Identification of Lilium ledebourii antiproliferative compounds against skin, bone, and oral cancer cells

Nastaran Partovi
University of Guilan
Hassan Hassani Kumleh (kumleh@guilan.ac.ir)
University of Guilan https://orcid.org/0000-0002-8781-4853
Ebrahim Mirzajani
University of Guilan
Mohsen Farhadpour
National Institute for Genetic Engineering and Biotechnology

Research Article

Keywords: Apoptosis, A431 cell line, KB cell line, G292 cell line, Phytochemicals

Posted Date: May 31st, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1672655/v1

Abstract

Cancers are one of the leading causes of death worldwide, and in spite of the progress in anticancer drug development, it is still imperative to find novel natural medicines. Therefore, this study was aimed at the evaluation of cell cycle arrest, apoptosis, and cytotoxicity induction via *Lonicera nummularifolia*, *Lilium ledebourii*, *Campsis radicans* and *Parthenocissus quinquefolia* extracts. Each extract was treated on G292, A431, and KB cancer cell lines, which compared to the HGF-1 normal cell line. From the MTT assay, all extracts significantly enhanced the cell death rate in three cancer cell lines more than the HGF-1 line. Based on evidence from the cell cycle, MTT and apoptosis tests, the Methanolic extract of *L. ledebourii* bulbs exhibited a considerable efficacy on apoptosis induction in the cancer cell lines. It seems that the mode of action for *L. ledebourii* Methanolic extract is due to the increased *BID/MAPK14* expression and the decreased *MDM2/BCL2/ MYC* expression, which lead to activation of the p53 protein-induced apoptosis. The crude extract was separated into fractions by preparative HPLC and effective fraction was characterized by Triple-Quad LC/MS connected to the UHPLC system. Overall, the findings suggest that *L. ledebourii* is a promising source of bioactive compounds, which are proven natural anticancer compounds.

1. Introduction

Cancers are one of the important drivers of death globally, therefore, challenges public health. The outbreak of this disease is rising, especially in South and Central America, Asia, and Africa that make up approximately 75% of cancer deaths globally [1]. A lot of research has been focusing on the establishment of different agents for tumor therapeutic approaches. The progress in chemical anticancer drugs has improved patient health and thereby chemotherapy has appeared as one of the potential options to treat a wide range of tumors [2]. However, these chemicals in turn lead to adverse side-effects on human tissues/cells, such as alopecia, vomiting, nausea, and bone marrow function inhibition [3]. In contrast, many phytochemicals and natural compounds have been proved as anticancer adjuvant therapy because of their proapoptotic, antioxidative, and antiproliferative features [4]. Accordingly, the continuing research for anticancer medicines from herbs plays an important role to determine the feasible options to have safe and to decline the side-effect activated via chemical drugs since natural phytochemicals have some promising benefits [5].

From previous reports, most biologically active compounds act as anticancer agents via their potential to induce apoptosis [6]. The programmed cell death as a potential target for tumor therapy plays a crucial role in the establishment, homeostasis, and removal of any abnormal cells by using a variety of cellular signaling pathways [7]. Since the apoptosis repression in carcinogenesis can disturb the balance between cell death and proliferation, thus, it leads to the establishment and progression of cancers [6]. Albeit the apoptosis induction in tumor cells is an interesting remedy towards tumor chemotherapy, the variety of molecular mechanisms in such abnormal cells made a complexity to clinical attempts [8].

Numerous therapeutic properties have been reported for *Lonicera nummularifolia, Lilium ledebourii, Campsis radicans*, and *Parthenocissus quinquefolia*. For instance, Farboodniay Jahromi et al., (2020) demonstrated the high antimicrobial property and ferric reducing activity along with free radical scavenging in *L. nummularifolia* [9]. Islam et al., (2019) presented evidence on the hypoglycemic, analgesic, membrane stabilizing, thrombolytic, and antioxidant activities in *C. radicans* [10]. Faisal et al., (2018) recorded the antioxidant activity of *P. quinquefolia* due to alkaloids, flavonoids, and terpenoids [11]. Shokrollahi et al., (2018) indicated that the antioxidant capacity of *L. ledebourii* is relatively high when compared to other medicinal herbs [12]. Despite such studies, there is a lack of information on the cytotoxicity effects of the medicinal herbs and their mechanism of action on cancer cells.

In the current research, we aimed at the cytotoxicity efficacy of extracts derived from four plants, including *L. nummularifolia, L. ledebourii, C. radicans*, and *P. quinquefolia*, on some cancer cells including oral squamous cell carcinoma and osteosarcoma (KB cell line), skin cancer (A431 cell line), and bone cancer (G292 cell line) in comparison to HGF-1 as normal cell line.

2. Material And Methods

2.1. Plant material and extract preparation

The leaves of three herbs including *L. nummularifolia* (voucher number: CAL4225), *C. radicans* (voucher number: BIC2267), and *P. quinquefolia* (voucher number: VIP2246) along with the bulbs of *L. ledebourii* (voucher number: LIL1228) were collected from the Guilan forests, Iran. 100 g of each sample was washed three times by sterile distilled water and their extraction was provided via pure methanol and ethanol (80%) through maceration method at 50 rpm, 25°C, and 24 h in darkness. Each extract was filtered by using Whatman® Grade 42 Ashless Filter Papers (Sigma-Aldrich) and then dried via a freeze dryer. Then, 1 mg of resulted material was solved in 1 ml of 0.1% (w/v) dimethyl sulfoxide (DMSO) through the medium RPMI to prepare the stock solution. Eventually, the resulted extractions were incorporated into the cell culture media.

2.2. Cell culture

KB, A431 and G292 cell lines along with HGF-1 as normal cell line were provided from the Biological Resource Center, Iran. These cell lines were grown in RPMI-1640 medium containing 1% (w/v) streptomycin-penicillin antibiotics and 10% (w/v) fetal bovine serum (Sigma-Aldrich) and kept in an incubator under 5% Co_2 at 37°C [13].

2.3. MTT assay

First, the intended cell lines were trypsinized and counted via Neubauer Chamber. Then, 7×10^3 cells were cultured in 100 µl of RPMI-1640 medium containing 10% (w/v) fetal bovine serum and 1% (w/v) streptomycin-penicillin antibiotics in a 96-well microplate and incubated 24 h at 37°C under 5% CO_2. To achieve a sufficient number of cells per plate, the cells were permitted to adhere to the plate bottom, and

then supernatants were removed. The 50 µg ml^{-1} (dissolved in RPMI-1640 medium containing 1% (w/v) streptomycin-penicillin antibiotics and 10% (w/v) fetal bovine serum) of each herb extract was incorporated into each well. Control cells of each line were treated only with 0.1% (w/v) DMSO. Following 24 h, the culture medium was removed and 20 µl of fetal bovine serum including 0.2 mg ml^{-1} of MTT reagent was incorporated into wells. Eventually, the insoluble formazan crystals were dissolved in 0.1% dimethyl sulfoxide and the absorbance was read via a spectrophotometer at 570 nm [14].

2.4. Flow cytometry

Flow cytometry technique was utilized to assay cell cycle and apoptosis in cancer cells. For achieving this goal, 50 µg mL^{-1} of each cell line was collected overnight and homogenized in 0.5 mL of phosphate-buffered saline through the vortex. The 70% ethanol (1 mL) was mixed with the solution and maintained on ice for 120 min. The resulted mixtures were centrifuged for 15 min at 300 ×g, and then supernatants were discarded. Then, 5 mL of phosphate-buffered saline was incorporated into each pellet for 30 s and the mixture was centrifuged for 5 min at 300 ×g. After the supernatant being discarded, the residue was utilized to evaluate cell cycle and apoptosis through the Annexin V -propidium iodide (PI) mixture along with a PI solution (according to the manufacturer's instruction, Sigma Kit). After 30 min, the mixtures were injected into the flow cytometry instrument [15].

