

Exploring the mode of action of the hydroalcoholic extract of *Balanites aegyptiaca* (L.) Del, (Ingudi) on cancer cell lines using proteomic tools

Shridhar Chougule

Regional Ayurveda Institute for Fundamental Research

Shraddha Bharsakale

Regional Ayurveda Institute for Fundamental Research

Amey Shirolkar

Regional Ayurveda Institute for Fundamental Research

Sangeeta Sangvikar

Regional Ayurveda Institute for Fundamental Research

Anagha Ranade

Regional Ayurveda Institute for Fundamental Research

Sudesh Gaidhani

Central Council for Research in Ayurvedic Sciences (CCRAS), Institutional Area, Opp. 'D' Block

Shruti Khanduri

Central Council for Research in Ayurvedic Sciences (CCRAS), Institutional Area, Opp. 'D' Block

Rajesh Gacche

Savitribai Phule Pune University

N. Srikanth

Central Council for Research in Ayurvedic Sciences (CCRAS), Institutional Area, Opp. 'D' Block

Sharad Pawar

sd_pawar@yahoo.com

Central Council for Research in Ayurvedic Sciences (CCRAS), Institutional Area, Opp. 'D' Block

Research Article

Keywords: *Balanites aegyptiaca* (L.) Del, anticancer, cell-based assays, proteomics, RRLC-ESI-QTOFMS, apoptosis

Posted Date: June 15th, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-9662468/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Additional Declarations:

No competing interests reported.

Table 1 is available in the Supplementary Files section.

Exploring the mode of action of the hydroalcoholic extract of *Balanites aegyptiaca* (L.) Del, (Ingudi) on cancer cell lines using proteomic tools.

Shridhar Chougule^{1,3}, Shraddha Bharsakale¹, Amey Shirolkar¹, Sangeeta Sangvikar¹, Anagha Ranade¹, Sudesh Gaidhani², Shruti Khanduri², Rajesh Gacche³, N. Srikanth², Sharad Pawar^{1*}

¹Regional Ayurveda Institute for Fundamental Research (CCRAS, New Delhi under Ministry of AYUSH, Govt. of India), Nehru Garden, Gandhi Bhavan, Kothrud, Pune-411038.

²Central Council for Research in Ayurvedic Sciences (CCRAS), Institutional Area, Opp. 'D' Block, Janakpuri, New Delhi - 110058, India.

³Department of Biotechnology, Savitribai Phule Pune University, Pune-411007, Maharashtra, India.

*Author for correspondence:

1. Dr Sharad Pawar

Scientist, Dept. of Pharmacology, RAIFR, Pune

email: sd_pawar@yahoo.com

ABSTRACT

Numerous plants are documented in ancient Ayurvedic literature to possess anticancer properties, yet their mode of pharmacological action is not completely understood. The present study explores the course of action of the hydroalcoholic extract of *Balanites aegyptiaca* (L.) Del. (Ingudi) on cancer cell lines using cell-based assays and proteomic tools.

Treatment of multiple cell lines (MCF-7, Chang Liver, HeLa, RIN-5F, and mesenchymal stem cells) resulted in a dose-dependent decline in cell viability. The IC₅₀ values were approximately 30 µg/mL for most cancer cell lines, whereas HeLa cells exhibited lower sensitivity (60 µg/mL). Induction of programmed cell death was validated by DNA laddering and increased caspase activity, confirming apoptosis as the primary mode of action.

Proteomic analysis of HeLa cells revealed substantial alterations in protein expression patterns following HI extract treatment. A large proportion of proteins associated with cytoskeletal architecture, extracellular matrix organization, and adhesion signalling were reduced. Enrichment analyses indicated suppression of pathways related to focal adhesion and actin cytoskeleton regulation. In contrast, proteins involved in RNA processing, DNA repair, and intracellular transport showed increased expression, suggesting activation of compensatory stress responses.

Network-based analysis highlighted central regulatory proteins, including AKT1, SRC, FN1, and CD44, indicating disruption of survival and adhesion-associated signalling pathways. Comparison with camptothecin-treated cells revealed distinct molecular responses.

Overall, the findings indicate that *B. aegyptiaca* extract induces apoptosis and extensive proteomic remodelling, supporting its potential as a source of anticancer agents.

Keywords: *Balanites aegyptiaca* (L.) Del, anticancer, cell-based assays, proteomics, RRLC-ESI-QTOFMS, apoptosis

INTRODUCTION:

Cancer is a multifactorial disease, distinguished by unregulated cell division and proliferation. The onset and development of cancer are linked to the cumulative effects of various aberrant genetic and epigenetic events that interfere with orderly processes. Cellular anomalies such as insufficient apoptosis, alterations in oncogenes, tumour suppressor genes, and microRNA further

correlate with cancer progression (Abdul et al. 2009; Burstein and Schwartz 2008; Giri et al. 2006; Mbaveng et al. 2011). Heterogeneity is a fundamental characteristic of cancer. These differences manifest at genetic, epigenetic, and phenotypic levels, both between tumour types and within the same tumour. Hence, to overcome heterogeneity and genetic diversity, the use of different cancer cell lines in cytotoxicity assays becomes mandatory. Also, every cell line exhibits unique molecular characteristics, making it crucial to test compounds across multiple lines to assess their broader efficacy and potential clinical relevance (Gillet et al., 2013). Drugs display varied responses across cancer types (Barretina et al. 2012; Dagogo-Jack & Shaw 2018; Dienstmann et al. 2013; Garnett et al. 2012; Garraway et al. 2012; Iorio et al. 2016). For example, a compound that is effective in lung cancer cells may not work in liver cancer cells. Thus, the use of multiple cell lines facilitates the screening of cancer types that respond to treatment. If a drug is more effective in specific cell lines with particular mutations or gene expression patterns, it may lead to the identification of biomarkers for patient stratification in clinical trials. Moreover, studies have highlighted that selecting an appropriate cell line that closely resembles the genomic alterations of specific cancer subtypes is key in improving translational relevance (Garnett et al. 2012).

In the Ayurvedic classic, cancer and cancer-like conditions are described under *Arbuda* (tumours) and *Granthi* (abnormal swellings). Traditionally, various plants have been utilized for their medicinal properties, such as tumour-suppressing, immune-enhancing, and detoxifying. Modern research supports many of these claims by identifying bioactive compounds with anticancer potential.

Among the various plants reported to possess anticancer potential, *Balanites aegyptiaca* (L.) Del., belonging to the family Zygophyllaceae/Balanitaceae, was selected for screening. It is widely used in traditional African and Middle Eastern medicine for tumour treatment. It is a thorny, evergreen, multibranched tree widely distributed throughout the drier parts of India, Israel, Arabia, Africa, Pakistan, the Middle East and South Asia (Ambasta 1986; Hall and Walker 1991; Hardman and Sofowora 1972). *B. aegyptiaca* is commonly known as Hinganbet or Hingota, in Ayurveda, it is known as Ingudi (Kirtikar and Basu 1933; Kumar et al. 2014). Its use is mentioned in the treatment of *Granthi*, *Gulma* and *Vrana* (Anonymous 2016). Various parts of *B. aegyptiaca* have been traditionally used for diverse therapeutic applications, including hepatoprotection, anti-inflammatory and antimicrobial activity, antidiabetic effects, deworming effects, and wound healing, as supported by ethnobotanical surveys and pharmacological studies (Kirtikar and Basu 1918; Ambasta 1986; Al Ashaal et al. 2010; Yadav and Panghal 2010;

Chothani and Vaghasiya 2011; Kamel et al. 2011; Mbaveng et al. 2011; Elfeel and Warrag 2011; Abdallah 2012; Abdel-Farid et al. 2014; Mohamed et al. 2015).

Saponins and other bioactives from *Balanites aegyptiaca* exhibit cytotoxicity against various cancer cell lines, including breast and liver cancer cell lines (Abdelwahab et al. 2011). Antioxidant and anti-inflammatory properties contribute to its anticancer potential (Mbaveng et al. 2011). In the current study, the anticancer potential of *B. aegyptiaca* has been assessed using cell-based assays and proteomic tools.

MATERIALS AND METHODS:

Media, chemicals, Cell lines and Plant material:

Minimal Essential Medium MEM (Himedia), 0.25% trypsin and 0.01% EDTA (Himedia), RPMI-1640 (Himedia), HiMesoXL Mesenchymal Stem Cell Expansion Medium (Himedia), Fetal Bovine Serum (MP-Biomedicals), Multi caspases assay kit (Merck Millipore), Apoptotic DNA ladder kit (Abchem) of cell culture grade were procured. Cell culture plasticwares (Tissue culture flasks, plates, and serological pipettes) were procured from Eppendorf. Four cell lines, MCF7, Chang Liver, HeLa, and RIN5F, were obtained from NCCS (National Centre of Cell Science, Pune), whereas the mesenchymal Stem cells (MSC) cell line was procured from HIMEDIA. *B. aegyptiaca* stem bark was collected from Ahmednagar, Maharashtra, India. An in-house taxonomist authenticated plant material vide specimen No. 232.

