

## Proteomic analysis of the anticancer effect of various extracts of endemic *Thermopsis turcica* in human cervical cancer cells

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**Background/aim:** *Thermopsis turcica* is a perennial species endemic to Turkey and different extracts of *T. turcica* have an antiproliferative effect on cancer cells, but there has not been any report on HeLa (human cervical cancer) cells.

**Materials and methods:** To get a better understanding of the molecular mechanism of anticancer activity of methanolic extracts of leaves (LE) and flowers (FE) of *T. turcica*, we employed 2-DE-based proteomics to explore the proteins involved in anticancer activity in HeLa cells.

**Results:** *T. turcica* extracts showed a potent cytotoxic effect on HeLa cells with the IC<sub>50</sub> values of 1.75 mg/mL for LE and 3.25 mg/mL for FE. The induction of apoptosis by LE and FE was also consistent with increased expression of caspase mRNAs and DNA fragmentation. In terms of the proteomic approach, 27 differentially expressed proteins were detected and identified through MALDI-TOF/TOF mass spectrometry. These altered proteins were involved in cytoskeleton organization and movement, protein folding, proteolysis and translation, cell cycle and proliferation, signal transduction, cell redox homeostasis, and metabolism.

**Conclusion:** Up-regulation of protein disulfide isomerases and down-regulation of Rho GDP-dissociation inhibitor, heterogeneous nuclear ribonucleoproteins, and heat shock proteins may contribute to the induction of apoptosis and arresting of the cell cycle in HeLa cells.

**Key words:** Anticancer activity, apoptosis, human cervical cancer, proteomics, *Thermopsis turcica*

### 1. Introduction

Cervical cancer has been ranked as the fourth-most common type of cancer in women worldwide; 528,000 new cancer cases and 266,000 deaths are reported annually [1]. It is well-established that almost all cases of cervical cancer result from infections with carcinogenic human papillomavirus. Although prophylactic vaccines were developed for the prevention of this type of cancer, different methods including surgery, chemotherapy, and radiotherapy are used for cancer treatment in developing countries [2]. However, chemotherapeutic treatment in cervical cancer can lead to drug resistance and nonspecific cytotoxicity [3]. Considering that metastatic cervical cancer is incurable, effective anticancer agents are needed.

Plants have been used for thousands of years because they have a large number of secondary metabolites with pharmacological and anticancer properties [4]. Due to their bioactivity, plant secondary metabolites

may have a potential action in cancer therapy [5]. Anticancer drugs from plant-derived compounds with pharmacological activities may also improve the efficiency of chemotherapeutic treatments [6]. The *Thermopsis* (*Fabaceae*) genus has some species with proven biological activity. For example, various extracts of *T. lanceolata* have potentially toxic effects on mice [7]. Ethanolic extract of *T. rhombifolia* has been shown to have antitumor activity against colon and brain cancers [8]. *Thermopsis turcica*, also known as *Vuralia turcica*, is an endemic species in Turkey and is considered a medicinal plant. Different extracts of *T. turcica* have been shown to have biological activities such as free radical scavenging, antiinflammatory, mutagenic, and antimicrobial activity [9–11]. Laboratory studies on *T. turcica* indicate the antitumor activity against liver cancer, acute promyelocytic leukemia, and prostate cancer [11,12].

Proteomics is a powerful tool in the investigation of several biological processes including cancer biology. The

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anticancer mechanisms of plant-derived compounds have been investigated by performing comparative proteome analysis on various types of cancer [13–15]. Xu et al. [13] reported that betulinic acid triggers rapid apoptosis in HeLa cells by invoking ER stress and the mitochondrial-mediated pathway. In a recent study, Khazaei et al. [16] showed that flower extract of *Allium atrovioleaceum* had cytotoxicity and induce caspase-dependent apoptosis in HeLa cells. However, there has been no report on *T. turcica* extracts affecting human cervical cancer cells, and more research is needed to understand the molecular mechanisms of apoptotic effect. The main purpose of this research was to investigate the potential anticancer effects of the methanolic extracts of *T. turcica* on the human cervical cancer cell line (HeLa) by evaluating cytotoxicity, caspase activity, and apoptosis-related proteins. In this study, a gel-based proteomics approach was used to characterize the proteins involved in apoptosis of HeLa cells treated with *T. turcica* extracts.

## 2. Materials and methods

### 2.1. Plant sample and extract preparation

*Thermopsis turcica* plants were collected from the southern coast of Lake Eber of Turkey in May. Flowers and leaves were separated, shade-dried at laboratory conditions, and thoroughly ground. The extraction of samples was performed by an accelerated solvent extraction system (ASE 300, Dionex, USA) with a 100 mL stainless steel cell. The powdered materials were placed in the stainless steel cell and extracted with 70% methanol. Methanolic extracts of flowers (FE) and leaves (LE) were evaporated to remove the solvent in a rotary evaporator and lyophilized in a freeze dryer (Labconco, USA). The dry extracts were solubilized in dimethyl sulfoxide (DMSO, 1%) to obtain a stock solution.

