscopy.[23] In this study, we evaluated apoptotic effect of vulpinic acid a wider research at molecular level. Although the most gene expression fold change on CaCo2, HepG2, Hep2C, RD, Wehi cancer cells were shown in the *Bax* gene, the lowest gene expression level was on L929 normal cell line. This study confirmed that vulpinic acid plays a major role in the apoptosis of cancer cells. Therefore, studies focusing on specific mechanisms at a molecular level have shown promising results for lichen secondary metabolite based drug therapies.

Lin *et al.* (2003) reported the role of polysaccharide (CFP-2) extracted from *Cladonia furcate* lichen species.[24] After the biologic validation of CFP-2, telomerase inhibition and apoptotic effect of CFP-2 were determined. HL-60 and K562 cells were treated with 50, 100, 200, 400 and 800 mg/L concentrations of CFP-2 for 4, 5 and 6 days using the MTT assay. DNA fragment analysis
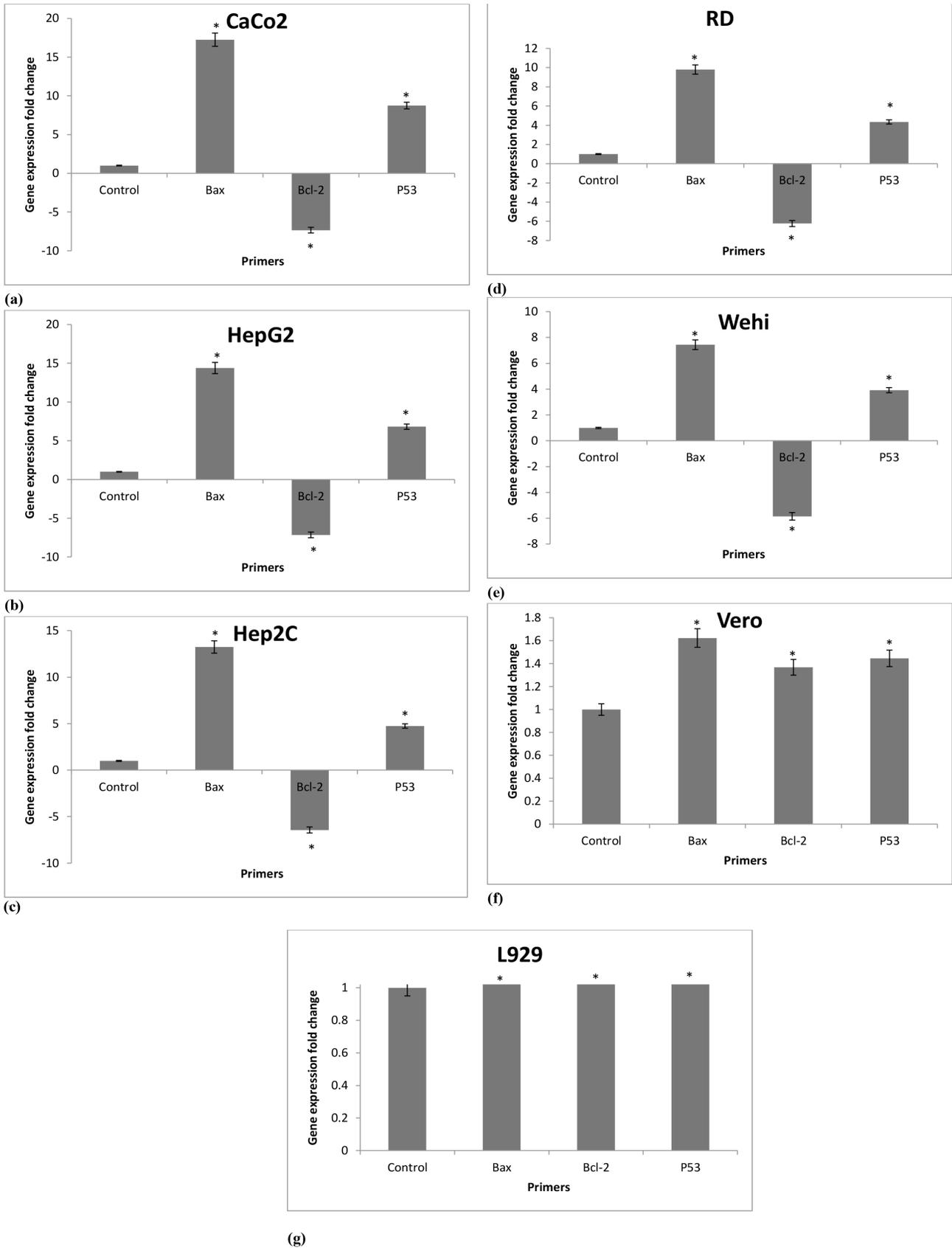
Figure 5: Vulpinic acid on the mRNA levels of CaCo2, HepG2, Hep2C, RD, Wehi, Vero and L929 cell lines (Values are statistically significant at * p < 0.05 respective control).

and western blot assay were applied for the determination of apoptotic effect on cancer cell lines. According to DNA fragment analysis, the intensity of DNA bands in gel decreased on cancer cell lines exposed to CFP-2. It was found that although Bcl-2 expression was at the same level withthe unexposed to CFL-2, Bax expression was increased in exposure to 300 mg/L CFP-2 application of the western blot assay.[24] We also demonstrated the effect of vulpinic acid on cancer and normal cell with apoptosis related protein based data. Similar observation with Lin *et al.* (2003) was obtained on increase of Bax protein by western blot assay for vulpinic acid ($p<0.05$). According to our qRT-PCR results, we also found that vulpinic acid was significantly increased in *Bax* compared with *P53* and *Bcl-2* levels ($p<0.05$). The study of Lin *et al.* and our study showed comparable results regarding vulpinic acid as an alternative source by inducing apoptosis of cancer cells.

