Proteomic Analysis of *Terminalia chebula* Extract-Dependent Changes in Human Lymphoblastic T Cell Protein Expression

Nando Dulal Das,¹ Kyoung Hwa Jung,¹ Ji Hyun Park,¹ Mi Ran Choi,¹ Hyung Tae Lee,¹
Moo Sung Kim,² Sang Rin Lee,² and Young Gyu Chai¹

¹Division of Molecular & Life Science, Hanyang University, Ansan, Korea.

²MacroCare Company, Ochang, Korea.

ABSTRACT *Terminalia chebula* is a native plant from southern Asia to southwestern China that is used in traditional medicine for the treatment of malignant tumors and diabetes. This plant also has antibacterial and immunomodulatory properties. The present study assessed *T. chebula* extract-dependent protein expression changes in Jurkat cells. Matrix-assisted laser desorption/ionization–time-of-flight mass spectrometry and Ingenuity Pathways Analysis (IPA) were performed to assess protein expression and networks, respectively. A comparative proteomic profile was determined in *T. chebula* extract (50 µg/mL)-treated and control cells; the expressions of β -tubulin, ring finger and CHY zinc finger domain containing 1, and insulin-like growth factor 1 receptor kinase were significantly down-regulated in *T. chebula* extract-treated Jurkat cells. Moreover, the molecular basis for the *T. chebula* extract-dependent protein expression changes in Jurkat cells was determined by IPA. Treatment with the *T. chebula* extract significantly inhibited nuclear factor- κ B activity and affected the proteomic profile of Jurkat cells. The molecular network signatures and functional proteomics obtained in this study may facilitate the evaluation of potential antitumor therapeutic targets and elucidate the molecular mechanism of *T. chebula* extract-dependent effects in Jurkat cells.

KEY WORDS: • gallic acid • Ingenuity Pathways Analysis • Jurkat cells • nuclear factor- κ B • *Terminalia chebula* extract

INTRODUCTION

TERMINALIA CHEBULA is a medicinal plant native mainly to tropical regions.¹ It has traditionally been used as an alternative medicine to treat cancers. *T. chebula* extract also exhibits a variety of biological functions, including anticancer, antidiabetic, and antibacterial activities.^{2,3} Recently, it was reported that chebulagic acid isolated from *T. chebula* extract inhibits cyclooxygenase and 5-lipoxygenase, key enzymes involved in inflammation and carcinogenesis.⁴

In this study, we used Jurkat cells, an immortalized T lymphocyte cell line. Jurkat cells have been used to determine the molecular mechanism of action of anticancer drugs and radiation.⁵ Our primary objective was to compare the protein expression profile of Jurkat cells treated with *T. chebula* extract with that of control cells. Proteomic techniques have emerged as a powerful tool to associate the broad-spectrum protein expression with specific cellular responses. In our study, a comparative protein expression signature was established for Jurkat cells treated with *T. chebula* extract alone or combined with tumor necrosis factor α (TNF α).

Recently, we reported the inhibitory effect of gallic acid from *T. chebula* extract on nuclear factor- κ B (NF- κ B) activity in Jurkat cells.⁶ NF- κ B, a transcription factor that is considered a master regulator of inflammation, is a promising drug target because it regulates the expression of inflammatory cytokines, adhesion molecules, and other proteins in several cancers. Most NF- κ B inhibitors act by compromising NF- κ B interaction with target DNA. Many antioxidants inhibit NF- κ B by reducing the phosphorylation of inhibitory I κ B.⁷ We reported that *T. chebula* extract containing gallic acid can inhibit NF- κ B activity and down-regulates interleukin-8 and monocyte chemotactic protein-1 transcription in Jurkat-NF- κ B- β -lactamase (bla) cells.⁶ It is interesting that the molecular network characterized by Ingenuity Pathways Analysis (IPA) analysis showed that *T. chebula* extract treatment affected the protein degradation, amino acid metabolism, and behavior networks, whereas NF- κ B and mitogen-activated protein kinase 1 (extracellular signal-regulated kinase/mitogen-activated protein kinase) linked to Akt, protein kinase C isoforms, insulin, and phosphoinositide 3-kinase, whose expressions were not up-regulated. This suggests that the *T. chebula* extract containing gallic acid is a direct, potent inhibitor of NF- κ B and can reduce the NF- κ B-dependent activity in Jurkat cells.