### 2.2. Cell culture and cytotoxicity assay

The HeLa cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. Cells were incubated in 5% CO<sub>2</sub> at 37 °C. The cytotoxicity of *T. turcica* on HeLa cells was determined using the WST-1 assay (Roche, Germany). The cervical carcinoma cells (1 × 10<sup>5</sup>/well) were subjected to different concentrations of *T. turcica* extracts or DMSO in 96 well plates for 24 h. At the end of the incubation at 37 °C in 5% CO<sub>2</sub>, WST-1 solution (10 µL) was added into each well. Optical absorbance was measured at 450 nm with a microplate spectrophotometer (Multiskan GO, Thermo Fisher Scientific, Germany) after incubation at 37 °C for 4 h.

### 2.3. Expression levels of caspase genes

HeLa cells (1 × 10<sup>6</sup>/well) were placed within 6 well plates and subjected to a medium containing IC<sub>50</sub> concentrations of FE (3.25 mg/L) and LE (1.75 mg/L)

for 24 h. After incubation, the isolation of total RNA was performed using a TriPure isolation reagent (Roche, USA). cDNA was synthesized with an RT<sup>2</sup> HT First Strand cDNA synthesis kit (Qiagen, Germany). An RT<sup>2</sup> SYBR Green qPCR Green I Master Kit (Qiagen) was used for quantitative analysis of gene expression. The *GAPDH* gene was used to normalize the expression level of apoptosis-related genes *CASP3*, *CASP8*, and *CASP9*. The primers used were 5' -TGGAATTGATGCGTGATGTT-3' and 5' -TGGCTCAGAAGCACACAAAC-3' for *CASP3*, 5' -TCCAAATGCAAAGTGGATGA-3' and 5' -TCCCAGGA TGACC CTCTTCT-3' for *CASP8*, 5' -CCA TATGATCGA GGACAT CCA-3' and 5' -GACTCCCTC GAGTCTCCA GAT-3' for *CASP9*, and 5' -AGCCACATC GCTCAGACAC-3' and 5' -GCCCAATACGACCAAATC C-3' for *GAPDH*.

### 2.4. DNA fragmentation

DNA fragmentation was determined in HeLa cells exposed to *T. turcica* extracts for 24 h. An apoptotic DNA Ladder Kit (Roche Diagnostics International AG, Rotkreuz, ZG, Switzerland) was used for the isolation of fragmented DNA. The DNA fragments were separated on a 2% agarose gel by electrophoresis.

### 2.5. Protein preparation and 2-D gel electrophoresis

HeLa cells (2 × 10<sup>6</sup>) treated with *T. turcica* extracts were harvested by trypsinization and washed three times with cold PBS. The cells were solubilized in 500 µL of a lysis buffer (7 M urea, 2 M thiourea, 60 mM DTT, 4% CHAPS, 1% PMSE, and 0.4% IPG buffer) for 2 h at 4 °C. The suspensions were centrifuged at 16,000 × g for 30 min. After centrifugation, supernatants were collected and stored at -80 °C. Proteins in supernatants were quantified by Bradford assay [17]. In the first dimension of 2-D gel electrophoresis, 50 µg of cell protein was diluted in 300 µL rehydration buffer (lysis buffer with 0.001% bromophenol blue) and was loaded onto IPG strips (17 cm, pH 4–7). Proteins were focused in a Protean IEF system (Bio-Rad) at 80 kVh. Two-dimensional SDS–PAGE was performed in 12% gel on the Protean II XL system (Bio-Rad Laboratories, Inc., Philadelphia, PA, USA). Gels were run under constant amperage (24 mA/gel). After electrophoresis, silver staining was used to visualize proteins in analytical gels [18], whereas protein spots in preparative gels were stained according to Candiano et al. [19].

### 2.6. Image analysis and mass spectrometry

Protein spots were detected and quantified using PDQuest software (Philadelphia, PA, USA). Spots showing significantly and consistently different intensities (more than 1.5-fold and Student's t-test, P < 0.05) were excised manually from 2-DE gels. The peptide fragments were obtained using an in-gel digestion kit (Thermo Fisher Scientific, Massachusetts, United States). The peptide

extracts were concentrated using a ZipTip (Millipore) and eluted in 1  $\mu$ L of matrix solution ( $\alpha$ -cyano-hydroxycinnamic acid). The analyte was spotted onto a MALDI plate and analyzed by AB Sciex TOF/TOF 5800 MS (Applied Biosystems, USA). MS/MS data were submitted to MASCOT for protein identification. The search parameters were as follows: Swiss-Prot; taxonomy, human; trypsin as the digestion enzyme; one missing cleavage; 50 ppm for mass accuracy; and  $\pm 0.4$  Da for MS/MS tolerance. Carbamidomethylation for cysteine and oxidation for methionine was also considered. A 95% or higher confidence interval was accepted as criteria for protein identifications. Program STRING 9.1 was employed to generate a protein interaction network [20].