Liu *et al.* (2010) and Ren *et al.* (2009) also identified a mitochondria mediated apoptosis pathway by showing changes in ratio of Bax/Bcl-2 proteins after treatment with retigeric acid.[25,26] They found retigeric acid was significantly changed in Bax and Bcl-2 levels. The studies by Liu *et al.*, Ren *et al.* and our study provide similar Bax/Bcl-2 proteins results for an alternative source inducing apoptosis of cancer cells.

Hsu *et al.* (2015) emphasized several triterpenoids including botulin (K02) and its derivatives (K03, K04 and K06) extracted from *Hibiscus syriacus* inhibited breast cancer cell and migration.[27] Moreover, they investigated cytotoxic effects on normal mammalian epithelial cells and these molecules inhibited very little on normal cells. This study also showed that betulin and its derivatives induced apoptosis by activating apoptosis-related genes. These results indicate that in the MDA-MB-231 cells, K02 and K06 induced apoptotic gene expression in a Tap63 associated manner. In H184B5F5/M10 normal breast cell, the expression of *Bax, Noxa, Puma* and *Perp* showed no change with K02, K03, K04 and K06 treatment. In this study, the examined cancer cell lines had the highest expression exposed to vulpinic acid, while the lowest expression of normal cell lines was observed. Our study revealed similar results to their studies. Similar observation was made by Zu *et al.* by qRT PCR for emodin.[28] Zu *et al.* havefound emodin significantly increases in *Bax* levels and decreases in *P53* and *Bcl-2* ($p<0.05$). In our studies, vulpinic acid exposed cancer cells showed significantly higher *Bax* expression than normal cells. The study by Zu *et al.* and our study provide similar results for alternative candidate drug molecules on the industrial area. Alhazmi *et al.* (2014) examined molecular mechanism of NS induced apop-tosis in MCF-7cell line.[29] *Casp-3, Casp-8,Casp-9* and *P53* gene expression changes were determined by dose-and time dependent NS. The expression levels of *Casp-3, Casp-8, Casp-9* and *P53* in MCF-7 cells treated with 100 µL/mL NS for 48 h markedly increased by 17.37, 4.82, 10.06 and 6.48 fold when compared with control untreated cells, respectively. In our study, *Bax* gene was the top significantly up-regulated gene at 17.2 fold in CaCo2 cancer cell line while *Bcl-2* was the most significantly down-regulated gene at 7 fold in response to vulpinic acid on CaCo2 and Bcl-2 cell lines, respectively.

CONCLUSION

The present study demonstrates that vulpinic acid significantly inhibits the growth of cancer cells and provides underlying apoptosis mechanism as an anticancer activity. Vulpinic acid could potentially be an alternative source for cancer therapy in the pharmaceutical industry. Our study determined the expression changes of *Bax, Bcl-2* and *P53* genes and proteins on exposed cancer and normal cells after vulpinic acid application. The molecular mechanism of vulpinic acid secondary metabolite needs to be investigated in greater detail. Further molecular based research is required for vulpinic acid before developing a promising chemotherapeutic agent for cancer treatment.

ACKNOWLEDGEMENT

CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

ABBREVIATIONS

VA: Vulpinic acid; **DMSO:** Dimethyl Sulfoxide; **qRT-PCR:** Quantitative Real Time PCR.

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PICTORIAL ABSTRACT
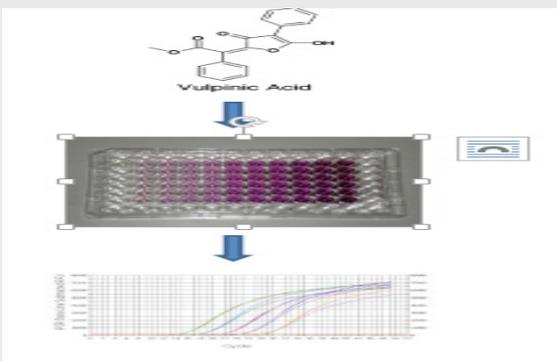
SUMMARY

Researchers are increasingly studied on new effective candidate drug molecules from biological organisms. Lichens are known to produce secondary metabolites that find uses as natural drug molecule. Lichen seconder metabolites could play a significantly role in especially cancer treatment because they are usually less toxic than traditional chemotherapy agents effective, inexpensive and easily available. In this study, we evaluated cytotoxic effect of vulpinic acid and their apoptotic effect. As a result of this study, vulpinic acid could be play an important role in cancer treatment.

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