Extract preparation:

The gathered stem bark was shade-dried at room temperature prior to grinding it to a coarse powder. The 25 gm of powdered material was used to make the hydro-alcoholic extract as per the guidelines mentioned in the API (Anonymous 2016). The prepared extract was applied to Whatman filter paper No.1; the filtrate was lyophilized (for obtaining dried extract powder) (Labconco). The yield obtained was 2.548 gm. A code **HI** derived from **Hinganbet**, one of the common names of *B. aegyptiaca*, has been used in all further assays.

In vitro Cytotoxicity assay:

A 96-well plate was seeded with cells (7.5×10^3 cells/well for Chang Liver, 8×10^3 cells/well for HeLa, 1×10^4 cells/well for MCF-7, 3.5×10^4 cells/well for RIN-5f and 1×10^4 cells/well for MSC). The seeded cells were treated for 24 hours with HI extract at various concentrations ranging from 20 to 200 $\mu\text{g/mL}$. The cytotoxicity was assessed based on the capability of live and active cells to metabolize yellow coloured MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] into formazan crystals (purple colored) via mitochondrial NAD(P)H-dependent oxidoreductase enzymes (Stockert et al. 2018; Stockert et al. 2012). The readings were recorded

at 550 nm (after multiple scanning experiments, the absorbance maximum was determined to be at 550 nm) using an ELISA plate reader (Tecan-EPOCH).

DNA fragmentation assay:

The DNA fragmentation or Ladder formation assay is a key method for distinguishing apoptosis (programmed cell death) from necrosis. In apoptosis, endonucleases cleave chromatin DNA into oligonucleosomal fragments (180–200 bp and multiples thereof), forming a characteristic ladder pattern when analyzed by agarose gel electrophoresis (Elmore 2007). Apoptotic DNA fragmentation occurs due to the activation of caspase-dependent DNases, leading to Oligonucleosomal fragmentation (Li and Yuan 2008; Nagata 2018). When analyzed via agarose gel electrophoresis, DNA smearing occurs in necrotic cell death due to random DNA degradation (Susin et al. 1999), whereas ladder formation occurs in apoptotic cell death.

The cells were seeded (at a cell density set according to the surface area requirement) into a 6-well plate. Different treatment concentrations of the HI extract (60 µg/mL for HeLa and 30 µg/mL for Chang Liver, MCF7, RIN-5f, and MSC cell lines) and a standard drug (4 µM & 7 µM Camptothecin) were applied, and an untreated control was maintained. The cells were harvested post 24 hours treatment using 0.25% Trypsin EDTA solution (Li and Yuan 2008). The cells were then harvested and processed for extracting DNA using an Apoptotic DNA ladder kit (Invitrogen). The images were acquired using the Gel Doc system (BioRad).

Caspase assay:

A Multi-Caspase Assay is utilized for detecting the triggering of multiple caspases during apoptosis and programmed cell death. Caspases are a family of cysteine proteases classified into Initiator caspases (caspase-8 and 9) that trigger apoptosis, and Effector caspases (caspase-3, 6, & 7) that execute apoptosis by cleaving cellular proteins (Elmore 2007). The multi-caspases assay was conducted for detecting the activation of caspase and ultimately apoptosis (Matassov et al. 2004; McIlwain et al. 2015). The assay procedure was as per the Muse™ Multi Caspase Kit (Cat. No. MCH100109) manual. The samples were prepared as follows for the RIN-5f cell line: untreated control, standard drug (7 µM Camptothecin), and HI extract (30 µg/mL). For Chang Liver, MCF-7, and MSC cell lines, 2 samples were prepared, namely, Untreated control and HI extract (30 µg/mL). For the HeLa cell line, untreated control and HI extract (60 µg/mL) were prepared.

Protein Extraction:

The HeLa cells (passage 93; H93N) were harvested from 6-well plates (seeding density was 3×10^5 cells /well) after 24 hours of treatment (Control, CPT 3 µM, and 34 µg/mL HI extract) using 0.25% Trypsin-EDTA solution. The cell pellet obtained after centrifugation (1500 rpm for

5 minutes) was washed with PBS to remove any residual media, then pelleted again. Protein extraction was done using the Methanol: Chloroform method (Fic et al. 2010). In brief, the cell pellet was resuspended in fresh PBS, vortexed or pipetted to ensure uniform suspension. Methanol addition (4:1), 4 volumes of methanol to the cell suspension (e.g., 100 μ L sample + 400 μ L MeOH) and vortex for 5-10 seconds. Chloroform addition (1:1), 1 volume of chloroform (e.g., 100 μ L) and vortex for 5 seconds. Water addition (3:1): 3 volumes of water (e.g., 300 μ L) and vortex and centrifuge at $14,000 \times g$ for 10 minutes at 4°C. Without disturbing the protein pellet, the upper aqueous phase (which contains metabolites and nucleic acids) was removed meticulously. 300 μ L methanol was added, vortexed briefly, and centrifuged at $14,000 \times g$ for 5 minutes. The obtained pellet was air-dried for 5-10 minutes, and the supernatant was disposed of. The dried pellet was solubilized using an 8 M Urea solution for further use.

Protein digestion:

Proteins were estimated using the Bradford method (Kruger 2009). With certain adjustments, the protein digestion procedure followed Promega's Technical Bulletin TB373. After precipitating the 100 μ g protein, the pellet was allowed to air dry for three to five minutes. and dissolved using ProteaseMAXTM Surfactant (0.1% in 50 mM NH_4HCO_3). 50 mM NH_4HCO_3 was used to adjust the final volume to 93.5 μ L. After adding 1 μ L of 0.5 M DTT, the mixture was incubated for 20 minutes at 56°C. to break disulfide bonds. After adding 2.7 μ L of 0.55 M iodoacetamide, the mixture was incubated for 15 minutes at room temperature in the dark to alkylate proteins decreased cysteine residues. After adding 1 μ L of trypsin (1 μ g/ μ L), the mixture was incubated at 37°C overnight. Samples were incubated and then kept at -20°C until additional analysis (Chougule et al. 2025).

Liquid Chromatography and Mass Spectrometry (LC-MS):

LC-MS analysis was conducted using Agilent 1290 Infinity Series RRLC coupled to Agilent 6545B AccurateMass Q-TOF MS. 10 μ L volumes of sample solutions were introduced into a C18 ZORBAX 300SB, 5 μ M (4.6 x 150 mm) column maintained at 40°C, and the autosampler was set at 5°C. The solvent system consisted of organic (0.1% Formic acid Acetonitrile) and aqueous (0.1% Formic acid water) in a 95:5 proportion. The solvent B (organic) was applied in gradient mode; the used solvent scheme (%/time, minutes) was as follows: 2%/5; 50%/45; 98%/50; 98%/52.5; 2%/55; 2%/58. The flow rate used was 0.3 mL/min. Mass Spectrometry was conducted using ion mode, positive polarity. Other parameters included: dual ESI ionization; gas temp. 325 °C; 45 psi nebulizer pressure; 10.0 L/min gas flow rate; 3500 V capillary voltage; 1000 V nozzle voltage; Fragmentor voltage 175V; Mass range for data acquisition was set to 100-3200m/z.

Calibration runs were done using BSA. The reverse-phase chromatographic separation was performed using a 5 μ M particle-size C18 column, with acetonitrile: water as mobile phase, and the sample run time was 60 minutes in positive mode. The mass range 100-3200 Da was selected for MS1, 100-3200 Da range for MS2. Samples were duplicated (Chougule et al. 2025; Gaikwad et al. 2020).

Peptide identification:

The differentially expressed proteins were identified using Spectrum Mill MS Proteomics Workbench software (Rev B.06.00.201 from Agilent Technologies). This software compares the experimental masses of tryptic peptides to the theoretical masses of peptides from the proteins listed in protein databases. $M+H^+$ 600–60,000 m/z , extraction time 0-60 minutes, the selected charge state range 2 to 4, max ambiguity in precursor charge 3, precursor mass tolerance ± 1.0 Da, product mass tolerance ± 0.5 Da, maximum allowed missed cleavages 2, length of sequence tag >7 , and maximum reported hits 5 per spectrum. The False Discovery Rate (FDR) in Spectrum Mill auto-validation was set at 1.2% (Chougule et al. 2025).

Bioinformatics and Data Analysis

Following protein identification using Spectrum Mill software, downstream bioinformatics analyses were performed to characterize differential proteomic profiles between experimental groups. Protein identifiers were mapped to gene symbols using the UniProt ID mapping tool (UniProt Consortium 2023).