2.5. Instrumental analysis

An UHPLC system was used for separating the extracts' species. This system includes an autosampler, a binary pump, and a vacuum degasser. It consisted of a reversed-phase rapid resolution C18 analytical column of 50 mm × 4.6 mm i.d. and 1.8 µm particle size (RR Zorbax Eclipse XDB-C18). In each sample, there was a 10 µL injection of the extract. The elution was conducted in gradient mode as the following: water with 0.1% formic acid served as eluent A, and acetonitrile was used as eluent B. Using the chromatographic technique, the primary mobile phase composition (10% eluent B) constant was kept for one minute, and then a linear gradient followed to 100% eluent B at 11 minutes. Then, 100% eluent B was passed through the column for four minutes. The overall run time was 20 minutes, with 0.6 mL min^{-1} as a flow rate.

Crude extract was separated by HPLC into 9 fractions (C1-C9) based on peak and retention time in different time ranges including 21 (C1), 30.5 (C2), 35.5 (C3), 38(C4), 40 (C5) 41.5 (C6), 50.5 (C7), 48 (C8), 60 (C9) min. The effective ingredient was identified by mass spectrometry of Fraction9.

2.6. Triple-Quad LC/MS Analysis

A triple quadrupole mass spectrometer Agilent 6410 Triple-Quad LC/MS was connected to the UHPLC system was used as the detector. The Spectrometer consisted of an electrospray interface working in positive ion mode and used these operating factors: nebulizer gad: 50 psi, capillary voltage: 5000V, gas temperature: 325 $^{\circ}C$, gas flow: 12 L min^{-1}. Nitrogen was used as the collision into ESI source in positive mode, specific MRM transition, collision energy, and fragment voltage ware optimized. Agilent Mass

Hunter Software was employed to develop the method, and data acquisition and processing, including MRM Mode Software and Mass Hunter Optimizer feature.

2.6. RNA extraction and qRT-PCR

The gene expression assay was directly accomplished on a total of 10^6 cells treated with the IC25 of doxorubicin and *L. ledebourii* Methanolic extract for 20 min. Total RNA from the cells was extracted via RNX-Plus solution (CinnaGen, Iran) based on the manufacturer's instruction. The First Strand cDNA Synthesis Kit was utilized to synthesize cDNA from RNA (Thermo Fisher Scientific). Primer design for MYC proto-oncogene, bHLH transcription factor (MYC; ID), p53 (P53; ID), BH3 interacting-domain death agonist (BID; ID), Map Kinase 14 (MAPK14; ID), MDM2 proto-oncogene (MDM2; NM_001145337), and B-cell lymphoma 2 (BCL2; NM_000633) was accomplished based on NCBI database and via primer3 software (Table 1.). qRT-PCR was run through SYBR Green PCR MasterMix (Thermo Fisher Scientific) in 40 cycles, including denaturation for 60s at 95°C, annealing for 20s at 55–60°C, and extension for 20s at 72°C. The *GAPDH* (NM_001256799) was used as a reference gene to estimate the steady-state transcript level of each gene. The $2^{-\Delta\Delta Ct}$ method was followed to calculate the fold change [16].

Table 1
primers utilized for qRT-PCR

Gene name	Primers	Tm°C
MYC	5′-GGAACTTACAACACCCGAGC-3′	56
	5′-GCTGCCATCACTGTTAAGCT-3′	
P53	5′-TGGCCATCTACAAGCAGTCA-3′	56
	5′-GGTACAGTCAGAGCCAACCT-3′	
BID	5′-CCTACTGGTGTTTGGCTTCC-3′	56
	5′-GCCTCTATTCTTCCCAAGCG-3′	
MAPK14	5′-CTGGATTTTGGACTGGCTCG-3′	57
	5′-AGTCAACAGCTCGGCCATTA-3′	
MDM2	5′-TCACAGATTCCAGCTTCGGA-3′	56
	5′-GCACGCCAAACAAATCTCCT-3′	
BCL2	5′-TTCTTTGAGTTCGGTGGGGT-3′	56
	5′-CTTCAGAGACAGCCAGGAGA-3′	
GAPDH	5′-TCACCAGGGCTGCTTTTAAC-3′	56
	5′-GGACTCCACGACGTACTCAG-3′	

2.7. Statistical analysis

Doxorubicin was used as a standard drug to compare with the herb extracts. IC50 for each extract was calculated through GraphPad Prism 7. All experiments were performed in a completely randomized factorial design with three replicates. Data analyses were calculated using SPSS 20 (SPSS Inc., Chicago, IL, USA). Data are presented as mean values ± standard error of the mean (SE). one-way analysis of variance test (ANOVA) was used for statistical analysis of data and Duncan post hoc test was used to compare the means of the groups. The differences were considered to be statistically significant at $P < 0.05$.

3. Results

3.1. Cytotoxicity of the herb extracts

The cytotoxicity rate was compared among HGF-1 and cancer cell lines because of two reasons: i) the increasingly concentration of the herb extracts changed the toxicity rate in a dose-dependent way and ii) the decrease of toxicity in a normal cell line is considered as an optimum safety index. From the results, The IC50 value of the Methanolic extract of *L. ledebourii* bulbs (Lm) showed the most cytotoxicity in the G292 cell line and less cytotoxicity in the HGF-1 line. In spite of the higher cytotoxicity of other extracts than Lm extract on the A431 line, this extract is superior because of lower toxicity in the HGF-1 line (Fig. 1b).

3.2. Apoptosis induction by herb extracts

To determine the potential pathways of cell death in the tumor lines treated with the extracts, staining with PI and Annexin V were utilized for recognizing the stages of apoptosis. From Fig. 2, the number of live cells in response to herb extracts could be a parameter of apoptosis efficiency in comparison with control. All extracts significantly decreased the number of live HGF-1 cells over control. The maximum number of live cells (67.4%) in the HGF-1 line was recorded for Lm extract. This extract hiked the number of early apoptosis (EA) cells than other extracts in the HGF-1 line. Because the Ethanolic extract of *L. nummularifolia* leaf (Le)-treated HGF-1 line presented the high percentage (40.3%) of (late apoptosis) LA cells when compared to Lm extract, which was ~ 0%, thus it displays a non-selective feature of apoptosis for Le. Besides, the Ethanolic extract of *C. radicans* leaves (Ce) exhibited a significant increment of dead cells in the HGF-1 line than control that it expresses a higher non-apoptotic lethal efficacy (Fig. 2a).

In the G292 line, albeit four herb extracts significantly enhanced the EA cells over control, Lm extract demonstrated the maximum rate of LA cells than control. Moreover, it seems that the lethal efficacy of Lm on the G292 cell line is associated with necrosis, not apoptosis (Fig. 2b). In the A431 line, the maximum rate of EA cells was observed in the lines treated with the Ethanolic extract of *P. quinquefolia* leaves (Pe); whereas the Lm extract significantly increased the rate of LA cells and dead cells than control and other herb extracts. The high death of the A431 cell line treated with Lm extract (58.3%) suggests the necrotic death (Fig. 2c). In the KB line, the Lm extract demonstrated the highest toxicity by EA and LA cells and the lowest rate of living cells (8.9%), while the Ce extract displayed the least toxicity among all extracts. When evaluating early apoptosis, Lm extract exhibited the highest level with a striking

percentage of 47.4%, followed by Ce with 28.1%; while the Le extract derived the lowest rate of early apoptosis among all herb extracts (Fig. 2d). additional data are given in Online Resource 1 (Fig S1).