In this study, we used proteomic techniques to identify novel molecular targets of the gallic acid fractionated

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Address correspondence to: Young Gyu Chai, Ph.D., Division of Molecular & Life Science, Hanyang University, 1271 Sa 3-dong, Ansan, Gyeonggi-do, 426-791, Korea, E-mail: ygchai@hanyang.ac.kr

T. chebula extract in Jurkat cells. Jurkat cells are used for studies of various biological phenomena, such as apoptosis and cell engulfment.^{8,9} In particular, we detected differential expression of proteins, including β -tubulin, ring finger and CHY zinc finger domain containing 1 (RCHY1), and insulin-like growth factor 1 receptor kinase (IGF1R) in *T. chebula* extract-treated Jurkat cells. Overall, the effects suggest that the components of *T. chebula* extract including gallic acid may be a novel drug candidate that has possible research and clinical value.

MATERIALS AND METHODS

Acquisition of T. chebula extract and reagents

Hot water (at 60°C for 4 h) *T. chebula* extract solution (10 mg/mL) was obtained from Sunten Pharmaceutical Co. (Taipei, Taiwan). Isolation and identification of gallic acid were described in our previous article.⁶ In brief, gallic acid was isolated from *T. chebula* extract by reversed-phase high-performance liquid chromatography (C₁₈, YMC-ODS-A, 250 × 10 mm, i.d.) with a solvent system of 15% acetonitrile in water at a flow rate of 1.5 mL/min. The purity of gallic acid so obtained was greater than 98.0% by high-performance liquid chromatography. Mouse recombinant TNF α was acquired from Sigma-Aldrich (USA). Cell culture medium, RPMI 1640 medium, fetal bovine serum, and penicillin–streptomycin were purchased from Gibco Life Technologies (UK).

Cell culture and treatments

Jurkat cells were obtained from the American Type Culture Collection (Manassas, VA, USA). Cell cultures were maintained at subconfluence in a 95% air/5% CO₂ humidified atmosphere at 37°C in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin–streptomycin. Initial screening studies were performed to determine the concentration of *T. chebula* extract. Here, *T. chebula* extract (50 μ g/mL) and TNF α (10 ng/mL), a 5-h treatment was used for all experiments.

Transfection and selection of cell line

Jurkat cells were transfected following the microporation procedure (iNCYTO, Korea) using the pLenti-bsd/NF- κ B-bla CellSensor™ vector (catalog number K1128, Invitrogen). The total procedure was described previously.⁶

Protein sample preparation and two-dimensional gel electrophoresis

Protein sample preparation and two-dimensional gel electrophoresis (2DE) were performed on the whole-cell lysate protein fraction using our previously published methods.¹⁰ In brief, IPG dry strips (pH 4.0–7.0, 7 cm, Bio-Rad Laboratories, Hercules, CA, USA) were rehydrated for 12–16 h with 9.5 M urea containing 2% CHAPS and 40 mM dithiothreitol and loaded with 200 μ g of the sample. Isoelectric focusing and stepwise electrophoresis were per-

formed. Gel electrophoresis was performed in an Ettan DALTsix electrophoresis unit (GE Healthcare) at a constant 3 W per gel at 15°C until the blue dye exited the bottom of the gel. Next, the 2DE gels were stained with Coomassie Brilliant Blue dye. The resulting spots were imaged, and densitometry was performed using PDQuest software (Bio-Rad Laboratories). We performed three independent 2DE analyses and found similar gel images for individual experiment. The relative densitometry of each band was normalized to protein volume to determine the relative expression of each protein spot.