Comparative analysis across control and treatment groups was carried out using normalized quantitative values. Differentially expressed proteins were identified based on $\log_2$ fold change thresholds and statistical significance criteria. Overlap and unique protein distributions among groups were visualized using Venny (Oliveros 2007-2015).

Heat maps were generated based on $\log_2$ fold change values to visualize global expression patterns and clustering of differentially expressed proteins across experimental conditions. Statistical significance for enrichment analyses was inferred using adjusted p -values (Benjamini–Hochberg correction) (Benjamini and Hochberg 1995), with $p < 0.05$ considered significant.

Functional enrichment analyses were conducted to find out the biological significance of differentially expressed proteins. Pathway enrichment analysis was performed using KEGG databases (Kanehisa and Goto 2000) through DAVID (Huang et al. 2009; Sherman et al. 2021) and Enrichr (Kuleshov et al. 2016), while Gene Ontology (GO) enrichment analysis (Aleksander et al. 2025) was used to classify proteins into biological processes, molecular functions, and cellular components. Enrichment results were visualized using SRplot (Tang et al. 2023).

Protein–protein interaction (PPI) networks were constructed using the STRING database (Szklarczyk 2021) with a high-confidence interaction score cutoff of 0.7, and only connected nodes were retained for further analysis. For visualization and topological analysis, the complex STRING networks were brought into Cytoscape (Shannon et al. 2003). Hub proteins were identified based on degree centrality. Functional modules (seed proteins) were identified using the clustering algorithm of MCODE (Molecular Complex Detection) (Bader and Hogue 2003) to detect densely connected sub-networks. For clarity, only hub proteins and MCODE-derived seed proteins were visualized in the final network representations.

RESULTS:

Cytotoxicity assay:

The cytotoxicity of *B. aegyptiaca* (Hingenbet-HI) was evaluated across five cell lines (MCF-7, MSC, Chang Liver, HeLa, and RIN-5F) in three biological replicates ($n = 3$). The cytotoxicity values for Chang Liver, MCF-7, RIN-5F, and MSC ranged between 20 and 40 $\mu\text{g/mL}$, whereas those against HeLa cells exhibited values between 40 and 80 $\mu\text{g/mL}$. Based on observations after expanding the respective scale, the IC_{50} was determined to be 30 $\mu\text{g/mL}$ for Chang Liver, MCF-7, RIN-5F, and MSC cell lines, and 60 $\mu\text{g/mL}$ for HeLa cells. The results are presented in Figures 1-5.

DNA fragmentation assay:

DNA fragmentation analysis confirmed the induction of programmed cell death following HI-treatment, as DNA fragmentation is a hallmark of apoptosis. The assay was executed using Camptothecin (CPT) as a positive control (4 μM and 7 μM) and HI (30 $\mu\text{g/mL}$ for Chang Liver, MSC, RIN-5F, and MCF-7; 60 $\mu\text{g/mL}$ for HeLa). The result of the DNA fragmentation assay for HeLa cell line has been depicted in Figure 6 (results for Chang Liver, MSC, RIN-5F, and MCF-7 cell lines have been recorded but not shown here).

Multi-caspase analysis:

Caspases are a family of endoproteases that play a critical role in regulating inflammation and apoptosis. Multi-caspase analysis was conducted using HI (30 $\mu\text{g/mL}$ for Chang Liver, RIN-5F, MCF-7, and MSC; 60 $\mu\text{g/mL}$ for HeLa). Analysis using the *Muse™ Cell Analyzer* demonstrated activation of apoptotic pathways across all cell lines. Figure 7 depicts multicaspase activation for the HeLa cell line (results for rest four cell lines have not been shown here).

Peptide and Protein Identification:

Proteomic data acquisition and analysis were performed for HeLa cell line samples (Figure 8). The untreated control samples showed the highest protein

identification, with 1,807 proteins identified with ≥ 2 distinct peptides and a total of 5,968 proteins. In CPT-treated samples, 251 proteins (≥ 2 peptides) and 1,359 total proteins were identified, whereas HI-treated samples yielded 1,223 proteins (≥ 2 peptides) and 4,873 total proteins.

The number of proteins identified across all samples is shown in Table 1. All the proteins identified with ≥ 2 distinct peptides have been described in the Supplementary Table 1.

Bioinformatics and Data Analysis:

- Comparative analysis between study groups

Comparative proteomic analysis across control, CPT-treated, and HI-treated groups revealed both unique and shared protein populations (Figure 9). A total of 1,351 proteins were unique to the control group, 128 to CPT-treatment, and 796 to HI-treatment. Additionally, 405 proteins were shared between control and HI-treated samples, 101 between control and CPT-treated samples, and 72 between both treatment groups. Notably, 50 proteins were common across all three groups, representing a conserved proteomic core. These findings highlight both treatment-specific and shared proteomic signatures, suggesting that CPT and HI-treatments induce distinct and overlapping changes in the cellular proteome relative to the control condition.

- Differential Expression analysis

To further examine the global proteomic alterations among the study groups, hierarchical clustering was performed and visualized using a heat map (Figure 10).

Hierarchical clustering revealed a clear separation between control, CPT-treated, and HI-treated samples, highlighting distinct proteomic responses to each condition. While the heatmap reflects relative expression patterns, the extent of these changes was further examined through fold change analysis, with detailed values provided in Supplementary Table 2.

HI-treatment was found to selectively induce a small group of proteins. Among the most prominent were A0A494C071 (4.70) and Q5TBA9 (4.29), along with TNXB (3.03), supported by moderate increases in proteins such as Q8NEZ4, P46013, and Q8IZC6. This pattern suggests that HI does not broadly activate the proteome but instead triggers specific pathways, possibly linked to extracellular matrix remodelling and adaptive cellular responses.

At the same time, several proteins showed a clear reduction in abundance following HI-treatment. Notable among these were Q9BXP5 (−4.36), Q9NYQ7 (−3.92), RABGEF1 (−

2.47), and FLNA (-2.02), indicating suppression of proteins involved in cytoskeletal organization and intracellular regulation. Together, these changes point toward a coordinated but selective inhibitory effect of the plant extract on structural and signalling components.

In contrast, CPT-treatment produced a much broader and more pronounced response. A large number of proteins showed strong increases, including Q96P44 (11.84), P42694 (10.63), Q7Z3T8 (10.01), and several others consistently above 8-fold change. This widespread upregulation suggests a global distortion of cellular processes, consistent with the strong cytotoxic nature of CPT.

Interestingly, some proteins behaved differently under the two treatments. For example, Q14315 shifted from a decrease under CPT to an increase under HI, while Q9NYF8 and Q9NZJ4 showed clear induction with CPT but slight suppression with HI. These opposing trends indicate that the two treatments influence distinct molecular pathways rather than simply varying in intensity.

Notably, a smaller group of proteins showed consistent downregulation across both treatments, including Q9BXP5, Q9NYQ7, RABGEF1, and FLNA, suggesting that certain structural and regulatory processes are commonly affected, regardless of treatment type.

Overall, HI-treatment induced a proteomic profile dominated by widespread downregulation of cytoskeletal and regulatory proteins, accompanied by strong induction of a defined subset of extracellular and stress-responsive proteins, consistent with substantial cellular remodelling under HI conditions (Supplementary Table 2).

- Pathway analysis

To investigate pathways affected by HI and CPT-treatments, KEGG and Gene Ontology (GO) enrichment analyses were performed using DAVID and SRplot for both upregulated and downregulated proteins. 1470 proteins [1399 unique proteins to untreated + 71 proteins (out of 405 common between untreated and HI-treated) downregulated with $\log_2FC$ values ≤ -1] were significantly downregulated post HI-treatment, whereas 874 proteins [816 proteins unique to HI-treated + 58 proteins (out of 405 common proteins) upregulated with values ≥ 1] were significantly upregulated.

1715 proteins [1702 unique to untreated + 13 proteins (out of 101 common between untreated and CPT-treated) downregulated

with log2FC values ≤ -1] were significantly downregulated post CPT-treatment, whereas 174 proteins [150 proteins unique to CPT-treated + 24 proteins (out of 101 common proteins) upregulated with values ≥ 1] were significantly upregulated.

❖ Downregulated Proteins:

➤ KEGG enrichment analysis:

Pathway enrichment analysis of the downregulated proteins revealed significant enrichment in pathways primarily associated with cytoskeletal organization, cell adhesion, and extracellular matrix interactions (Figure 11). The most significantly enriched pathways included motor proteins ($p = 4.12 \times 10^{-6}$) and focal adhesion ($p = 4.15 \times 10^{-6}$), indicating suppression of proteins involved in cellular motility and adhesion (Supplementary Table 3). Additional enriched pathways such as cytoskeleton in muscle cells, ECM-receptor interaction, and integrin signalling further highlight the downregulation of proteins associated with cell structural integrity and cell-matrix communication. Pathways related to regulation of actin cytoskeleton, Hedgehog signalling, and cardiomyopathy-related pathways were also significantly represented, suggesting that treatment may influence cytoskeletal remodelling and signalling processes linked to cellular architecture and contractility.