3.3. Cell cycle assay

The various steps of the cell cycle were assayed in response to the herb extracts and the subG1 step was regarded as a marker of apoptotic status on cancer status. As demonstrated in Fig. 3c, the Methanolic extract of *L. ledebourii* bulbs (Lm) exhibited the most accumulation of cells in the subG1 step of G292 and KB lines than control. This extract resulted in the highest rate of apoptosis in G292 with a remarkable quantity of 36.3% of total cells in the subG1 step over control. Besides, the Ethanolic extract of *P. quinquefolia* leaves (Pe) significantly enhanced the a mount of cells in the subG1 in KB line than control (Fig. 3d). However, both of these herb extracts (Lm and Pe) displayed a very slight efficacy on the HGF-1 and A431 lines and their entrance into the subG1 step. In the presence of Ce extract, although the number of the sub-G1 cells in all cancer lines was more than control, the number of the HGF-1 cells was more only at the G2 step than other treatments (Fig. 3a). Moreover, the Lm and Le extracts could increase the number of cells stopped in the S phase in all cancer cells (Fig. 3a–d). From our observations, the apoptosis, cell cycle, and MTT tests revealed that the Lm extract plays a vital role in death acceleration of tumor cells. additional data are given in Online Resource 2 (Fig S2).

3.4. The expression of genes involved in apoptosis

L. ledebourii extract exhibited a significant change in the transcript level of some genes in cancer cells. However, the expression patterns of other genes were similar to doxorubicin. The transcript level of *P53, BID, and MAPK14* genes enhanced significantly over doxorubicin (Fig. 4a-c). In contrast to the *L. ledebourii* extract, doxorubicin significantly induced the transcript level of the *MYC* in the tumor and normal cell lines (Fig. 4d). The transcript level of *MDM2* and *BCL2* apoptotic inhibitors genes was significantly decreased in response to Lm extract over doxorubicin (Fig. 4e-f).

3.5. Characterization of antiproliferative compounds

To select the optimum extract with high level of lethal efficiency on cancer cells, several factors were considered with together including IC50, cell number in sub-G1, and scatter gating of cell population in histograms of Annexin V and PI. Therefore, the Methanolic extract of *L. ledebourii bulbs* that was include a balance of all these factors and approximately indicated the high cytotoxicity level in cancer cell lines were selected as significant anticancer agent. After separating the Fractions of the extract by chromatography, In accordance with chromatogram provided in Fig. 5, the most effective Fraction determination of the toxicity effect of each fraction on the G292 cell line was identified. The results showed that 8th fraction among 9 fractions had the highest toxicity on cell line G292 (Table. 2). In order to investigate the purity of the fraction8, an analytical chromatography was used when its chromatogram is in accordance with Fig. 6. The results showed that purity of the purity of purified compound was high to identify and determine the structure. According to the results of the mass of chromatography, fraction8 was except the form of phenolic compounds, a list of compounds identified based on the metabolite profile of the fraction8 (Fig. 7.(a)), in Table 3 is presented and the matching of m/z results with similar

articles showed that an effective substance with anti-cancer properties is likely to be Catechin and Coumarin, Caffeic acid, ferulic acid, Kaempferol, Apigenin. The Mass spectrum chromatogram for the materials identified in Fig. 7 (b). is presented.

Table 2
Percentages of Cancer (G292) and Normal (HGF-1) Cell Death (Cell Toxicity) Induced by Different Fractions of *L. ledebourii* after 24 h Treatment with 50 µg ml^{-1} Concentration

Fraction	Cell lines (Mean ± SE)	
	G292	HGF-1
Lm1	8.15	7.52
Lm2	12.87	8.36
Lm3	29.34	11.25
Lm4	43.16	13.62
Lm5	52.13	14.91
Lm6	56.43	17.32
Lm7	67.24	18.75
Lm8	94.23	23.37
Lm9	74.65	18.14

Table 3 List of major phenolic compounds identified by Triple-Quad LC/MS present in the Fraction 8 obtained from *L. ledebourii* Methanolic extract

Compounds	m/z	Reference
Caffeic acid	166-329-667-511	Zhao et al., 2021; Ben Said et al., 2017
ferulic acid	200	Zhao et al., 2021
coumarin acid	295-277-365-205-269-541-280	Zhao et al., 2021; Ben Said et al., 2017
Catechin	290-289-271-247-245-306-205-458-425-578-	Pe´rez-Magarino et al., 1999; Ben Said et al., 2017
Kaempferol	241	Pe´rez-Magarino., 1999
Apigenin	269–271	Tsimogiannis et al., 2007; Bouaziz et al., 2005

4. Discussion

Phytochemicals from a range of medicinal herbs are gaining broad attention for their anticancer properties. Many researchers have found that plant bioactive ingredients can improve the efficiency of chemotherapy and ameliorate the side-effect of chemical drugs utilized as chemotherapeutic agents [17–19]. Thus, we evaluated the efficacy of *L. nummularifolia*, *L. ledebourii*, *C. radicans*, and *P. quinquefolia* extracts on cell cycle arrest, apoptosis, and cytotoxicity induction in the skin, bone, and oral cancer cells.

From the MTT assay, four extracts significantly enhanced the cell death in the cancer cell lines when compared to the HGF-1 cell line. The high toxicity levels in the cancer cell lines were recorded for Le on G292 and A431, Pe on KB and A431, Ce on A431, as well as Lm on G292 and KB. Similarly, Farboodniay-Jahromi et al., (2020) demonstrated that alkaloids, flavonoids, and phenols can justify clearly the biological activity of *L. nummularifolia* on cell lines [9].

As our observations revealed, the Methanolic extract of *L. ledebourii* bulbs (Le) could induce the programmed cell death and enhanced the cell accumulation in subG1 step and the arrest of the cell cycle in the G0/G1 step. *L. ledebourii* has been proved as an herb containing lectin as carbohydrate-binding proteins [20]. Several researches demonstrated the anticancer efficacy of these proteins in apoptosis induction, cell accumulation in G0/G1 and/or G2/M phases, and ribosomal attachment [21].

From previous reports, it was well-known that the *BH3 interacting-domain death agonist (BID)* gene is up-regulated via *p53* tumor suppressor and is involved in *p53*-induced apoptosis [31]. Moreover, apoptotic motivators induce *caspase8* and its substrate, *BH3* interacting-domain death agonist, in a death receptor-independent way [31]. Our observations are in line with these reports, suggesting the *L. ledebourii* extract can increase *BID* and subsequent P53 genes expression, thereby makes help to induce apoptosis in the KB cell line, A431 cell line, and G292 cell line.

Map Kinase 14, a crucial member of the MAPK family *P38*, play a dual function in some tumors [27]. Several researchers have indicated that *Map Kinase 14* can promote the onset and progress of breast tumors via inducing its downstream genes [28]. In addition, *Map Kinase 14* has also been exhibited to play as an inhibitor in lung, colon, liver, and cancer [29]. Given the inhibitory role of *MAPK14* in skin cancer [30], it seems that the increased level of *MAPK14* transcript derived by Le extract can be effective in controlling cell growth through apoptosis.

Cancer cells enhance the MYC gene expression, usually as a consequence of tonic WNT signaling, *MYC* gene amplification, and/or chromosomal translocation [25]. By gain of prosurvival signals (e.g., NF-κB and *BCL2*) and/or by loss of surveillance mechanism (e.g., *MDM2* and *P53)*, cancer cells can tolerate the enhanced *MYC* level and thereby avoid programmed cell death [26]. As proved in this study, the *L. ledebourii* Methanolic extract decreased the transcript level of *MYC*. Thus, it seems that *L. ledebourii* induces negative auto-regulation that in turn decreases *MYC* expression.