In-gel digestion

Protein spots were enzymatically digested in-gel in a manner similar to that previously described,¹¹ using modified porcine trypsin (Promega, Madison, WI, USA). Gel pieces were washed with 50% acetonitrile to remove sodium dodecyl sulfate, salt and Coomassie stain. After that, gel pieces were dried to remove the solvent followed to rehydration with trypsin (8–10 ng/ μ L) and incubated for 8–10 h at 37°C. The proteolytic reaction was terminated by adding 5 μ L of 0.5% trifluoroacetic acid. Tryptic peptides were recovered by extraction with 50% aqueous acetonitrile, and the aqueous-phase extractions of replicate gel pieces were combined. An aliquot of this solution was mixed with an equal volume of a saturated solution of α -cyano-4-hydroxycinnamic acid in 50% aqueous acetonitrile, and 1 μ L of the mixture was spotted on the target plate.

Matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry and database analysis

Peptide analysis was performed using an Ultraflex matrix-assisted laser desorption/ionization (MALDI)–time-of-flight (TOF) mass spectrometer (Bruker Daltonics, Bremen, Germany). In brief, peptides were evaporated using an N₂ laser (337 nm) in a delayed extraction approach and were accelerated with a 20-kV injection pulse for duration of flight analysis. Each spectrum represented the cumulative average of 300 laser shots. The ProFound search program was developed at Rockefeller University (<http://prowl.rockefeller.edu/prowl-cgi/profound.exe>) and was used for protein identification employing a Bayesian algorithm by peptide mass fingerprinting data. The presence of signal peptide is considered when such information is available in the corresponding National Center for Biotechnology Information GenPept format flatfile (www.ncbi.nlm.nih.gov/Entrez/batch.html). The following input parameters (mass tolerance ± 1 ; iodoacetamide modifications for amino acid residues) were applied. Spectra were internally calibrated using trypsin autodigestion ion peaks (m/z 842.510 and 2211.1046).

Western blotting assay

Western blotting was performed according to standard procedures.⁶ In brief, equal amounts of protein from whole-cell lysates (10 μ g) were resolved by electrophoresis on 12%

polyacrylamide gels. Proteins were then transferred to polyvinylidene difluoride membranes by electroblotting using the immersion method followed by blocking with 5% skimmed milk in 1% Tris-buffered saline containing Tween for 1 h and incubating overnight with primary antibodies at 4°C. After washing, the membranes were incubated for 1 h with the secondary antibodies. Blots were visualized with enhanced chemiluminescence (ECLTM Plus kit, GE Healthcare). The antibodies used were Rchy1/Pirh2 (sc-166293) and heat shock 70-kDa protein (HSP70) (sc-1060) from Santa Cruz Biotechnology Inc.

Canonical pathway analysis of datasets

IPA (Ingenuity[®] Systems [www.ingenuity.com], Mountain View, CA, USA) was conducted to determine the most significant canonical pathways in the datasets. Detailed methods have been described previously.¹² In brief, genes from the dataset were considered for literature review. The significance of the association between the dataset and the canonical pathway was measured in two ways: (1) the ratio of the number of genes from the dataset that map to a given canonical pathway was divided by the total number of genes that map to the same canonical pathway; and (2) Fisher's exact test was used to calculate a *P* value to determine the probability that the association between the genes in the dataset and the canonical pathway could be explained by chance alone. After the datasets were uploaded, each gene identifier was mapped to its corresponding gene object in the IPA Knowledge Base, and these genes were overlaid onto a global molecular network. Gene networks were then algorithmically generated based on their connectivity.

Graphical representation of networks/pathways

Genes or gene products are represented as nodes, and the biological relationship between two nodes is represented as an edge (line). All edges are supported by the reference literature, from the canonical information stored in the IPA Knowledge Base. The intensity of the node color indicates the degree of up-regulation (red) or down-regulation (green).