Pathway enrichment analysis of proteins downregulated following CPT-treatment revealed significant suppression of cytoskeletal organization and cell-matrix interaction pathways (Figure 12). The most enriched pathway was Cytoskeleton in muscle cells, followed by Focal adhesion and ECM-receptor interaction, indicating disruption of integrin-mediated signalling and cytoskeletal integrity. Downregulation of Motor proteins further suggests impaired intracellular transport and cytoskeleton-dependent movement.

Additional enriched pathways, including Human papillomavirus infection, Amoebiasis, and Small cell lung cancer, reflect shared components involved in adhesion, signalling, and structural regulation rather than disease-specific processes. Modulation of the Hedgehog signalling pathway suggests potential effects on developmental signalling networks. Collectively, these findings indicate that CPT-treatment suppresses cytoskeletal dynamics, adhesion signalling, and

intracellular transport mechanisms, consistent with impaired cellular structure and reduced migratory potential.

➤ GO enrichment analysis:

Gene Ontology biological process enrichment analysis of proteins downregulated following HI-treatment revealed significant suppression of cytoskeleton- and adhesion-related processes (Figure 13). The most enriched terms included microtubule-based movement and regulation of small GTPase-mediated signal transduction, indicating disruption of cytoskeletal dynamics and Rho GTPase-driven signaling pathways. Processes such as cell–substrate adhesion, cell–substrate junction organization, and actomyosin structure organization were also significantly enriched, suggesting attenuation of integrin-mediated adhesion and actin–myosin contractility.

Additionally, enrichment of the regulation of cell morphogenesis and non-membrane-bounded organelle assembly points to impaired cellular structural organization and intracellular architecture. The downregulation of the establishment of organelle localization further indicates disruption of intracellular trafficking and spatial organization. Collectively, these findings suggest that HI-treatment suppresses cytoskeleton-dependent signalling, adhesion dynamics, and mechanotransduction pathways, potentially limiting cellular motility and invasive behaviour.

GO biological process enrichment analysis of proteins downregulated following CPT-treatment revealed significant suppression of extracellular matrix (ECM) organization and cytoskeletal signalling pathways (Figure 14). Highly enriched terms such as extracellular matrix organization, external encapsulating structure organization, and cell–substrate junction organization indicate disruption of cell–matrix interactions and adhesion complexes. Enrichment of microtubule-based movement and regulation of small GTPase-mediated signal transduction further suggests impairment of cytoskeletal dynamics and Rho GTPase-dependent signalling.

Notably, enrichment of double-strand break repair and DNA duplex unwinding indicates modulation of DNA damage response pathways, consistent with CPT-induced genotoxic stress. Collectively, these findings suggest that CPT-treatment suppresses ECM remodelling, cytoskeletal organization, and adhesion

signalling while concurrently affecting DNA repair mechanisms, reflecting combined structural and genomic stress responses.

❖ Upregulated Proteins

➤ KEGG enrichment analysis:

In contrast, pathway enrichment analysis of the upregulated proteins revealed enrichment in pathways associated with protein processing, epigenetic regulation, and cellular transport mechanisms. The most significantly enriched pathway was motor proteins ($p = 1.51 \times 10^{-7}$), followed by protein digestion and absorption and polycomb repressive complex, suggesting increased activity in protein turnover and chromatin regulatory mechanisms (Supplementary Table 4 and Figure 15). Other enriched pathways included the cytoskeleton in muscle cells, ABC transporters, the spliceosome, and lysine degradation, indicating enhanced involvement of proteins related to transport, RNA processing, and metabolic regulation. Additionally, pathways such as cobalamin transport and metabolism and renin secretion suggest potential alterations in metabolic and signalling pathways following treatment.

Pathway enrichment analysis of proteins upregulated following CPT-treatment revealed modest enrichment of pathways associated with cellular stress responses and metabolic regulation (Figure 16). The most prominent pathways included Amyotrophic lateral sclerosis and Pathways of neurodegeneration – multiple diseases, reflecting shared components involved in protein homeostasis, cytoskeletal regulation, and stress signalling rather than disease-specific processes. Enrichment of Arginine biosynthesis suggests alterations in amino acid metabolism, while pathways such as ABC transporters and Nicotine addiction likely represent shared signalling and transport-related components. Collectively, these findings indicate that CPT-treatment induces selective activation of stress-responsive and metabolic pathways, with comparatively lower enrichment and protein representation than the corresponding downregulated pathways.

➤ GO enrichment analysis:

GO biological process enrichment analysis of proteins upregulated following HI-treatment revealed significant activation of cytoskeleton-dependent transport and intracellular trafficking processes (Figure 17). Highly enriched terms included cytoskeleton-dependent intracellular transport, microtubule-based

movement, and microtubule-based transport, indicating enhanced microtubule-driven trafficking and motor protein activity. Enrichment of vesicle cytoskeletal trafficking and vesicle transport along actin filaments further suggests coordinated regulation of vesicular transport systems.

Additionally, processes such as the regulation of the mRNA metabolic process point to increased post-transcriptional regulatory activity, while terms related to morphogenesis likely reflect shared cytoskeletal and transport components rather than developmental processes per se. Collectively, these findings indicate that HI-treatment promotes intracellular transport, cytoskeletal dynamics, and vesicle-mediated trafficking, suggesting adaptive cellular reorganization and enhanced intracellular logistics.

GO biological process enrichment analysis of proteins upregulated following CPT-treatment revealed activation of processes related to protein synthesis, signalling, and vesicle dynamics (Figure 18). The most enriched term, translational elongation, indicates increased protein biosynthesis. Enrichment of regulation of GTPase activity and regulation of lipid kinase activity suggests modulation of intracellular signalling pathways, particularly those linked to small GTPase-mediated regulation.

Additionally, processes such as synaptic vesicle endosomal processing and synaptic vesicle recycling point to enhanced vesicle trafficking and membrane turnover. Other enriched terms, including bile acid biosynthetic process and vitamin D receptor signalling, likely reflect metabolic and signalling adaptations. Collectively, these findings indicate that CPT-treatment induces selective upregulation of protein synthesis, signalling pathways, and vesicle-mediated transport processes, consistent with an adaptive cellular response to treatment-induced stress.

❖ Protein–protein interaction (PPI) Network Analysis:

➤ PPI among downregulated proteins:

Protein–protein interaction (PPI) networks were constructed using STRING and visualized in Cytoscape to identify key regulatory proteins. The networks exhibited high connectivity, indicating coordinated functional relationships among differentially expressed proteins.

For HI-downregulated proteins, the network (Figure 19) revealed a central cluster of hub proteins, including AKT1, SRC, FN1, CD44, PIK3CD, ITGA7, and ITGB4, enriched in integrin-mediated signalling, PI3K/AKT signalling, and cytoskeletal organization. Seed proteins were distributed within core and peripheral modules, with proteins such as BRCA2, ITGA5, and UTP14A showing both hub and seed characteristics, suggesting key regulatory roles. Overall, the network indicates suppression of cell adhesion, cytoskeletal dynamics, and survival signalling.

In contrast, the CPT-downregulated network (Figure 20) displayed a more complex modular architecture. Distinct clusters included DNA replication and repair proteins (e.g., TOP2A, BRCA2, ATM), ribosomal proteins (e.g., RPS20, RPL13A), and epigenetic regulators (e.g., KMT2A, SETD2), alongside ECM and adhesion-associated proteins. These findings suggest coordinated inhibition of genomic maintenance, protein synthesis, transcriptional regulation, and cell–matrix signaling.

Collectively, HI-treatment primarily suppresses cytoskeletal and signalling networks, whereas CPT-treatment induces broader inhibition of nuclear and structural pathways.

➤ PPI among upregulated proteins:

PPI network analysis of proteins upregulated following HI-treatment revealed a moderately interconnected network (Figure 21) with distinct functional clusters. Hub proteins, including DHX9, WRN, SNRNP200, PRPF6, PRPF3, and NCBP1, formed the central backbone and were predominantly associated with RNA processing and spliceosomal machinery. A major cluster comprised RNA splicing factors such as SRSF4, SRSF7, RBMX, and DHX helicases, indicating enhanced post-transcriptional regulation. Concurrent enrichment of DNA damage response proteins, including ATR, FANCD2, FANCI, and WRN, suggests activation of genomic maintenance pathways. Additionally, a cluster of mitotic regulators (KIF20A, KIF4A, ESPL1, CENPA, SGO1) highlights increased cell cycle and chromosome segregation activity. Proteins exhibiting both hub and seed characteristics, such as SUPT5H, FANCI, ATP5F1A, and SUCLG2, likely function as key integrators across these processes. Overall, the network indicates

coordinated upregulation of RNA processing, DNA repair, and cell cycle pathways in response to HI-treatment.