### 2.7. Statistical analysis

WST-1 and gene expression analyses data were expressed as mean  $\pm$  SD. The results were analyzed by the SPSS statistical software package using an analysis of variance (ANOVA). To determine significant differences among means at the significance level of  $P < 0.05$ , Duncan's multiple range test was used.

## 3. Results

### 3.1. Cytotoxic effects of *T. turcica* extracts in HeLa cells

The antiproliferative effects of flower and leaf extracts of *T. turcica* against HeLa cells are shown in Figure 1. We observed that *T. turcica* extracts induced a significant decrease in the viability of HeLa cells with increasing concentration. The  $IC_{50}$  value of FE in HeLa cells was determined as 3.25 mg/mL while the  $IC_{50}$  value of LE was determined as 1.75 mg/mL.

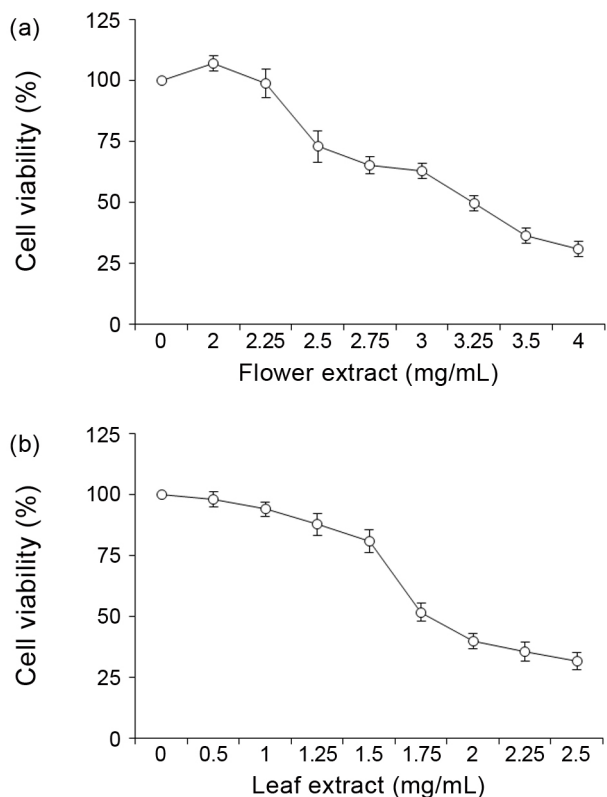
### 3.2. *T. turcica* extracts induce DNA fragmentation and expression of caspase genes

HeLa cells exposed to  $IC_{50}$  of FE and LE for 24 h exhibited DNA laddering patterns in the agarose gels, indicating apoptotic DNA fragmentation (Figure 2). In contrast, no DNA ladder was observed in DMSO treated control.

The effect of FE and LE on the expression levels of caspase-8 (*CASP8*) and caspase-9 (*CASP9*) genes as well as the executioner caspase-3 (*CASP3*) is shown in Figure 3. The elevated transcript abundance of caspase genes was found in HeLa cells treated with  $IC_{50}$  of crude extracts. The expression level of *CASP8* was found higher as compared to *CASP9*. Additionally, a significant increase in the expression level of *CASP3* occurred in treated cells compared to untreated cells.

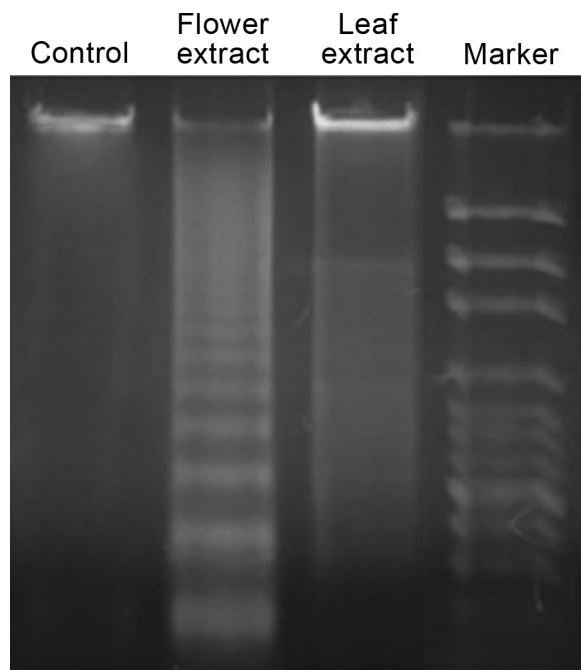
### 3.3. Proteomic analysis of HeLa cells exposed to *T. turcica* extracts

To better characterize the effects of *T. turcica* flower and leaf extracts on cervical carcinoma (HeLa) cells, proteomic analysis was used to identify specific proteins related to apoptosis. The alterations in proteomes of FE- and LE-