It is well-known that the *MDM2/BCL2* dual repression makes an opportunity for a bioactive compound to act as an antitumor agent [22, 23]. These statements are in line with our findings when the *L. ledebourii* Methanolic declined the transcript levels of *BCL2* and *MDM2*. It has been presented the evidence on the activating role of Le extract on *p53* expression. Since the induction of *p53* tumor suppressor indirectly decreases several cell cycle genes and eventually result in cell cycle arrest [24], the *L. ledebourii* extract regulates a plethora of genes involved in cell cycle by *MDM2/BCL2* and *p53* pathways and can contribute to cell cycle arrest.

Based on the results of Triple-Quad LC/MS connected to the UHPLC analysis for *L. ledebourii* methanolic extract, it was determined that anti-cancer substance is a phenolic compound. The phenolic compounds are secondary metabolites of plants, which have a wide range of biological activities, such as anti-inflammatory, antioxidant, anti-aging, and antidepressant properties [32]. According to the report of Luo et al. [33], the phenolic compounds in L. brownii are mainly phenylpropanoids, which have significant antioxidant activity.

The results of identifying the sample structure of fraction 8 showed that was mainly composed of coumarin acid, Catechin, caffeic and ferulic acid, Kaempferol and Apigenin which are connected by one molecule of glycerol, and the glycerol group are formed by para/ortho substitution or glucose substitution/acetylation. Hence, these compounds belong to phenolic glycerides/glycosides (phenylpropanoid compounds), and are called “regalosides” in some studies. coumaric acid, a Phenolic Compounds with anti-cancer effects. They can downregulate amyloid β-induced the overexpression of *COX-2* and *iNOS* in *PC12* cells through inhibiting *MAPK* signaling pathway and *NF-κB* activity [34]. Coumarin was capable of down-regulating *MDM2* and the anti-apoptosis proteins *Bcl-2* and *Bcl-xL*, up-regulating the level of *P53* and the pro-apoptosis protein *BAX*, causing cell cycle arrest at G2/M phase and activating Caspase-9 to induce apoptosis [35]. catechin can neutralize reactive oxygen species (ROS) present in the cell and also can reduce abnormal cell replication by altering cell signaling pathways (ex. MAP kinase pathway). catechin is effectively used as a preventative agent in cancer therapy. For better outcome catechin can be used as an adjunct therapy with radiation and chemotherapy during cancer treatment [36]. Research results of Serafim et al., showed that results show that the new caffeic and ferulic acid lipophilic derivatives show increased cytotoxicity toward human breast cancer cell lines [37]. Kaempferol and apigenin were investigated as their anti-SIRTs potential. Sharma et al., showed in their paper, KMP and API inhibits cellular proliferation by DNA damage and S-phase cell cycle arrest in TNBC Cells. KMP and API inhibited *SIRT3* and *SIRT6* proteins, and as promising candidates to be further developed as sirtuin modulators against *TNBCs* [38].

5. Conclusion

As shown in this study, the Methanolic extract of *L. ledebourii* significantly enhances the cell death rate in G292, A431, and KB cancer cells when compared to the HGF-1 cell line. Moreover, this herb extract can be a novel medicine for cancer therapy because of high level of apoptosis activation in tumor cells and low cytotoxicity on normal cells. It seems that the mechanism of action for *L. ledebourii* Methanolic extract is

modulating *BID/MAPK14* along with *MDM2/BCL2/MYC* expression, which in turn, leads to the *P53* protein-induced apoptosis.

Declarations

Author contribution:

The experiment was designed by [Hassan Hassani Kumleh]. Chromatographic analyzes were performed by [Mohsen Farhadpour]. Experiments and data analysis were performed by [Nastaran Partovi]. The article was written by [Ebrahim Mirzajani].

Acknowledgements

This research has been supported by Plant Biotechnology Department, Faculty of Agricultural Sciences, University of Guilan, Iran.

References

1. Siegel RL, Miller KD, Jemal A (2020) Cancer statistics, 2020. CA Cancer J Clin 70:7–30
2. Pucci C, Martinelli C, Ciofani G (2019) Innovative approaches for cancer treatment: Current perspectives and new challenges. Ecancermedicalscience 13:961. https://doi.org/10.3332/ecancer.2019.961
3. Chikara S, Nagaprashantha LD, Singhal J, Horne D, Awasthi S, Singhal SS (2018) Oxidative stress and dietary phytochemicals: Role in cancer chemoprevention and treatment. Cancer Lett 413:122–134
4. Seca AML, Pinto DCGA (2018) Plant Secondary Metabolites as Anticancer Agents: Successes in Clinical Trials and Therapeutic Application. Int J Mol Sci 19:263. https://doi.org/10.3390/ijms19010263
5. Greenwell M, Rahman PK (2015) Medicinal Plants: Their Use in Anticancer Treatment. Int J Pharm Sci Res 6(10):4103–4112
6. D'Arcy MS (2019) Cell death: a review of the major forms of apoptosis, necrosis and autophagy. Cell Biol Int 43(6):582–592. https://doi.org/10.1002/cbin.11137
7. Elmore S (2007) Apoptosis: a review of programmed cell death. Toxicol Pathol 35(4):495–516
8. Renehan AG, Booth C, Potten CS (2001) What is apoptosis, and why is it important? BMJ 322:1536–1538. https://doi.org/10.1136/bmj.322.7301.1536
9. Farboodniay Jahromi MA, Emami A, Nazeri R, Pirbonyeh N (2020) Antibacterial and Antioxidant Activity of extract and fractions of *Lonicera nummularifolia* Jaub & Spach. Trends Pharmacol Sci 6:131–142

10. Islam M, Jannat T, Kuddu MR et al (2019) *In vitro* and *in vivo* evaluation of pharmacological potentials of *Campsis radicans* L. Clin Phytosci 5:42. https://doi.org/10.1186/s40816-019-0144-9
11. Faisal S, Perveen A, Khan ZU, Sardar AA, Shaheen S, Manzoor A (2018) Phytochemical screening and antioxidant potential of *Parthenocissus quinquefolia* (L.) planch extracts of bark and stem. Pak J Pharm Sci 31(5):1813–1816
12. Shokrollahi S, Heshmati G, Yosef Zadeh H (2018) Study the Phenolic Compounds and Antioxidant Activity of the Extract Lily (*Lilium ledebourii* (Baker) Boiss). J Fasa Univ Med Sci 8(2):727–734
13. Ackermann T, Tardito S (2019) Cell Culture Medium Formulation and Its Implications in Cancer Metabolism. Trends Cancer 5(6):329–332. https://doi.org/10.1016/j.trecan.2019.05.004
14. Florento L, Matias R, Tuaño E, Santiago K, Dela Cruz F, Tuazon A (2012) Comparison of cytotoxic activity of anticancer drugs against various human tumor cell lines using in vitro cell-based approach. Int J Biomed Sci 8(1):76–80
15. Gupta V, Zhang QJ, Liu YY (2011) Evaluation of anticancer agents using flow cytometry analysis of cancer stem cells. Methods Mol Biol 716:179–191. https://doi.org/10.1007/978-1-61779-012-6_11
16. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2 – ΔΔCT method. Methods 25(4):402–408
17. Subramaniam S, Selvaduray KR, Radhakrishnan AK (2019) Bioactive Compounds: Natural Defense Against Cancer? Biomolecules 9(12):758. https://doi.org/10.3390/biom9120758
18. Abotaleb M, Samuel SM, Varghese E et al (2018) Flavonoids in Cancer and Apoptosis. Cancers (Basel) 11(1):28
19. Patlolla JM, Rao CV (2012) Triterpenoids for cancer prevention and treatment: current status and future prospects. Curr Pharm Biotechnol 13(1):147–155. https://doi.org/10.2174/138920112798868719
20. Mahdinezhad N, Fakheri BA, Ghanbari S (2018) Phytochemical compounds of *Lilium ledebourii* bioss using bulb explants. Bangladesh J Bot 4:911–920
21. Mishra A, Behura A, Mawatwal S et al (2019) Structure-function and application of plant lectins in disease biology and immunity. Food Chem Toxicol 134:110827. https://doi.org/10.1016/j.fct.2019.110827
22. Billen L, Shamas-Din A, Andrews D (2008) Bid: a Bax-like BH3 protein. Oncogene 27:S93–S104
23. Dhillon A, Hagan S, Rath O et al (2007) MAP kinase signalling pathways in cancer Oncogene 26:3279–3290
24. Anwar T, Arellano-Garcia C, Ropa J et al (2018) p38-mediated phosphorylation at T367 induces EZH2 cytoplasmic localization to promote breast cancer metastasis. Nat Commun 9:2801. https://doi.org/10.1038/s41467-018-05078-8
25. Gupta J, del Barco Barrantes I, Igea A et al (2014) Dual function of p38α MAPK in colon cancer: suppression of colitis-associated tumor initiation but requirement for cancer cell survival. Cancer Cell 25:484–500