In contrast, the PPI network of proteins upregulated following CPT-treatment was relatively sparse but functionally organized (Figure 22). A central module of ribosomal and translational proteins (RPL21, RPL29, EEF1G, GFM1) indicated activation of protein synthesis, with GFM1 acting as a key hub–seed connector. Additional clusters included RNA processing and transport proteins (DHX38, CWC25, THOC2, NUP54), suggesting modulation of RNA metabolism. Hub proteins such as MTOR and UHRF1 point to activation of signalling and epigenetic regulation, while KIF2C indicates involvement of microtubule dynamics. Seed proteins, including AKAP9 and GFM1, were distributed across modules, with peripheral nodes representing specialized functions. Collectively, CPT-treatment induces selective upregulation of translation, RNA processing, and signalling pathways, reflecting an adaptive cellular response.

DISCUSSION:

The present study integrates cytotoxic, apoptotic, and proteomic analyses to elucidate the cellular response to HI and CPT-treatments. Cytotoxicity and apoptosis assays confirmed that HI induces programmed cell death across multiple cell lines, comparable to CPT, as evidenced by DNA fragmentation and caspase activation, which are characteristics of apoptosis (Elmore 2007; Li and Yuan 2008; McIlwain et al. 2015).

Proteomic profiling revealed distinct yet partially overlapping responses between HI and CPT treatments. A key observation was the predominant downregulation of proteins following HI treatment, particularly those associated with cytoskeletal organization and extracellular matrix (ECM) interactions, including FLNA, COL1A1, COL1A2, and RABGEF1, which are key components of collagen-rich extracellular matrix architecture and tumour microenvironment remodelling (Lu et al. 2012; Pickup et al. 2014). Collagen type I proteins such as COL1A1 and COL1A2 play critical roles in maintaining ECM integrity and regulating cell adhesion, migration, and mechanotransduction in cancer progression (Lu et al. 2012; Pickup et al. 2014; Ricard-Blum 2011). These proteins are critical components of integrin-mediated adhesion and mechanotransduction pathways (Geiger et al. 2009; Hynes 2002). Consistently, pathway enrichment analysis demonstrated suppression of focal adhesion, ECM–receptor interaction, and actin cytoskeleton pathways, indicating disruption of cell–matrix communication and structural integrity.

Mechanistically, integrin signalling is closely linked to the PI3K/AKT pathway, which regulates cell survival, migration, and cytoskeletal dynamics (Manning and Toker 2017). The identification of hub proteins such as AKT1, SRC, FN1, and CD44 within the HI-downregulated PPI network suggests that HI-treatment attenuates integrin-mediated activation of PI3K/AKT signalling, thereby impairing survival signalling and cellular motility. In addition, cytoskeletal regulators such as FLNA are essential for actin organization and mechanosensing (Stossel et al. 2001), and their downregulation indicates compromised structural stability. Suppression of processes involving Rho GTPase signalling and actomyosin organization further supports inhibition of cytoskeletal remodelling and migration-associated pathways (Ridley 2001). Given the established role of integrin–cytoskeleton signaling in regulating YAP/TAZ-mediated transcription (Dupont et al. 2011), disruption of this axis may contribute to reduced proliferative and invasive capacity.

In contrast, CPT treatment exhibited a broader impact on nuclear and biosynthetic processes, consistent with its role as a topoisomerase I inhibitor (Pommier 2006). Downregulation of proteins involved in DNA replication and repair (TOP2A, BRCA2, ATM), which are central regulators of DNA damage response and homologous recombination repair pathways (Jackson and Bartek 2009; Lord and Ashworth 2012; Shiloh and Ziv 2013; Roy et al. 2012), reflects activation of DNA damage response mechanisms and genomic instability. ATM kinase functions as a master regulator of DNA damage signalling, while BRCA2 plays a crucial role in homologous recombination-mediated repair of double-strand breaks (Shiloh and Ziv 2013; Roy et al. 2012). Concurrent suppression of ribosomal proteins and epigenetic regulators (KMT2A, SETD2) suggests inhibition of protein synthesis and transcriptional regulation, indicating a global cytotoxic effect.

Upregulated protein networks further highlight adaptive cellular responses. HI-treatment induced upregulation of proteins involved in RNA processing, DNA repair, and cell cycle regulation, suggesting activation of compensatory mechanisms to maintain genomic integrity and transcriptional control. Enrichment of cytoskeleton-dependent transport and vesicular trafficking processes indicates active intracellular reorganization in response to treatment-induced stress. In contrast, CPT-treatment selectively upregulated pathways related to translation, RNA metabolism, and signalling, including mTOR-associated processes, which are known to regulate protein synthesis and stress adaptation (Saxton & Sabatini 2017).

Protein–protein interaction network analysis validates these findings by unveiling crucial regulatory hubs and functional modules. The HI-downregulated network was dominated by integrin–cytoskeleton signalling hubs, whereas the CPT-downregulated network exhibited

extensive modular organization involving DNA repair, ribosome biogenesis, and epigenetic regulation. These observations suggest that HI targets cellular architecture and adhesion signalling, while CPT exerts a broader inhibitory effect on genomic stability, transcription, and protein synthesis.

Collectively, this study demonstrates that HI and CPT modulate distinct yet interconnected cellular pathways. HI predominantly disrupts ECM–integrin–cytoskeleton signalling and mechanotransduction, potentially limiting cell adhesion and migration, whereas CPT induces widespread suppression of nuclear and biosynthetic processes through DNA damage–mediated mechanisms. These findings provide mechanistic insight into the differential modes of action of HI and CPT and highlight their potential implications in targeting cancer cell proliferation and invasiveness.

CONCLUSION:

In summary, the present study presents a complete proteomic and bioinformatic characterisation of cellular responses to HI and CPT-treatments. HI-treatment triggered strong downregulation of proteins that are associated with cytoskeletal organization, extracellular matrix interactions, and integrin-mediated signalling, indicating disturbance of cellular architecture and adhesion dynamics. In contrast, CPT-treatment had a larger inhibitory effect on nuclear and biosynthetic activities, including DNA replication, repair, transcriptional control, and protein synthesis.

Pathway enrichment and PPI network analysis further revealed that HI primarily targets ECM–integrin–cytoskeleton signalling and mechanotransduction pathways, whereas CPT produces genomic instability and broad inhibition of proliferative machinery. The observed increase of RNA processing, DNA repair, and transport-related pathways implies the activation of adaptive responses to treatment-induced stress.

Overall, these findings indicate unique yet complementary modes of action for HI and CPT, with HI working as a modulator of structural and signalling networks and CPT functioning as a traditional DNA-damaging agent. This study gives mechanistic insight into treatment-induced proteome remodelling and highlights the potential of HI as a candidate for targeting cellular adhesion and invasion pathways.

Acknowledgements The authors owe a debt of gratitude to DG-CCRAS, New Delhi, for moral support.

Author contribution SP designed the study and planned the experiments; SC and SB carried out the experiments, recorded the observations and compiled the results; AS assisted in omics data

compilation; SS helped in arranging necessary chemicals; AA, SS provided useful technical inputs in experimentation; SC wrote the manuscript draft; all the authors reviewed the manuscript and provided useful inputs.

Funding This research did not receive any specific grant from any funding agencies in the public, commercial, or not-for-profit sectors.

Data availability The mass spectrometry proteomics data will be deposited to the ProteomeXchange Consortium via the PRIDE partner repository.

Declarations The authors declare no competing interests.