**Figure 1.** Effects of the flower (a) and leaf (b) extracts of *T. turcica* on the viability of HeLa cells.

treated HeLa cells were analyzed by 2-DE (Figure 4). Afterimage analyses, a total of 27 proteins were identified by MALDI-TOF/TOF MS analysis and MASCOT database searches (Table). The identified proteins have different biological functions including cytoskeleton organization (25.9%), protein metabolism (25.9%), chaperone activity (11.1%), energy metabolism (7.4%), and gene regulation (7.4%). Other categories are involved in chromatin regulator (3.7%), redox regulation (3.7%), apoptosis (3.7%), signal transduction (3.7%), primer metabolism (3.7%), and mitochondria metabolism (3.7%). Protein profiling revealed 16 up-regulated proteins and 11 down-regulated proteins in FE- and/or LE-treated HeLa cells. Two proteins, namely proteasome subunit beta type-7 (PSMB7, spot 8) and tubulin beta chain (TUBB, spot 16), were up-regulated in only LE-treated HeLa cells, while 4 proteins, including inorganic pyrophosphatase (PPA1, spot 11), suppressor of G2 allele of SKP1 homolog (spot 17), and actin 1 (ACTB, spots 20 and 21), were up-regulated in only FE-treated cells. Additionally, peroxiredoxin-2 (PRDX2, spot 2), adapter molecule crk (CRK, spot 3), chloride intracellular channel protein 1 (CLIC1, spot 9), tubulin beta chain (TUBB, spot 10), protein disulfide-isomerase A3 (PDIA3, spot 13), eukaryotic translation

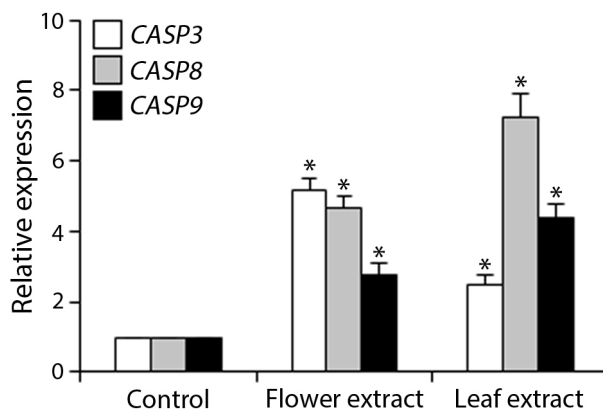


**Figure 2.** DNA fragmentation of HeLa cells exposed to flower and leaf extracts of *T. turcica*.

initiation factor 3 subunit I (EIF3I, spot 14), stomatin-like protein 2 (STOML2, spot 18), 40S ribosomal protein SA (RPSA, spot 19), actin 2 (ACTG1, spot 22), and protein disulfide isomerase (P4HB, spot 26) proteins were up-regulated in both FE- and LE-treated cells. However, Rho GDP-dissociation inhibitor 1 (RhoGDI, spot 4), heat shock protein beta-1 (HSBP1, spot 5), calpain small subunit 1 (CAPNS1, spot 3), heat shock cognate 71 kDa protein (HSPA8, spot 7), L-lactate dehydrogenase B chain (LDHB, spot 12), heterogeneous nuclear ribonucleoproteins C1/C2 (HNRNPC, spot 15) and F (HNRNPF, spot 23), tubulin alpha-1B chain (TUBA1B, spot 24), ATP synthase subunit beta mitochondrial (ATP5B, spot 25), and 60 kDa heat shock protein mitochondrial (HSPD1, spot 27) proteins were down-regulated in both FE- and LE-treated cells. One protein, namely chromobox protein homolog 3 (CBX3, spot 1), was down-regulated in only LE-treated cells. Furthermore, an interaction network of 27 differentially expressed proteins was constructed using STRING analysis (Figure 5).