RESULTS

The protein expression profiles of Jurkat-NF- κ B-bla cells treated with the *T. chebula* extract and the combination of *T. chebula* extract and TNF α were analyzed. In our previous study, we reported that *T. chebula* extract (50 μ g/mL) inhibited NF- κ B activity and strongly suppressed inhibitory I κ B α phosphorylation in Jurkat-NF- κ B-bla cells.⁶ Cells treated with *T. chebula* extract (50 μ g/mL) for 5 h and incubated with *T. chebula* extract exhibited 100% viability in comparison with untreated cells (data not shown). Therefore, this extract had a minimal effect on Jurkat-NF- κ B-bla cell viability, although effects on cell viability were observed at higher doses (100–200 μ g/mL) (data not shown). Proteins that showed changes more than twofold from three independent experiments were selected and analyzed sub-

sequently by MALDI-TOF mass spectrometry (MS) using trypsin. We compared the protein expression pattern of Jurkat-NF- κ B-bla cells with or without extract treatment. Based on the ProFound database results, we identified protein expression that was up-regulated and down-regulated in Jurkat-NF- κ B-bla cells.

Effects of *T. chebula* extract treatment

The effects of *T. chebula* extract treatment on protein expression were analyzed. The expressions of many proteins were up-regulated in *T. chebula* extract-treated Jurkat-NF- κ B-bla cells, such as cell growth regulator with EF-hand domain 1, ankyrin repeat and MYND domain containing 2, KIAA0518, DOCK2, 14-3-3 proteins, and tropomyosin 4. β -Tubulin, RCHY1, IGF1R, and human Dudp pyrophosphatase complex with Dudp, TBC1 domain family, member 13 were among the proteins with reduced expression (Table 1 and Supplementary Fig. S1A; Supplementary Data are available online at www.liebertonline.com/jmf), and the decreases were significant for β -tubulin, RCHY1, and IGF1R. β -Tubulin is necessary for cellular component movement, microtubule-based movement, spindle assembly, and protein polymerization. Therefore, *T. chebula* extract treatment may negatively affect Jurkat-NF- κ B-bla cells.

Effects of *T. chebula* extract treatment in combination with TNF α

To analyze the *T. chebula* extract effect in the presence of an inducer, we treated the cells with a combination of *T. chebula* extract and TNF α . The effects of *T. chebula* extract treatment in combination with TNF α are listed in Table 2. Indolethylamine *N*-methyltransferase, apolipoprotein B-100 precursor (APOB), C14orf21, ring finger protein 20, and R32184 expressions were significantly up-regulated. In addition, APOB expression was increased in treated cells. APO-B100 is a low-density lipoprotein that is synthesized exclusively in the liver. It modulates the inflammatory I κ B kinase–NF- κ B signaling cascade and the secretion of apolipoprotein B-100-containing lipoproteins.¹³ Proteins of the R32184/WDR18 family are involved in a variety of cellular processes, including cell cycle progression, signal transduction, apoptosis, and gene regulation. Among the proteins with decreased expression after treatment with *T. chebula* extract and TNF α , changes in expression of HSP70 8 isoform 1 (HSPA8), putative DNA binding protein, and polyribonucleotide nucleotidyltransferase 1 were significant (Supplementary Fig. S1B).

Comparative network analysis of drug effects using IPA software

IPA analysis was used to identify gene networks, canonical pathways, and diseases (biomarkers). This network analysis can provide primary information about physical connectivity and functional relationships between proteins. Comparative analysis of untreated Jurkat-NF- κ B-bla cells

TABLE 1. EFFECTS OF *T. CHEBULA* EXTRACT TREATMENT ON PROTEIN EXPRESSION IN JURKAT-NUCLEAR FACTOR- κ B- β -LACTAMASE CELLS