REFERENCES:

- Abdallah EM (2012) Wound healing activity of *Balanites aegyptiaca*. *J Med Plants Res.* 6(9):1606–1610
- Abdel-Farid IB, Sheded MG, Mohamed EA (2014) Metabolomic profiling and antioxidant activity of *Balanites aegyptiaca*. *J Pharm Biomed Anal.* 98:317–324. doi:10.1016/j.jpba.2014.06.003
- Abdelwahab SI, Mohan S, Abdulla MA, Sukari MA, Abdul AB, Taha MM, et al (2011) The methanolic extract of *Balanites aegyptiaca* induces apoptosis in cancer cells via caspase activation. *BMC Complement Altern Med.* 11:57. doi:10.1186/1472-6882-11-57
- Abdul AB, Abdel-Wahab SI, Fong HK, Mohan SM, Al-Zubairi AS, Elhassan MM (2009) In vitro Response of Cancer Cells to the Growth-Inhibitory Effects. *Research Journal of Pharmacology.* 3(1):1-6.
- Al Ashaal HA, Farghaly AA, Abd El Aziz MM, Ali MA (2010) Phytochemical investigation and medicinal evaluation of *Balanites aegyptiaca*. *J Ethnopharmacol.* 129(2):210–214. doi:10.1016/j.jep.2010.03.017
- Aleksander SA, Balhoff JP, Carbon S, Cherry JM et al (2025) The Gene Ontology knowledgebase in 2026. *Nucleic Acids Research.* 54(D1):gkaf1292. <https://doi.org/10.1093/nar/gkaf1292>
- Ambasta SP, editor (1986) *The useful plants of India.*
- Anonymous (2016) *Ayurvedic Pharmacopoeia of India, Part I, Vol IX, Pharmacopoeia Commission for Indian Medicine and Homeopathy, Ministry of AYUSH, Govt. of India*
- Bader GD, Hogue CW (2003) An automated method for finding molecular complexes in large protein interaction networks. *BMC bioinformatics.* 4(1):2. <https://doi.org/10.1186/1471-2105-4-2>

- Barretina J, Caponigro G, Stransky N, Venkatesan K, Margolin AA, Kim S et al (2012) The Cancer Cell Line Encyclopedia enables predictive modelling of anticancer drug sensitivity. *Nature*. 483(7391):603-607. <https://doi.org/10.1038/nature11003>
- Benjamini Y and Hochberg Y (1995) Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. *Journal of the Royal Statistical Society: Series B (Methodological)*, 57: 289-300. <https://doi.org/10.1111/j.2517-6161.1995.tb02031.x>
- Burstein HJ, Schwartz RS (2008) Molecular origins of cancer. *New England Journal of Medicine*. 358(5):527. <https://doi.org/10.1056/NEJMe0800065>
- Chothani DL, Vaghasiya HU (2011) A review on *Balanites aegyptiaca*: ethnobotany, phytochemistry and pharmacology. *Int J Pharm Sci Res*. 2(5):976–984.
- Chougule S, Shirolkar A, Gacche R et al (2025) Proteomics-based assessment of the antidiabetic activity by polyherbal formulation in streptozotocin-induced hyperglycemic rat model. *J Proteins Proteom* 16, 253–267. <https://doi.org/10.1007/s42485-025-00191-x>
- Dagogo-Jack I, Shaw AT (2018) Tumour heterogeneity and resistance to cancer therapies. *Nature reviews Clinical oncology*. 15(2):81-94. <https://doi.org/10.1038/nrclinonc.2017.166>
- Dienstmann R, Rodon J, Barretina J, Tabernero J (2013) Genomic medicine frontier in human solid tumors: prospects and challenges. *Journal of Clinical Oncology*. 31(15):1874-1884. <https://doi.org/10.1200/JCO.2012.45.2268>
- Dupont S, Morsut L, Aragona M et al (2011) Role of YAP/TAZ in mechanotransduction. *Nature*. 474(7350):179-83. <https://doi.org/10.1038/nature10137>
- Elfeel AA, Warrag EI (2011) Medicinal uses of *Balanites aegyptiaca* and its effect on blood glucose levels. *J Med Plants Res*. 5(16):3727–3731.
- Elmore S (2007) Apoptosis: a review of programmed cell death. *Toxicologic pathology*. 35(4):495-516. <https://doi.org/10.1080/01926230701320337>
- Garnett MJ, Edelman EJ, Heidorn SJ, Greenman CD, Dastur A, Lau KW et al (2012) Systematic identification of genomic markers of drug sensitivity in cancer cells. *Nature*. 483(7391):570-575. <https://doi.org/10.1038/nature11005>
- Garraway LA, Jänne PA (2012) Circumventing cancer drug resistance in the era of personalized medicine. *Cancer discovery*. 2(3):214-226. <https://doi.org/10.1158/2159-8290.CD-12-0012>
- Gayakwad S, Shirolkar A, Warkad S et al (2020) Proteomic and metabolomic analysis of *Nothapodytes nimmoniana* (J. Graham) extracts' treatment on HeLa cells. *J Proteins Proteom* 11, 27–62. <https://doi.org/10.1007/s42485-020-00031-0>

- Geiger B, Spatz JP, Bershadsky AD (2009) Environmental sensing through focal adhesions. *Nature reviews Molecular cell biology*. 10(1):21-33. <https://doi.org/10.1038/nrm2593>
- Gillet JP, Varma S, Gottesman MM (2013) The clinical relevance of cancer cell lines. *J Natl Cancer Inst*. 105(7):452–458. <https://doi.org/10.1093/jnci/djt007>
- Giri B, Gomes A, Debnath A, Saha A, Biswas AK, Dasgupta SC (2006) Antiproliferative, cytotoxic and apoptogenic activity of Indian toad (*Bufo melanostictus*, Schneider) skin extract on U937 and K562 cells. *Toxicon*. 48(4):388-400. <https://doi.org/10.1016/j.toxicon.2006.06.011>.
- Hall JB, Walker DH (1991) *B. aegyptiaca*—A Monograph. School of Agricultural and Forest Science. University of Wales. Bangor. 1991:1-2.
- Hardman R, Sofowora EA (1972) A reinvestigation of *Balanites aegyptiaca* as a source of steroidal sapogenins. *Economic Botany*. 26(2):169-173. <https://doi.org/10.1007/BF02860779>
- Huang DW, Sherman BT, Lempicki RA (2009) Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nature protocols*. 4(1):44-57. <https://doi.org/10.1038/nprot.2008.211>
- Hynes RO (2002) Integrins: bidirectional, allosteric signaling machines. *Cell*. 110(6):673-87. [https://doi.org/10.1016/S0092-8674\(02\)00971-6](https://doi.org/10.1016/S0092-8674(02)00971-6)
- Iorio F, Knijnenburg TA, Vis DJ, Bignell GR, Menden MP et al (2016) A landscape of pharmacogenomic interactions in cancer. *Cell*. 166(3):740-754. <https://doi.org/10.1016/j.cell.2016.06.017>
- Jackson SP, Bartek J (2009) The DNA-damage response in human biology and disease. *Nature*. 461(7267):1071-8. <https://doi.org/10.1038/nature08467>
- Kamel MS, et al (2011) Hepatoprotective activity of *Balanites aegyptiaca* extracts. *Phytother Res*. 25(4):567–571.
- Kanehisa M, Goto S (2000) KEGG: kyoto encyclopedia of genes and genomes. *Nucleic acids research*. 28(1):27-30. <https://doi.org/10.1093/nar/28.1.27>
- Kirtikar KR, Basu BD (1918) *Indian medicinal plants*. Allahabad: Lalit Mohan Basu, Bhuwaneśwari Āśrama.
- Kirtikar KR, Basu BD (1933) *Medicinal plants of India*. Pub. LM Basu, Allahabad. 3.
- Kruger NJ (2009) The Bradford Method For Protein Quantitation. In: Walker, J.M. (eds) *The Protein Protocols Handbook*. Springer Protocols Handbooks. Humana Press, Totowa, NJ. https://doi.org/10.1007/978-1-59745-198-7_4

- Kuleshov MV, Jones MR, Rouillard AD, Fernandez NF et al (2016) Enrichr: a comprehensive gene set enrichment analysis web server 2016 update. *Nucleic acids research*. 44(W1):W90-7. <https://doi.org/10.1093/nar/gkw377>
- Kumar S, Singh D, Dwivedi KN (2014) MEDICINAL IMPORTANCE OF INGUDI (BALANITES AEGYPTIACA LINN. DELILE): A HISTORICAL PLANT. *Journal of Pharmaceutical & Scientific Innovation*. 3. 275-277. <https://doi.org/10.7897/2277-4572.034155>.
- Li J, Yuan J (2008) Caspases in apoptosis and beyond. *Oncogene* 27, 6194–6206. <https://doi.org/10.1038/onc.2008.297>
- Lord CJ, Ashworth A (2012) The DNA damage response and cancer therapy. *Nature*. 481(7381):287-94. <https://doi.org/10.1038/nature10760>
- Lu P, Weaver VM, Werb Z (2012) The extracellular matrix: a dynamic niche in cancer progression. *Journal of cell biology*. 196(4):395-406. <https://doi.org/10.1083/jcb.201102147>
- Manning BD, Toker A (2017) AKT/PKB signaling: navigating the network. *Cell*. 169(3):381-405. <https://doi.org/10.1016/j.cell.2017.04.001>
- Matassov D, Kagan T, Leblanc J, Sikorska M, Zakeri Z (2004) Measurement of Apoptosis by DNA Fragmentation. In: Brady, H.J.M. (eds) *Apoptosis Methods and Protocols*. *Methods in Molecular Biology*, vol 282. Humana Press. <https://doi.org/10.1385/1-59259-812-9:001>
- Mbaveng AT, Kuete V, Mapunya BM, Beng VP, Nkengfack AE, Meyer JJ, Lall N (2011) Evaluation of four Cameroonian medicinal plants for anticancer, antigonorrheal and antireverse transcriptase activities. *Environmental toxicology and pharmacology*. 32(2):162-7. <https://doi.org/10.1016/j.etap.2011.04.006>
- McIlwain DR, Berger T, Mak TW (2015) Caspase functions in cell death and disease. *Cold Spring Harbor Perspectives in Biology*. 7(4), a026716. <https://doi.org/10.1101/cshperspect.a026716>
- Mohamed AM, et al (2015) Antidiabetic activity of *Balanites aegyptiaca* fruit extract in experimental models. *Pharmacognosy Res*. 7(2):170–175.
- Nagata S (2018) Apoptotic DNA fragmentation. *Experimental cell research*. 256(1):12-8. <https://doi.org/10.1006/excr.2000.4834>
- Oliveros, J.C. (2007-2015) Venny. An interactive tool for comparing lists with Venn's diagrams. <https://bioinfogp.cnb.csic.es/tools/venny/index.html>