#### 4. Discussion

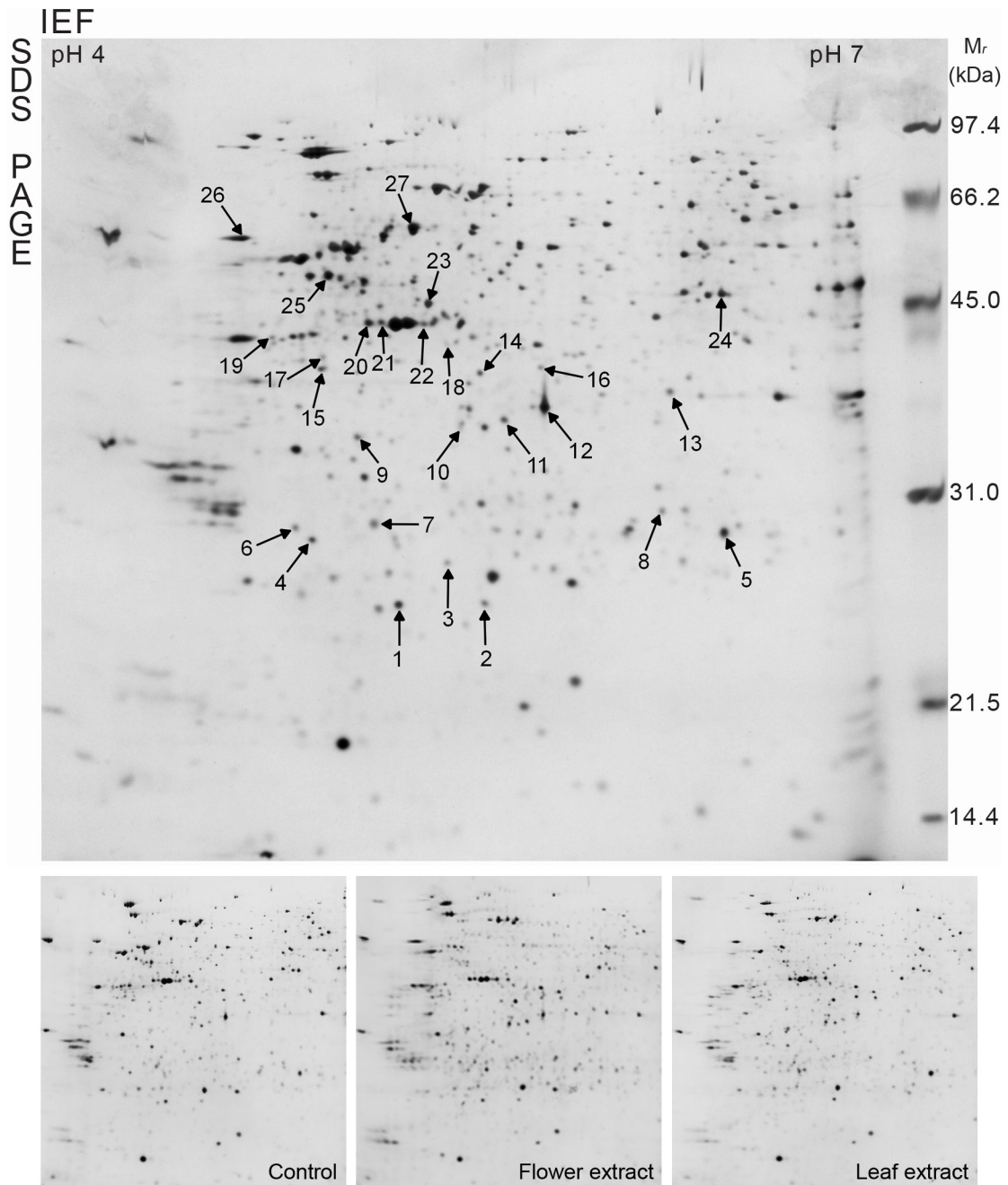
Natural products from plants with a broad range of biological activity potential can be a source of effective anticancer drugs [21]. Although a few studies reported the cytotoxicity of *T. turcica* extracts on some cancer types [11,12], there has been no research on the cytotoxic effects of different extracts in human cervical cancer. The



**Figure 3.** Gene expression levels of caspase genes in HeLa cells treated with  $IC_{50}$  of *T. turcica* extracts for 24 h. The asterisk (\*) indicates significant differences as compared to control ( $P < 0.05$ ).

results of the present study showed a significant inhibition of proliferation in *T. turcica* leaf and flower extracts, associated with induction of apoptosis as measured by DNA fragmentation and up-regulation of apoptotic genes. *T. turcica* is a rich source of natural products such as secondary metabolites [10]. Antitumor activities of plant-derived metabolites have been attributed to the plant secondary metabolites [4,22,23]. The present study suggests that these biologically active compounds of *T. turcica* could be responsible for its antiproliferative activity in HeLa cells.

Apoptosis is tightly regulated by extrinsic and intrinsic pathways. Caspase-8 is the initiator protease in the extrinsic pathway while caspase-9 is in the intrinsic pathway. These proteases promote the activity of the cascade of the effector caspases such as caspase-3 [24]. In the present study, crude extracts of *T. turcica* increased the gene expression of caspase-8, -9, and -3 in HeLa cells. Among the initiator caspases, the increase in the expression level of caspase-8 was more pronounced in an  $IC_{50}$  concentration. Caspase-3 is involved in the execution of the apoptotic pathway and activated by numerous death signals initiated by mitochondrial and nonmitochondrial pathways [25]. DNA fragmentation is largely mediated by the family of cysteinyl aspartate-specific protease caspases. The active form of caspase-3 protein occurs by post-translational cleavage [26]. Although the level of caspase-3 expression in the leaf extract is higher than the control, no apparent DNA fragmentation has been observed. This situation may be related to providing caspase-3 activity by the cleaved form of the protein. Additionally, proteomic analysis showed the down-regulation of Rho GDP-dissociation inhibitor 1 (RhoGDI) protein. RhoGDI is a major regulator of the activities of Rho GTPases and



**Figure 4.** Representative 2-DE gel of differentially expressed protein in HeLa cells treated with flower and leaf extracts of *T. turcica* for 24 h. Total proteins were separated by 17 cm IPG strips (pH 4–7), following by 12% SDS-PAGE and silver staining.

increases cancer cell survival under drug treatments by inhibiting the degradation of GTPase via caspases [27]. In the present study, reduced RhoGDI may induce apoptosis with the increased caspase-3 in HeLa cells treated with *T. turcica* extracts. Additionally, we found down-regulation of calpain small subunit 1 protein by proteomic analysis. Calpain is a calcium-dependent cysteine protease family, and it is implicated in the apoptotic pathway through

interaction with caspases [28]. Atencio et al. [29] reported that inactivation of calpain in various cancer cells led to p53-dependent apoptosis, cell cycle arrest, and activation of caspase including caspase-8 and -9. It has been also shown that calpain inactivates caspase-9 by the degradation of enzymes [30]. Taken together, opposite expression of caspase genes and calpain in treated HeLa cells may be related to decreased cell proliferation.