<i>Protein</i>	<i>Spot number</i>	<i>NC</i>	<i>TC</i>	<i>Ratio</i>	<i>Est'd Z</i>	<i>Accession number</i>
DOCK2 protein	605	1	5193	5193.00	0.34	gi54261667
Bip protein	2407	1	3219	3219.00	2.03	gi6470150
Transmembrane and coiled-coil domains 4	1307	1	2853	2853.00	0.96	gi31565770
Tropomyosin 4 isoform 2	2212	1	2584	2584.00	2.43	gi4507651
β 4 spectrin isoform σ 2	3606	1	2195	2195.00	0.58	gi11602887
Lactamase, β 2	7113	1	2145	2145.00	0.22	gi7705793
Cell growth regulator with EF-hand domain 1	111	1	2061	2061.00	2.43	gi21961322
14-3-3n	1106	1	1408	1408.00	1.33	gi437363
Chain A, cryogenic crystal structure of human myeloperoxidase isoform C	1105	1	1406	1406.00	0.94	gi7766941
NLR5 protein	302	1	1313	1313.00	0.79	gi30048125
Chain A, Cryogenic crystal structure of human co-chaperone P23	1107	1	1225	1225.00	1.34	gi9257073
Transmembrane protein 74	1108	1	1056	1056.00	0.99	gi23308545
Ankyrin repeat and MYND domain containing 2	205	1	930	930.00	2.43	gi28461129
Laminin B1	2108	1	703	703.00	0.39	gi186915
KIAA0518 protein	407	1	560	560.00	1.17	gi3043560
Sarcomeric tropomyosin κ	1104	702	1	0.00	2.43	gi48660012
LOH12CR1	6108	1515	1	0.00	0.28	gi15072483
Chain A, human Dudp pyrophosphatase complex with Dudp	8108	1620	1	0.00	2.43	gi34810576
Chain A, structure of insulin-like growth factor 1 receptor kinase	7101	1627	1	0.00	0.17	gi762941
Ring finger and CHY zinc finger domain containing 1	6202	1840	1	0.00	1.28	gi28703891
v-crk sarcoma virus CT10 oncogene homolog (avian)-like	7302	2060	1	0.00	2.3	gi4885153
HDCMC28P	7401	2189	1	0.00	0.22	gi7643778
TBC1 domain family, member13	4106	2215	1	0.00	2.43	gi55960036
Tubulin, β	1511	7678	1	0.00	1.42	gi55961564

Est'd Z, estimated Z score as a measure of confidence for Profound-based identification; NC, negative (untreated control); TC, *T. chebula* extract (50 μ g/mL)-treated.

TABLE 2. EFFECTS OF *T. CHEBULA* EXTRACT TREATMENT IN COMBINATION WITH TUMOR NECROSIS FACTOR α ON PROTEIN EXPRESSION IN JURKAT-NUCLEAR FACTOR- κ B- β -LACTAMASE CELLS