26. Wagner EF, Nebreda AR (2009) Signal integration by JNK and p38 MAPK pathways in cancer development. Nat Rev Cancer 9:537–549
27. Chen H, Liu H, Qing G (2018) Targeting oncogenic Myc as a strategy for cancer treatment. Sig Transduct Target Ther 3:5. https://doi.org/10.1038/s41392-018-0008-7
28. Phesse T, Myant K, Cole A et al (2014) Endogenous c-Myc is essential for p53-induced apoptosis in response to DNA damage *in vivo*. Cell Death Differ 21:956–966
29. Rezadoost MH, Ghasempour A, Hasani Komleh H (2019a) Apoptosis Induction by *Calystegia Sepium* HPLC Fraction can be via Dual Targeting of MDM2 and BCL-2 in Cancer Cells. BMMJ 5:83–95
30. Rezadoost MH, Kumleh HH, Ghasempour A (2019b) Cytotoxicity and apoptosis induction in breast cancer, skin cancer and glioblastoma cells by plant extracts. Mol Biol Rep 46(5):5131–5142
31. Mantovani F, Collavin L, Del Sal G (2019) Mutant p53 as a guardian of the cancer cell. Cell Death Differ 26:199–212. https://doi.org/10.1038/s41418-018-0246-9
32. Zhao K, Xiao Z, Zeng J, Xie H (2021) Effects of Different Storage Conditions on the Browning Degree, PPO Activity, and Content of Chemical Components in Fresh Lilium Bulbs (Lilium brownie F.E.Brown var. viridulum Baker.). Agriculture 2021, 11, 184. https:// doi.org/10.3390/agriculture11020184
33. Luo J, Li L, Kong L (2012) Preparative separation of phenylpropenoid glycerides from the bulbs of Lilium lancifolium by high-speed counter-current chromatography and evaluation of their antioxidant activities. Food Chem. 2012, 131, 1056–1062. [CrossRef]
34. Lu Q, Li R, Yang Y, Zhang Y, Zhao Q, Li J (2022) Ingredients with anti-inflammatory effect from medicine food homology plants. Food Chem 368:130610. doi:10.1016/j.foodchem.2021.130610.. 10.1016/j.foodchem.2021.130610
35. Ahmed EY, Abdel Latif NA, Nasr T, Awad H, Abdelhafez O (2022) Design, synthesis, and molecular modeling of coumarin derivatives as MDM2 inhibitors targeting breast cancer. Chemical biology & drog Design. https://doi.org/10.1111/cbdd.14028
36. Ahmed R, Pritha G (2022) A systematic investigation on flavonoids, catechin, β-sitosterol and lignin glycosides from *Saraca asoca* (ashoka) having anti-cancer & antioxidant properties with no side effect. J Indian Chem Soc 99(1). https://doi.org/10.1016/j.jics.2021.100293
37. Serafim TL, Carvalho FS, Marques MP, Calheiros R, Silva T, Garrido J, Milhazes N, Borges F, Roleira F, Silva ET, Holy J, Oliveira PJ (2011) Lipophilic caffeic and ferulic acid derivatives presenting cytotoxicity against human breast cancer cells. Chem Res Toxicol 16(5):763–774. doi: 10.1021/tx200126r. Epub 2011 Apr 28. PMID: 21504213
38. Sharma A, Sinha S, Keswani H, Shrivastava N (2021) Kaempferol and Apigenin suppresses the stemness properties of TNBC cells by modulating Sirtuins. Mol Divers. doi: 10.1007/s11030-022-10384-x. Epub ahead of print. PMID: 35129762

Figures

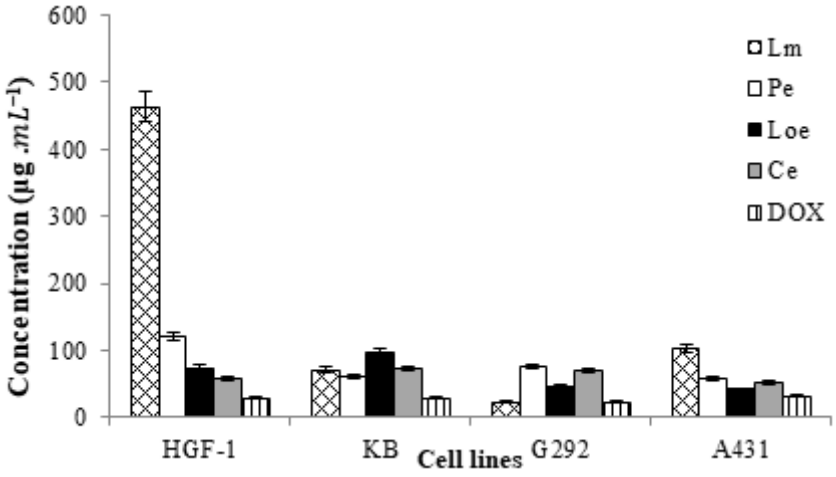

Figure 1

The IC50 value of Doxorubicin and the herb extracts on the cancer (G292, A431, and KB) and normal (HGF-1) cell lines. Lm: Methanolic extract of *L. ledebourii* bulbs; Pe: Ethanolic extract of *P. quinquefolia* leaves; Le: Ethanolic extract of *L. nummularifolia* leaves; Ce: Ethanolic extract of *C. radicans* leaves. The data are expressed as mean ± SEM (# $P < 0.01$ and $ $P < 0.05$ vs. control).