- Pickup MW, Mouw JK, Weaver VM (2014) The extracellular matrix modulates the hallmarks of cancer. *The EMBO Reports*. 15(12):1243-1253. <https://doi.org/10.15252/embr.201439246>
- Pommier Y (2006) Topoisomerase I inhibitors: camptothecins and beyond. *Nature Reviews Cancer*. 6(10):789-802. <https://doi.org/10.1038/nrc1977>
- Ricard-Blum S (2011) The collagen family. *Cold Spring Harbor perspectives in biology*. 3(1):a004978. <https://doi.org/10.1101/cshperspect.a004978>
- Ridley AJ (2001) Rho GTPases and cell migration. *Journal of cell science*. 114(15):2713-2722. <https://doi.org/10.1242/jcs.114.15.2713>
- Roy R, Chun J, Powell SN (2012) BRCA1 and BRCA2: different roles in a common pathway of genome protection. *Nature Reviews Cancer*. 12(1):68-78. <https://doi.org/10.1038/nrc3181>
- Saxton RA, Sabatini DM (2017) mTOR Signaling in Growth, Metabolism, and Disease. *Cell*, 168(6), 960–976. <https://doi.org/10.1016/j.cell.2017.02.004>
- Shannon P, Markiel A, Ozier O, Baliga NS, Wang JT et al (2003) Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome research*, 13(11), 2498–2504. <https://doi.org/10.1101/gr.1239303>
- Sherman BT, Hao M, Qiu J, Jiao X, Baseler MW, Lane HC, Imamichi T, Chang W (2021) DAVID: a web server for functional enrichment analysis and functional annotation of gene lists (2021 update). *Nucleic acids research*. 50(W1):W216-21. <https://doi.org/10.1093/nar/gkac194>
- Shiloh Y, Ziv Y (2013) The ATM protein kinase: regulating the cellular response to genotoxic stress, and more. *Nature reviews Molecular cell biology*. 14(4):197-210. <https://doi.org/10.1038/nrm3546>
- Stockert JC, Blázquez-Castro A, Cañete M, Horobin RW, and Villanueva Á (2012) MTT assay for cell viability: Intracellular localization of the formazan product is in lipid droplets. *Acta histochemica*. 114(8), 785-796. <https://doi.org/10.1016/j.acthis.2012.01.006>
- Stockert JC, Horobin RW, Colombo LL, Blázquez-Castro A (2018) Tetrazolium salts and formazan products in Cell Biology: Viability assessment, fluorescence imaging, and labeling perspectives. *Acta histochemica*. 120(3):159-67. <https://doi.org/10.1016/j.acthis.2018.02.005>
- Stossel TP, Condeelis J, Cooley L, Hartwig JH, Noegel A, Schleicher M, Shapiro SS (2001) Filamins as integrators of cell mechanics and signalling. *Nature reviews Molecular cell biology*. 2:138-145. <https://doi.org/10.1038/35052082>

- Susin SA, Lorenzo HK, Zamzami N, Marzo I, Snow BE et al. (1999) Molecular characterization of mitochondrial apoptosis-inducing factor. *Nature*. 397(6718):441-6. <https://doi.org/10.1038/17135>
- Szklarczyk D, Gable AL, Nastou KC, Lyon D, Kirsch R et al (2021) The STRING database in 2021: customizable protein–protein networks, and functional characterization of user-uploaded gene/measurement sets. *Nucleic acids research*. 49(D1):D605-12. <https://doi.org/10.1093/nar/gkaa1074>
- Tang D, Chen M, Huang X, Zhang G, Zeng L, Zhang G, Wu S, Wang Y (2023) SRplot: A free online platform for data visualization and graphing. *PloS one*. 18(11):e0294236. <https://doi.org/10.1371/journal.pone.0294236>
- UniProt Consortium (2023) UniProt: the universal protein knowledgebase. *Nucleic Acids Res*. 51:D523–D531. doi:10.1093/nar/gkac1052
- Yadav JP, Panghal M (2010) Antimicrobial activity of *Balanites aegyptiaca* extracts. *Indian J Pharm Sci*. 72(5):575–578.

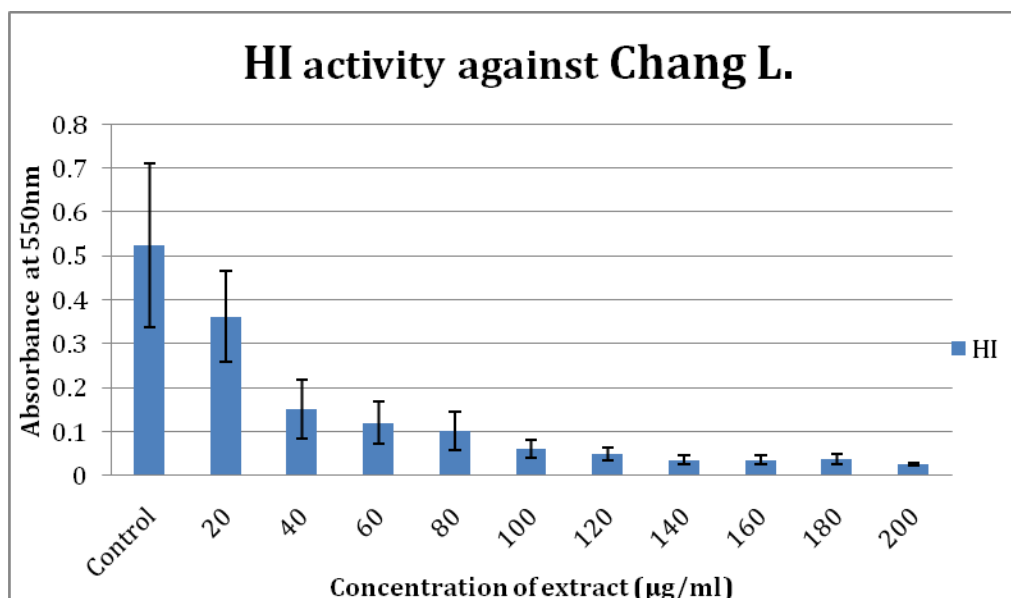


Figure 1: Cytotoxic effect of HI extract on Chang Liver cells assessed by MTT assay.

Bar graph representing the effect of hydroalcoholic extract (HI) on Chang Liver cells viability post 24 hours treatment at varying concentrations (20–200 µg/mL), as determined by MTT assay. Cell viability is expressed as absorbance measured at 550 nm, reflecting metabolically active cells. A concentration-dependent decrease in absorbance is observed with increasing extract concentration, indicating reduced cell viability. The data are depicted as mean $\pm$ SD (Standard Deviation) of three biological replicates. Error bars represent SD.

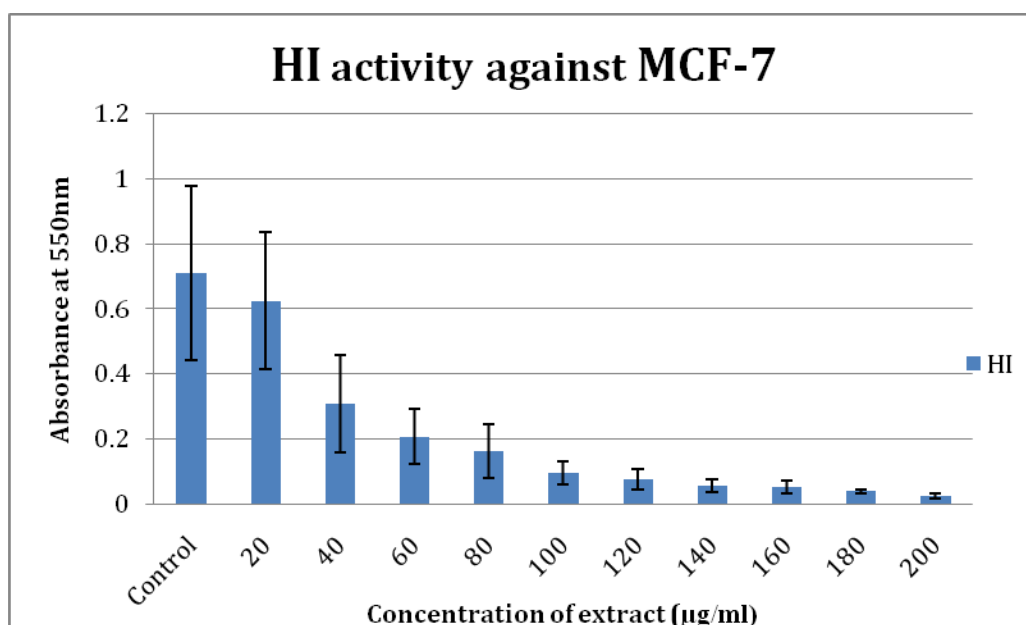


Figure 2: Cytotoxic effect of HI extract on MCF-7 cells assessed by MTT assay.