<i>Protein</i>	<i>Spot number</i>	<i>NC</i>	<i>TC + TNFα</i>	<i>Ratio</i>	<i>Est'd Z</i>	<i>Accession number</i>
R32184_1	8113	1	6439	6439.00	2.43	gi3025445
Indolethylamine <i>N</i> -methyltransferase	3107	1	1952	1952.00	2.43	gi6580817
Ring finger protein 20	6612	1	1143	1143.00	0.56	gi55665535
Apolipoprotein B100 precursor	5619	1	865	865.00	1.23	gi178792
C14orf21 protein	5512	1	817	817.00	0.44	gi19263717
Laminin B2	306	990	8012	8.09	1.05	gi34238
Hypothetical protein	4403	1454	5051	3.47	1.24	gi57999530
Kinesin light chain 1P	8107	1209	3936	3.26	1.4	gi30409772
p37 AUF1	8106	2900	9373	3.23	0.98	gi433344
Pre-B-cell leukemia homeobox 3 isoform 1	1306	734	1757	2.39	2.43	gi5453852
KIAA1125 protein	6601	1444	1044	0.72	1.17	gi6329749
Mitogen-activated protein kinase kinase 5 isoform B	2217	17,904	1770	0.10	2.43	gi4506101
Phosphoribosylaminoimidazole carboxylase, phosphoribosylaminoimidazole succinocarboxamide synthetase isoform 2	6211	542	1	0.00	1.35	gi5453539
FLJ10305	7309	600	1	0.00	0.58	gi48146803
Truncated tenascin XB	6208	709	1	0.00	2.43	gi6448761
Heat shock 70-kDa protein 8 isoform 1	6411	724	1	0.00	1.36	gi5729877
Solute carrier family 24 (sodium/potassium/calcium exchanger), member 1	6606	735	1	0.00	1.17	gi4759128
EH domain containing 4	6409	976	1	0.00	0.16	gi7212811
DDX60 protein	2206	1284	1	0.00	2.43	gi50959706
COG4 protein	2410	1553	1	0.00	0.66	gi39645057
PNPT1 protein	1206	1576	1	0.00	2.43	gi34785309
Putative DNA binding protein	3311	2137	1	0.00	0.66	gi15706422
Immunoglobulin heavy chain	6109	2608	1	0.00	0.23	gi4096428
Chain A, binary complex of human type-I IMP dehydrogenase with 6-Cl-Imp	7206	9278	1	0.00	1.32	gi33357127

6-Cl-IMP, 6-chloropurine riboside 5'-monophosphate; COG4, component of oligomeric Golgi complex 4; PNPT1, polyribonucleotide nucleotidyltransferase 1.

TABLE 3. INTERACTIVE MOLECULES OF NETWORKS THAT HAD THE TOP SCORES IN JURKAT-NUCLEAR FACTOR- κ B- β -LACTAMASE CELLS TREATED WITH *T. CHEBULA* EXTRACT AND TUMOR NECROSIS FACTOR α , ALONE AND IN COMBINATION

Analysis	Molecules in network	Score	Focus molecules	Top functions
NC versus TNF α +TC	APOB, ATR (includes EG:545), BCL6, C4ORF14, CBR3, COG4, CUL4B, EPO, FIG4, GAB1, GRWD1, HNF4A, HSPA8, MAP2K5, MAP3K, MAP3K2, NFKB1, ONECUT1, PACSIN3, PPP1CA, RFWD2 (includes EG:64326), RNF20, SCFD1, SLC24A1, SON, SQSTM1, STARD7, SYNE1, TNS1, TUBB6, VAC14, VCL, WDR18, WDR51B, ZMYND8	41	17	Immunological disease, inflammatory disease, carbohydrate metabolism
NC versus TC	ACTB, Akt, BIN1, BIRC5, CALR, CORO1B, CRK-CRKL-CBL, CRKL, DOCK2, GAB1/2, GCG, GRB2, Histone h3, Hsp90, HSP90B1, HSPA5, IFIT2, insulin, LDL, Lh, Met dimer, MT3, Ntrk1 dimer, PI3K, PIK3AP1, Pkc(s), Pld, RIN3, SCARF2, SHROOM2, TUBA1B, TUBB, YWHAD, YWHAG, YWHAH	32	14	Protein degradation, amino acid metabolism, behavior

APOB, apolipoprotein B-100 precursor; ATR, ataxia telangiectasia and Rad3 related; BCL6, B-cell CLL/lymphoma 6; BIN1, bridging integrator 1; CBR3, carbonyl reductase 3; COG4, component of oligomeric golgi complex 4; CRKL, v-crk sarcoma virus CT10 oncogene homolog; DOCK2, dedicator of cytokinesis 2; EPO, erythropoietin; GAB1, GRB2-associated binding protein 1; GRB2, growth factor receptor-bound protein 2; HNF4A, hepatocyte nuclear factor 4A; HSPA8, heat shock 70-kDa protein 8 isoform 1; Hsp90/HSP90, heat shock 90-kDa protein; LDL, low-density lipoprotein; MAPK, mitogen-activated protein kinase; NFKB1, nuclear factor- κ B 1; PI3K, phosphoinositide 3-kinase; TUBB, β -tubulin; WDR18, WD repeat domain 18; ZMYND8, zinc finger, MYND-type containing 8.