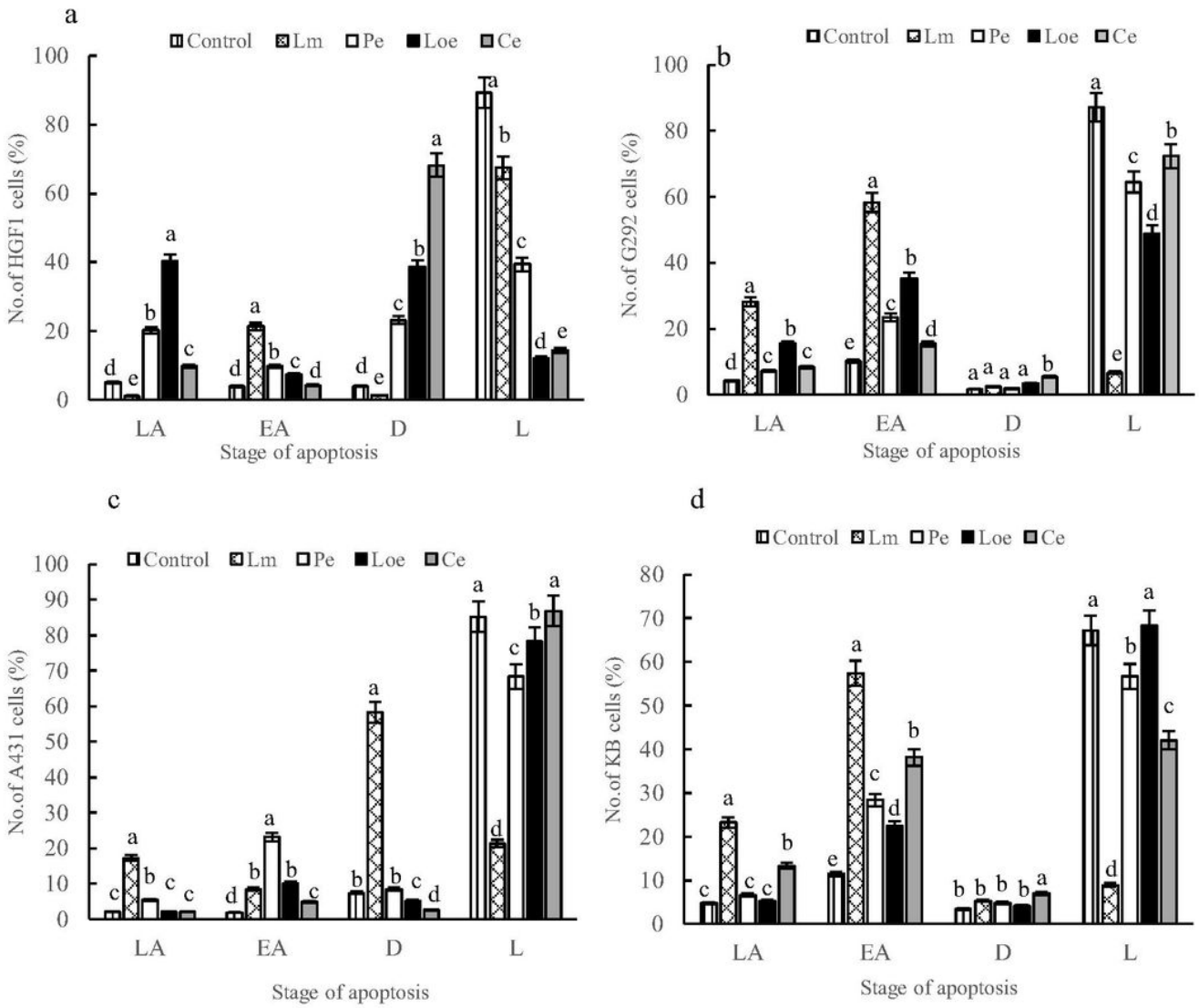

Figure 2

Flow cytometry of apoptosis in the HGF-1 normal cell line (a) along with G292 (b), A431 (c) and KB (d) cancer lines by 50 µg mL^{-1} of the herb extracts. The percentage of cells in various steps of apoptosis was tagged with LA: late apoptosis, EA: early apoptosis, D: dead, L: live. Lm: Methanolic extract of *L. ledebourii* bulbs; Pe: Ethanolic extract of *P. quinquefolia* leaves; Le: Ethanolic extract of *L. nummularifolia* leaves; Ce: Ethanolic extract of *C. radicans* leaves. The data are expressed as mean ± SEM (# P< 0.01 and $ P <0.05 vs. control).

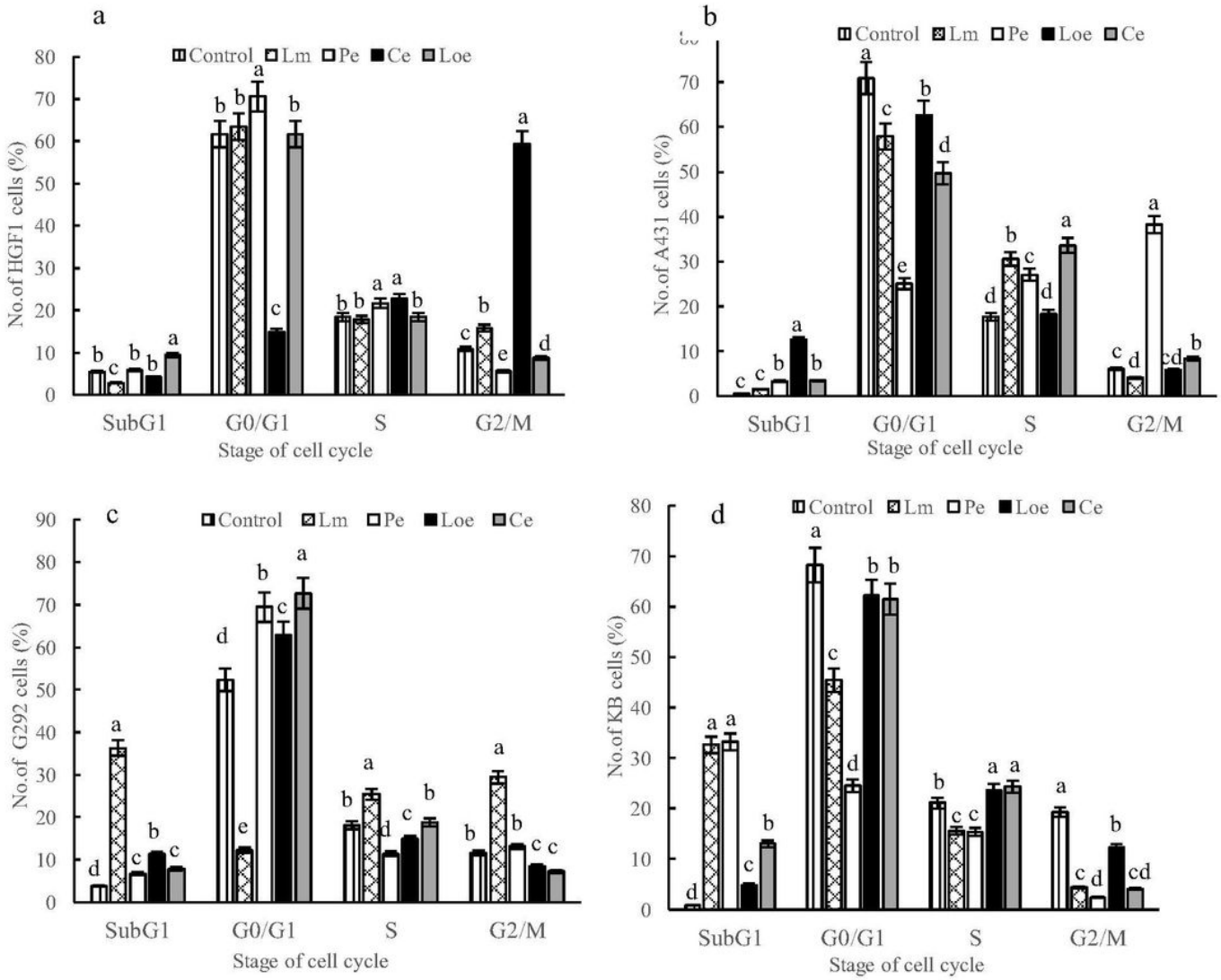

Figure 3

Flow cytometry of cell cycle stages in the HGF-1 normal cell line (a) along with G292 (b), A431 (c) and KB (d) cancer lines by 50 µg mL^{-1} of each herb extract. The percentage of cells in various steps of programmed cell death was tagged with LA: late apoptosis, EA: early apoptosis, D: dead, L: live. Lm: Methanolic extract of *L. ledebourii* bulbs; Pe: Ethanolic extract of *P. quinquefolia* leaves; Le: Ethanolic extract of *L. nummularifolia* leaves; Ce: Ethanolic extract of *C. radicans* leaves. The data are expressed as mean ± SEM (# P< 0.01 and $ P <0.05 vs. control).