**Table.** Differentially expressed 27 proteins identified by MALDI-TOF/TOF mass spectrometry in HeLa cells exposed to leaf (LE) and flower (FE) extracts of *T. turcica*.

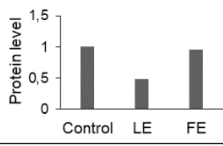
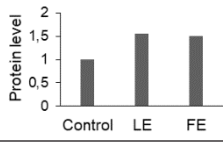
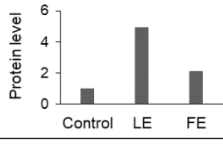
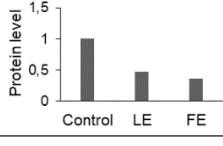
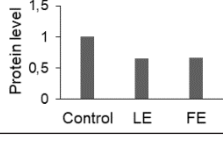
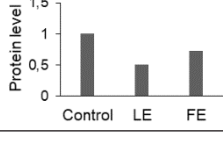
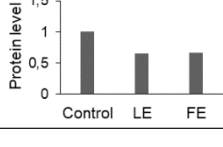
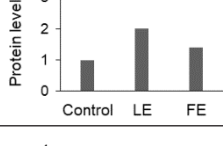
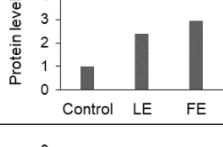
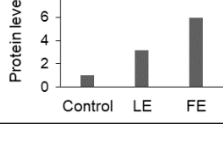
Spot <sup>a</sup>	Accession number <sup>b</sup>	Protein <sup>c</sup>	Score <sup>d</sup>	MW pI <sup>e</sup>	Cover. <sup>f</sup>	MP <sup>g</sup>	Protein level <sup>h</sup>
1	CBX3_HUMAN	Chromobox protein homolog 3	316	20.8 5.23	46%	19	
2	PRDX2_HUMAN	Peroxiredoxin-2	366	21.9 5.66	59%	22	
3	CRK_HUMAN	Adapter molecule crk	87	33.8 5.38	37%	15	
4	GDIR1_HUMAN	Rho GDP-dissociation inhibitor 1	532	23.2 5.02	47%	29	
5	HSPB1_HUMAN	Heat shock protein beta-1	452	22.8 5.98	37%	27	
6	CPNS1_HUMAN	Calpain small subunit 1	189	28.3 5.05	35%	21	
7	HSP7C_HUMAN	Heat shock cognate 71 kDa protein	543	70.9 5.37	33%	33	
8	PSB7_HUMAN	Proteasome subunit beta type-7	92	29.9 7.57	21%	14	
9	CLIC1_HUMAN	Chloride intracellular channel protein 1	527	26.9 5.09	63%	26	
10	TBB5_HUMAN	Tubulin beta chain	385	49.6 4.78	38%	32	

Table. (Continued).

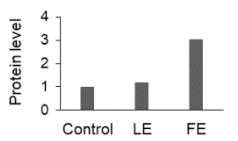
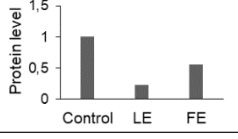
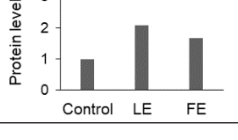
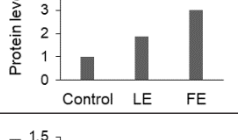
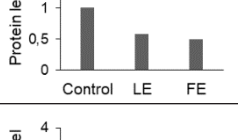
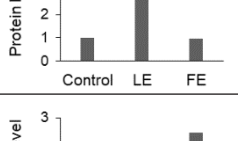
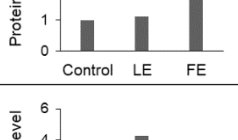
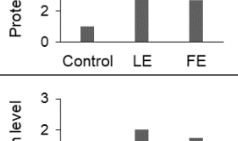
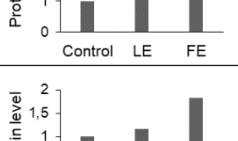
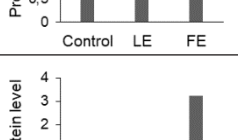