with cells treated with the *T. chebula* extract and TNF α combination is shown in Table 3. The top-scoring gene networks identified in the negative control and *T. chebula* extract-treated cells where interactive genes involved in protein degradation, amino acid metabolism, and behavior

(Fig. 1). For the negative control and *T. chebula*/TNF α combined treatment, the top-scoring proteins are involved in molecular networks of immunological disease, inflammatory disease, and carbohydrate metabolism (Supplementary Fig. S2). β -Tubulin expression was higher in the negative

FIG. 1. Molecular network of protein degradation, amino acid metabolism, and behavior for NC-treated cells in comparison with TC extract-treated cells. The direct (solid line) and indirect (broken line) interaction of both up-regulated (red) or down-regulated (green) genes associated with nuclear factor- κ B (NF- κ B) signaling were identified in Jurkat-NF- κ B-bla cells. This dataset was analyzed using Ingenuity Pathway Analysis. The node color is indicative of gene expression, and the color intensity is proportional to the gene expression fold change. ERK, extracellular signal-regulated kinase. Color images available online at www.liebertonline.com/jmf

FIG. 2. TC extract inhibited the expression of ring finger and CHY zinc finger domain containing 1 (RCHY1) and heat shock 70-kDa protein (HSP70). Cells were incubated for 5 h. The gallic acid fractionated TC extract (50 μg/mL) and combined treatment with TC extract and TNF α inhibits RCHY1/Pirh2 and HSP70 expression markedly than the untreated control and only TNF α . β -Actin expression was detected as a control. Data shown are representative of three independent experiments.

control-treated cells than in *T. chebula*-treated cells. We focused on the NF- κ B and extracellular signal-regulated kinase/mitogen-activated protein kinase signaling molecules in a network where the expression of Akt, protein kinase C isoforms, insulin, and phosphoinositide 3-kinase was not increased.

T. chebula extract component gallic acid inhibits RCHY1/Pirh2 and HSP70 in Jurkat cells

To validate the protein expression, we applied the gallic acid fraction of the *T. chebula* extract where the reduced expression of RCHY1/Pirh2 and HSP70 was found in Jurkat cells. The gallic acid fractionated *T. chebula* extract and combined treatment (5 h) with TNF α reduced the RCHY1/Pirh2 and HSP70 band compared to untreated control and only TNF α treatment, respectively (Fig. 2).

DISCUSSION

Total protein of the cells was extracted after treatment. *T. chebula* extract inhibited NF- κ B activity in Jurkat cells in a dose-dependent manner. After 2DE and MALDI-TOF-MS, proteins were identified in drug-treated Jurkat-NF- κ B-bla cells. The protein expression in cells treated with the *T. chebula* extract and TNF α in combination was compared with the negative control. For the *T. chebula* extract treatment, we focused on proteins whose expressions were down-regulated to determine novel protein drug targets in lymphoma cells. Among the proteins with down-regulated expression, the expression of β -tubulin, RCHY1, and IGF1R was significant (Table 1). β -Tubulin is necessary for cellular component movement and cell motility. Suppression of β -tubulin inhibits Jurkat cell proliferation and survival. We determined the down-regulation of RCHY1, which is a ubiquitin-protein E3 ligase and promotes p53 degradation. The *RCHY1* gene encodes a RING-H2 domain-containing protein with intrinsic ubiquitin-protein ligase activity. It was reported that RCHY1 (Pirh2) physically interacts with p53 and promotes ubiquitination of p53, independent of Mdm2.¹⁴ Furthermore, RCHY1 represses p53 activity, including p53-dependent transactivation and growth inhibi-