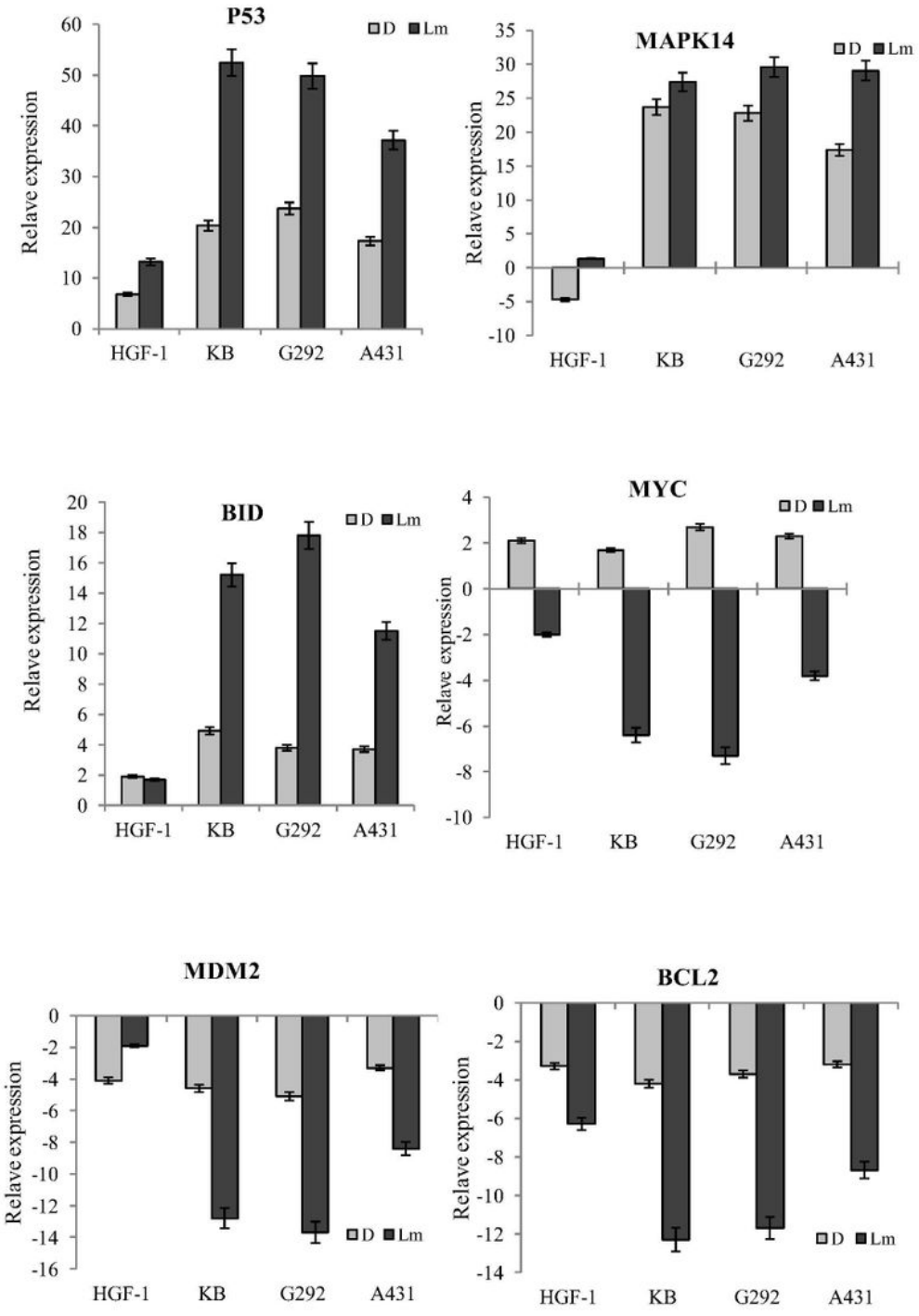

Figure 4

Expression of *MYC*, *P53*, *BID*, *MAPK14*, *MDM2*, and *BCL2* genes in response to the *L. ledebourii* Methanolic extract and Doxorubicin in the cancer (G292, A431, and KB) and normal (HGF-1) cell lines. The data are expressed as mean ± SEM (# $P < 0.01$ and $ $P < 0.05$ vs. control).

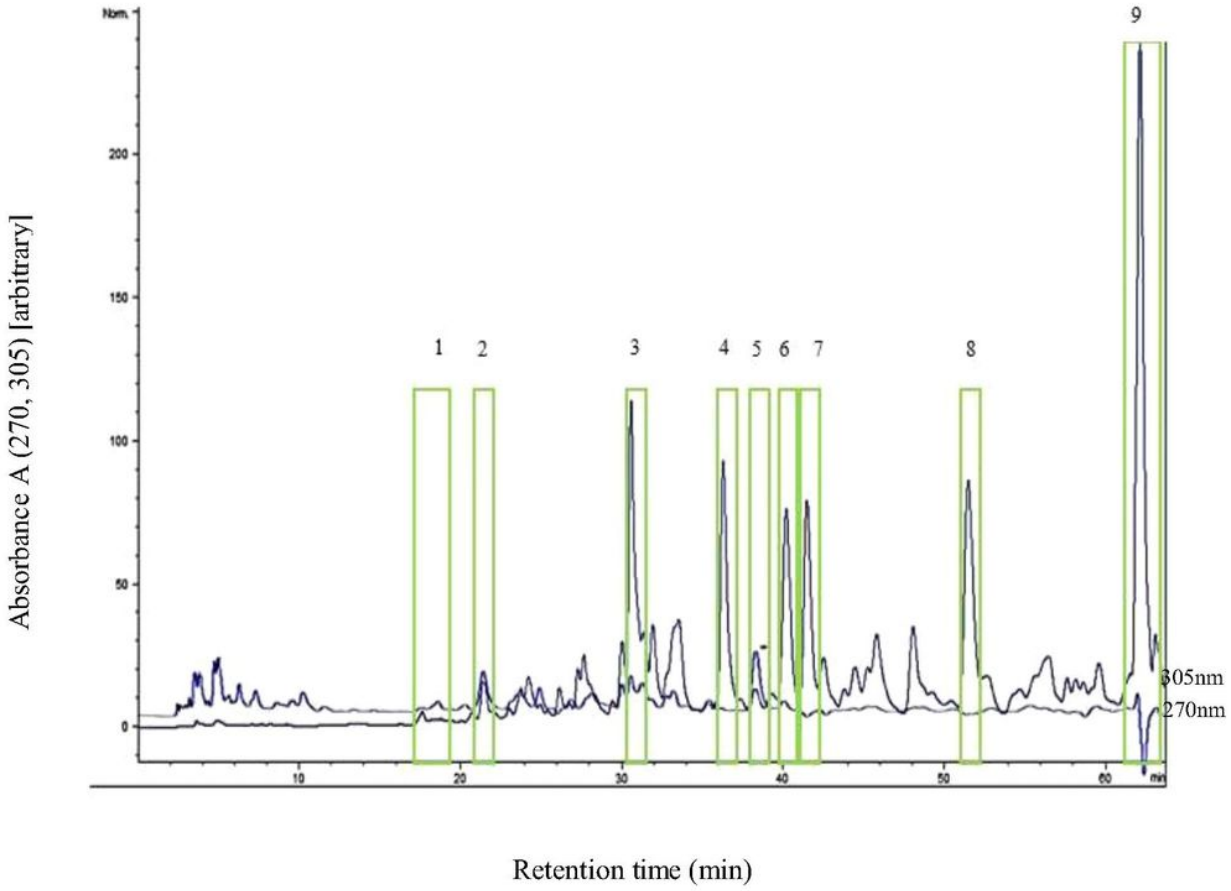

Figure 5

Analytical chromatogram for injection of Methanolic extract of *L. ledebourii* bulbs in two wavelengths 270 and 305nm