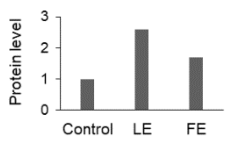
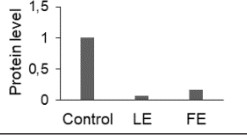
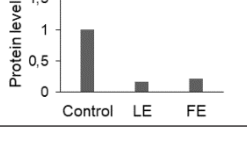
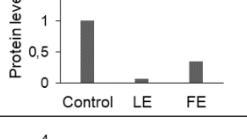
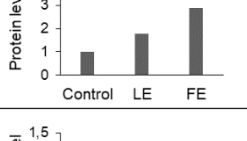
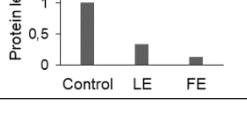
11	IPYR_HUMAN	Inorganic pyrophosphatase	389	32.6 5.53	54%	22	
12	LDHB_HUMAN	L-lactate dehydrogenase B chain	277	36.6 5.71	33%	20	
13	PDIA3_HUMAN	Protein disulfide-isomerase A3	686	56.7 5.98	54%	40	
14	EIF3I_HUMAN	Eukaryotic translation initiation factor 3 subunit I	371	36.5 5.38	63%	28	
15	HNRPC_HUMAN	Heterogeneous nuclear ribonucleoproteins C1/C2	363	33.7 4.95	48%	26	
16	TBB5_HUMAN	Tubulin beta chain	280	49.6 4.78	39%	34	
17	SUGT1_HUMAN	Suppressor of G2 allele of SKP1 homolog	155	40.9 5.07	35%	17	
18	STML2_HUMAN	Stomatin-like protein 2	281	38.8 5.50	30%	33	
19	RSSA_HUMAN	40S ribosomal protein SA	478	32.8 4.79	58%	28	
20	ACTB_HUMAN	Actin, cytoplasmic 1	468	41.7 5.29	56%	28	
21	ACTB_HUMAN	Actin, cytoplasmic 1	270	41.7 5.29	45%	22	

Table. (Continued).

22	ACTG_HUMAN	Actin, cytoplasmic 2	447	41.8 5.31	56%	26	
23	HNRPF_HUMAN	Heterogeneous nuclear ribonucleoprotein F	328	45.6 5.38	45%	28	
24	TBA1B_HUMAN	Tubulin alpha-1B chain	616	50.1 4.94	41%	27	
25	ATPB_HUMAN	ATP synthase subunit beta Mitochondrial	779	56.5 5.26	53%	39	
26	PDIA1_HUMAN	Protein disulfide-isomerase	369	57.1 4.76	41%	26	
27	CH60_HUMAN	60 kDa heat shock protein Mitochondrial	665	61.0 5.70	41%	33	

<sup>a</sup> Represents the spot number shown in 2-D gels (Figure 4).

<sup>b</sup> Refers to the accession number according to the MASCOT search against the SwissProt database.

<sup>c</sup> Refers to the protein name according to the MASCOT search against the SwissProt database.

<sup>d</sup> Refers to the probability score for protein identification based on MS analysis and MASCOT search.

<sup>e</sup> Refers to the molecular weight (MW, kDa) and isoelectric point (pI) for the protein identified.

<sup>f</sup> Refers to the percentage of the matching amino acid sequence.

<sup>g</sup> Refers to the number of matching peptide (MP).

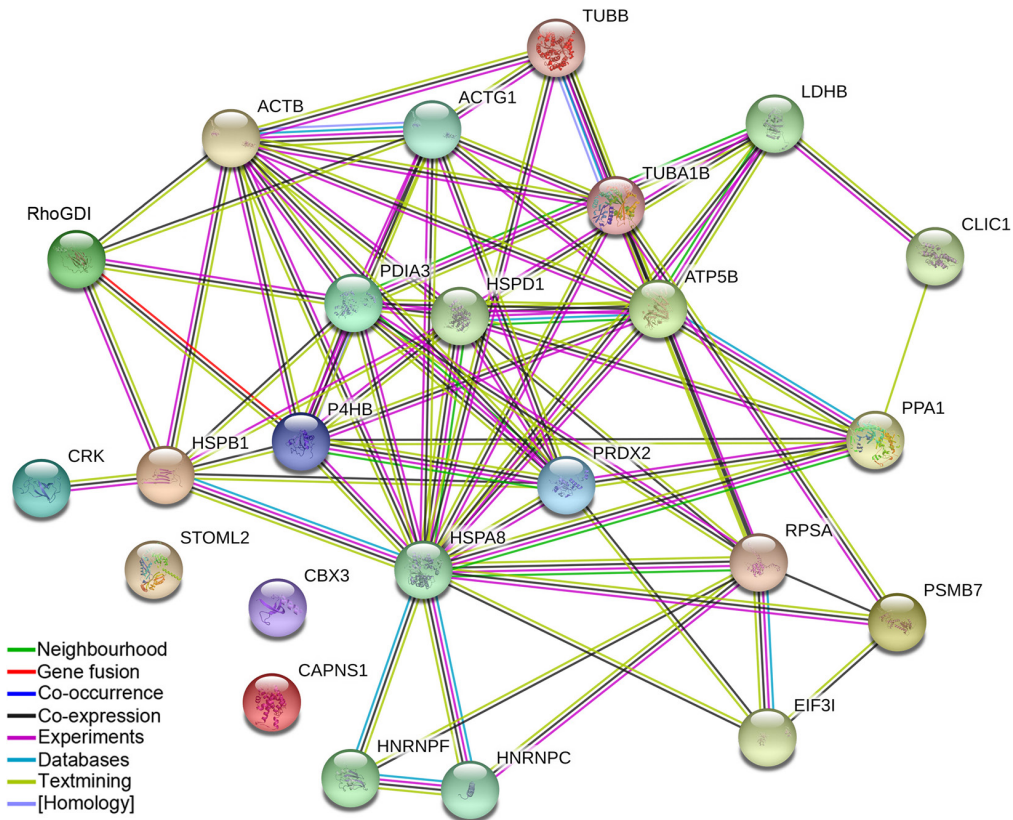
<sup>h</sup> Refers to changes in protein abundance.