tion. RCHY1 is overexpressed in both human and murine lung cancers as determined by Pirh2 mRNA and protein expression in lung neoplastic tissues and is uninvolved with adjacent lung tissue. Increased RCHY1 expression may degrade wild-type p53 and reduce its tumor suppression function in tumor cells.¹⁵ Regarding these findings, RCHY1 may be a potential target of the *T. chebula* extract. The expression of IGF1R was down-regulated in *T. chebula* extract-treated cells. IGF1R triggers a cascade of intracellular signaling via tyrosine autophosphorylation and promotes cell survival and proliferation.¹⁶ Previous studies have implicated IGF1R in several cancers, including breast and prostate cancer.¹⁷ In some scenarios, its anti-apoptotic properties allow cancerous cells to resist the cytotoxicity of chemotherapeutic drug treatment and radiotherapy. IGF1R expression is increased in the majority of primary and metastatic prostate cancers.¹⁸ Therefore, *T. chebula* extract-dependent reduction of IGF1R expression may represent a beneficial anticancer activity.

In addition, DOCK2 expression was up-regulated in *T. chebula* extract-treated Jurkat-NF- κ B-bla cells. The *DOCK2* gene encodes a hematopoietic cell-specific CDM family protein that is indispensable for Rac-dependent lymphocyte chemotaxis.¹⁹ The expression of proteins associated with the KIAA0518 protein complex and proteins encoded by the *MGA/MAX* gene was also increased. MLL1, ASH2L, HCFC1/HCF1 WDR5, and RBBP5 are components of the KIAA0518 protein complex that methylates lysine-4 of histone H3 and activates gene transcription.²⁰ The protein expression of two key components of this complex were up-regulated by *T. chebula* extract treatment.

In cells treated with a combination of the *T. chebula* extract and TNF α , the expression of HSPA8 was most significantly down-regulated. Previous studies have shown that HSP70 is present on the surface of many cancer cells, monocytes, and umbilical vein endothelial cell lines.^{21–23} Flow cytometric analysis confirmed the presence of HSP70 and HSPA8 on the surface of leukemic cancer cell lines.²⁴ The *T. chebula* extract-dependent suppression of HSPA8 expression in TNF α -treated Jurkat cells may represent a novel therapeutic approach to treat lymphoma. We also validated a decreased HSP70 expression in Jurkat cells treated with *T. chebula* extract and with combined treatment with *T. chebula* extract and TNF α (Fig. 2).

IPA analysis of protein expression in negative control cells and cells treated with the *T. chebula* extract determined that the proteins with the top scores are involved in protein degradation, amino acid metabolism, and behavior (Fig. 1). The NF- κ B and extracellular signal-regulated kinase/mitogen-activated protein kinase signaling pathways in this network were linked to Akt, protein kinase C isoforms, insulin, and phosphoinositide 3-kinase, whose expressions were not up-regulated. This suggests that treatment with the *T. chebula* extract can also inhibit NF- κ B as we reported in our previous study.⁶ A previous study reported that a *T. chebula* extract compound, chebulagic acid, also inhibits NF- κ B activity.²⁵

Overall, proteomic MALDI-TOF-MS and IPA analysis uncovered key proteins and signaling networks that may be responsible for the inhibitory effects of *T. chebula* extract treatment in Jurkat cells. Our results suggest that the *T. chebula* extract treatment mainly suppresses the expression of β -tubulin, RCHY1, IGF1R, and HSP70, which may negatively affect lymphoma cells. IPA analysis identified interacting proteins that were differentially expressed in *T. chebula* extract-treated Jurkat cells and suppressed NF- κ B signaling. Moreover, our study suggests that the *T. chebula* extract component gallic acid can inhibit RCHY1 and HSP70 in Jurkat cells, potentiating the anticancer effect of *T. chebula* extract. Future studies are necessary to identify the potential drug targets of other active compounds from *T. chebula* extract.

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AUTHOR DISCLOSURE STATEMENT

M.S.K. and S.L.L. are employees of Macro/care Co. The remaining authors have declared that no competing interests exist.

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