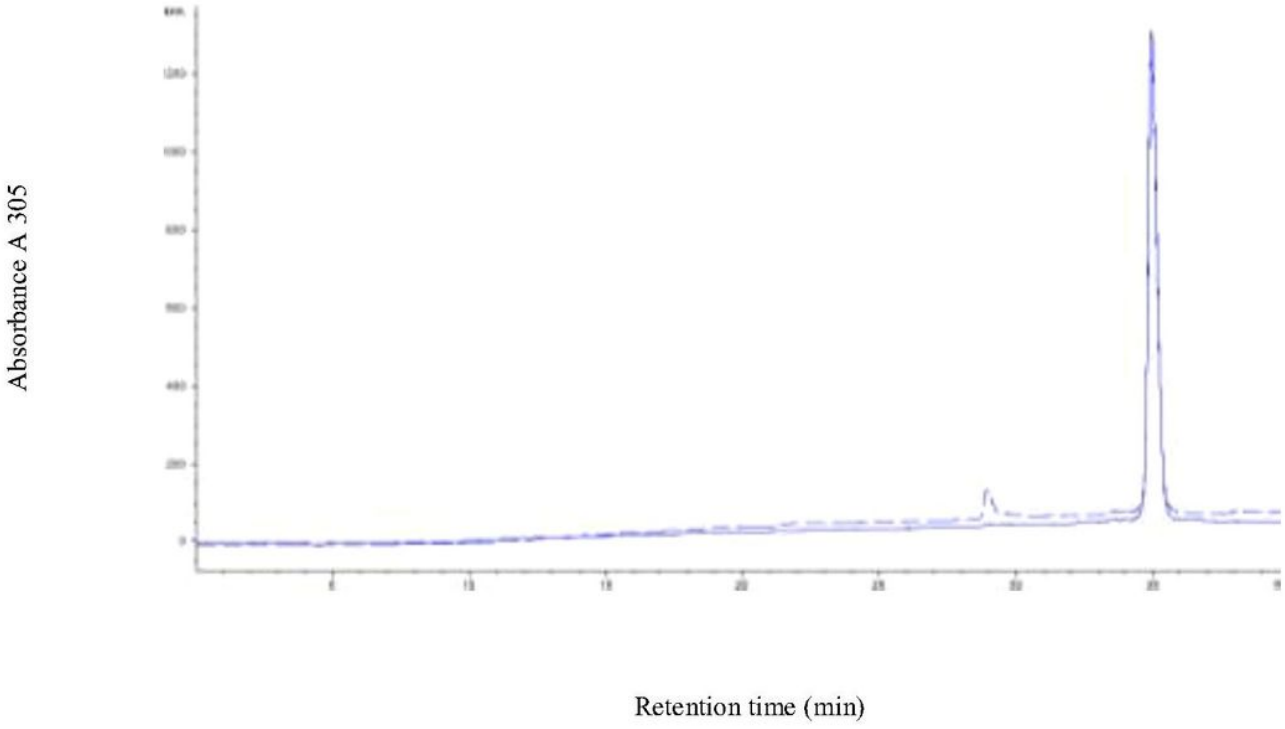

Figure 6

Analytical chromatogram for injection of fraction8 in wavelengths 305 nm.

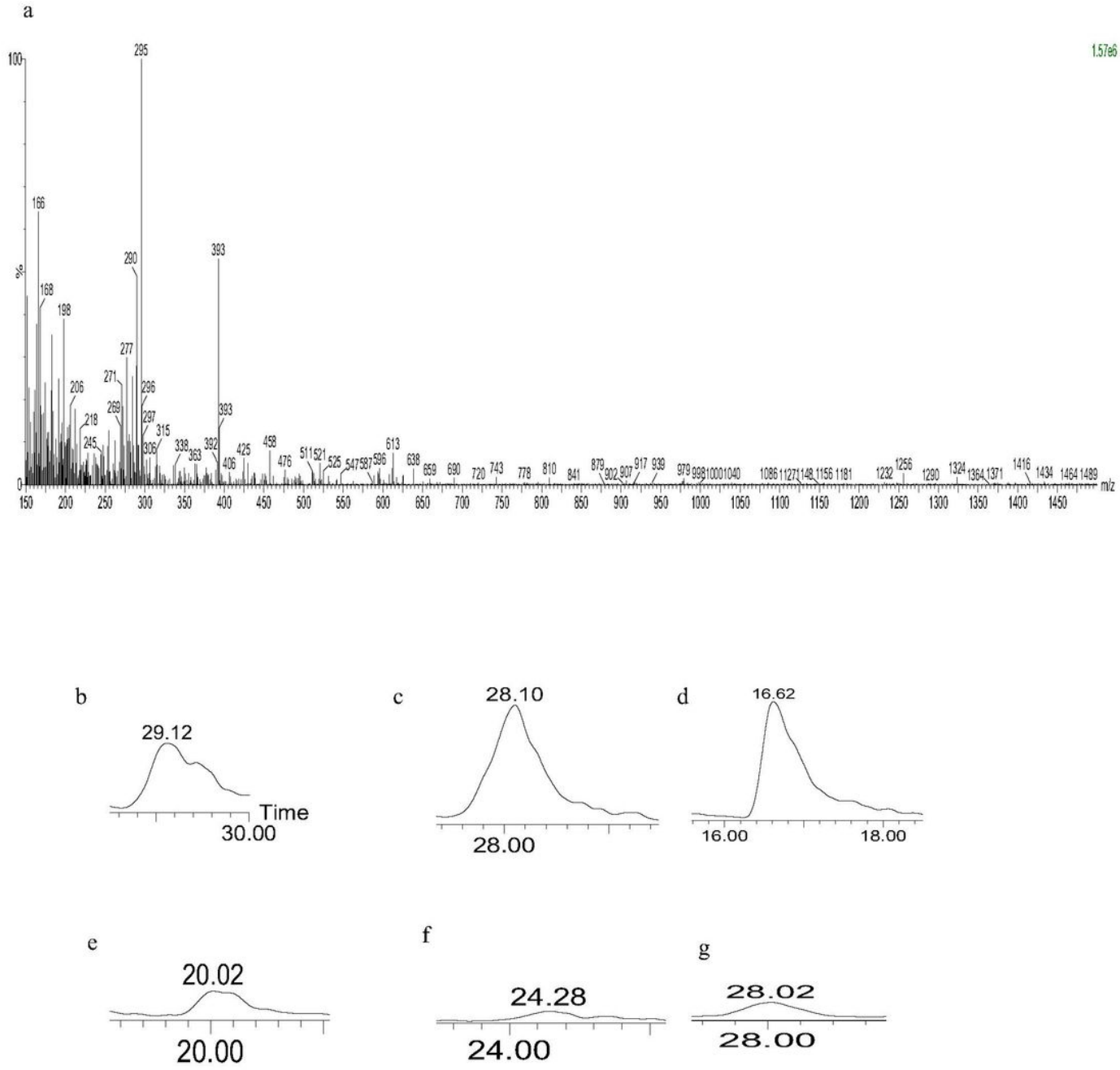

Figure 7

Mass spectrum chromatogram for the (a) Fraction 8 obtained from *L. ledebourii* Methanolic extract, (b) Catechin, (c) coumarin acid, (d) Caffeic acid, (e) ferulic acid, (f) Kaempferol, (g) Apigenin

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- ESM1.docx
- ESM2.docx