Chromobox protein homolog 3 (CBX3) has been involved in the regulation of gene transcription, and it is upregulated in many cancer types [31,32]. It is also suggested that CBX3 plays a crucial role in the progression of tumors [33]. CBX3 promotes cell proliferation in colon cancer and tongue squamous cell carcinoma by suppressing the p21 activity [31,34]. It is also stated that CBX3 mediates the recruitment of NIPBL (Nipped-B-like protein) to sites of DNA damage [35]. In our study, the abundance of CBX3 protein was down-regulated in LE-treated HeLa cells. This result suggests that leaf extract harms DNA repair mechanisms and cell proliferation in HeLa cells by suppressing the CBX3 protein.

Heat shock proteins (HSPs) function as chaperones that help the abnormal proteins formed under stress conditions to fold properly [36]. HSPs are essential for cell metabolism and are considered as the protector of oncoprotein related to cancer proliferation and progression [37]. HSPs function as antiapoptotic proteins and high levels of HSPs have been associated with aggressiveness of several tumors [38,39]. The down-regulation of three HSP proteins (HSBP1, HSPA8, and HSPD1) may associate with a cytotoxic response of *T. turcica* extracts in HeLa cells.

Protein disulfide isomerase (PDI) family proteins catalyze the formation of disulfide bonds [40]. ER stress often results in increased regulation of chaperones, such





**Figure 5.** Protein interaction network for the differentially expressed proteins generated by STRING 9.1.

as the PDI family, which serves as an important cellular defense against abnormal proteins [41]. Liu et al. [42] reported that the constant ER stress triggers the signaling switch from pro-survival to pro-apoptosis by the release of PDIs (PDIA1 and PDIA3) from the ER lumen to induce Bak-dependent mitochondrial outer membrane permeabilization. In our study, leaf and flower extracts of *T. turcica* up-regulated the expression levels of PDI proteins (PDIA1 and PDIA3) as well as proteasome subunit beta type-7. This result shows that *T. turcica* extracts can induce the Bak-dependent apoptotic pathway by releasing the PDIs from the ER lumen to the cytosol.

Heterogeneous nuclear ribonucleoprotein (HNRNP) has been reported to promote the expression of c-Myc and Bag-1 proteins [43]. Down-regulation of c-Myc is associated with the stimulation of apoptosis in some cancer cells [44,45]. It is also reported that suppression of c-Myc expression led to G2/M arrest in different cell types [46, 47]. In our study, the lower abundance of HNRNP (HNRNPC and HNRNPF) proteins may be responsible for the cell cycle inhibiting and apoptotic effects of *T. turcica* extracts by inhibiting the expression of c-Myc.

Stomatin-like protein 2 (STOML2) is a major component of the inner membrane of mitochondria [48]. Previous studies revealed the prognostic role of STOML2

protein in different cancer types including cervical cancer [49,50]. Suppression of STOML2 in esophageal cancer cells reduced the growth rate and cell attachment [51]. In the present study, the expression level of STOML2 protein was up-regulated in HeLa cells treated with *T. turcica* extracts. However, further studies are necessary for the determination of the mechanism of STOML2 protein in HeLa cells.

Under hypoxia, the pyruvate is converted to lactate in cancer cells by lactate dehydrogenase (LDHB) [52]. The expression of LDHB was significantly increased in some kinds of tumors [53,54]. The down-regulation of LDHB protein in *T. turcica* treated HeLa cells may suggest inhibition of cell proliferation. Since  $\text{NAD}^+$  is required for glycolysis, inhibition of LDHB could lead to cellular energy collapse and subsequent cell death.

In conclusion, the methanolic extracts of leaves and flowers of *T. turcica* cause cytotoxic effects in HeLa cancer cell lines. Gene expression levels of caspase-3, -8, and -9 proteases which play a role in apoptosis were significantly increased in HeLa cell lines treated with *T. turcica* extracts. This is the first comparative proteomic research providing molecular insights about the bioactivity of *T. turcica* extracts in HeLa cells. The expression levels of 27 proteins were regulated differently in HeLa cells treated with *T. turcica*

extracts. It can be suggested that different levels of proteins such as HSPs, PDI, RhoGDI, CBX3, and HNRNP can contribute to cell cycle inhibiting and apoptotic effects of *T. turcica* extracts. The results of this research have provided valuable proteomic information about the anticancer activity of *T. turcica* extracts in human cervical cancer.

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