Bar graph representing the effect of hydroalcoholic extract (HI) on MCF-7 cells viability post 24 hours treatment at varying concentrations (20–200 µg/mL), as determined by MTT assay. Cell viability is expressed as absorbance measured at 550 nm, reflecting metabolically active cells. A

concentration-dependent decrease in absorbance is observed with increasing extract concentration, indicating reduced cell viability. The data are depicted as mean $\pm$ SD (Standard Deviation) of three biological replicates. Error bars represent SD.

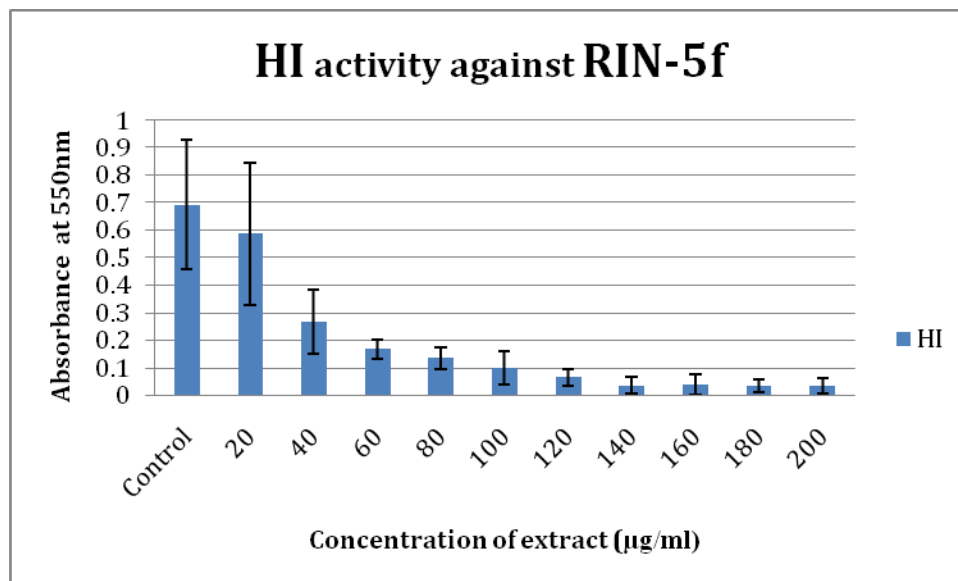


Figure 3: Cytotoxic effect of HI extract on RIN-5f cells assessed by MTT assay.

Bar graph representing the effect of hydroalcoholic extract (HI) on RIN-5f cells viability post 24 hours treatment at varying concentrations (20–200 µg/mL), as determined by MTT assay. Cell viability is expressed as absorbance measured at 550 nm, reflecting metabolically active cells. A concentration-dependent decrease in absorbance is observed with increasing extract concentration, indicating reduced cell viability. The data are depicted as mean $\pm$ SD (Standard Deviation) of three biological replicates. Error bars represent SD.

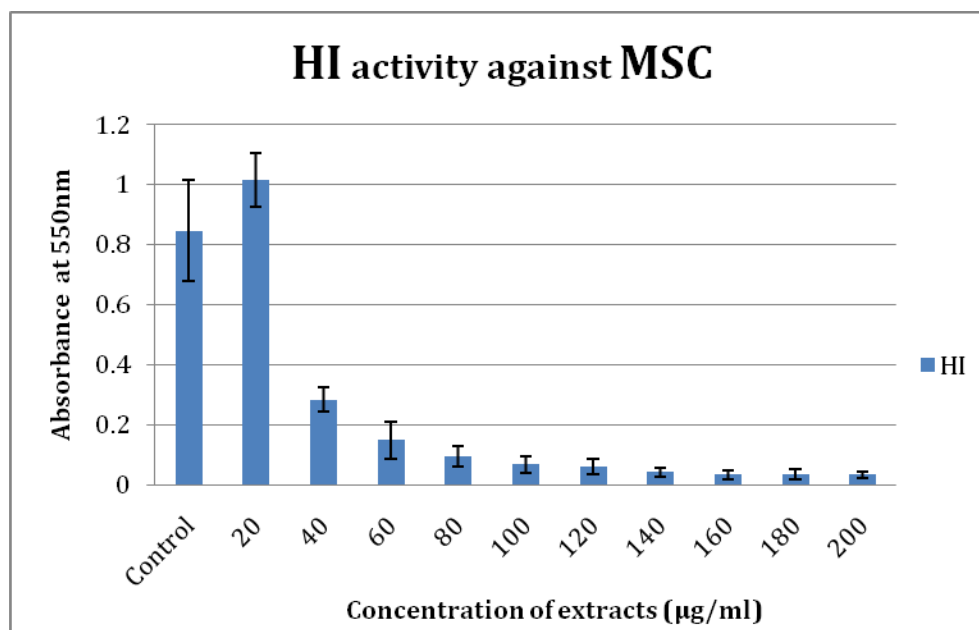


Figure 4: Cytotoxic effect of HI extract on MSC cells assessed by MTT assay.

Bar graph representing the effect of hydroalcoholic extract (HI) on MSC cells viability post 24 hours treatment at varying concentrations (20–200 µg/mL), as determined by MTT assay. Cell viability is expressed as absorbance measured at 550 nm, reflecting metabolically active cells. A concentration-dependent decrease in absorbance is observed with increasing extract concentration, indicating reduced cell viability. The data are depicted as mean $\pm$ SD (Standard Deviation) of three biological replicates. Error bars represent SD.

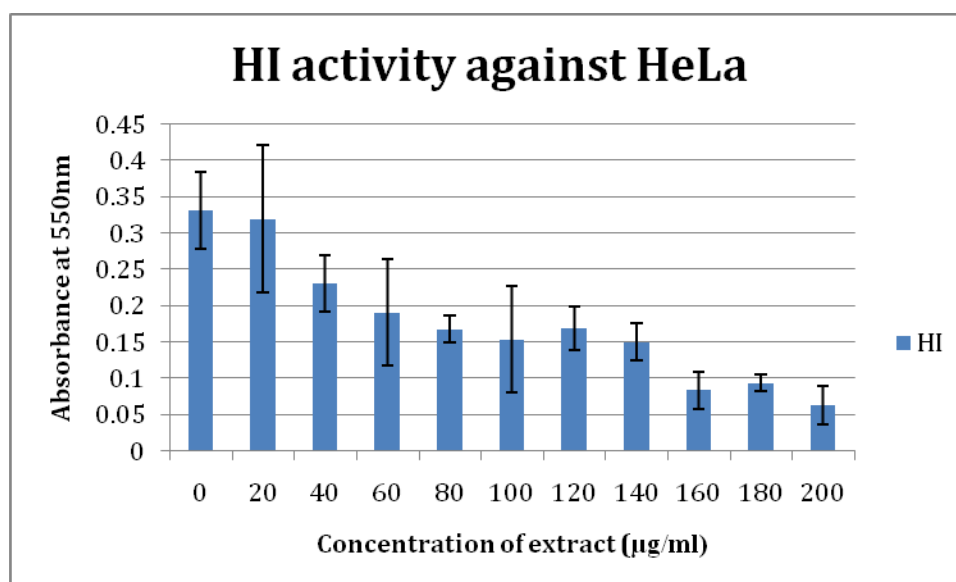


Figure 5: Cytotoxic effect of HI extract on HeLa cells assessed by MTT assay.

Bar graph representing the effect of hydroalcoholic extract (HI) on HeLa cells viability post 24 hours treatment at varying concentrations (20–200 µg/mL), as determined by MTT assay. Cell viability is expressed as absorbance measured at 550 nm, reflecting metabolically active cells. A

concentration-dependent decrease in absorbance is observed with increasing extract concentration, indicating reduced cell viability. The data are depicted as mean $\pm$ SD (Standard Deviation) of three biological replicates. Error bars represent SD.

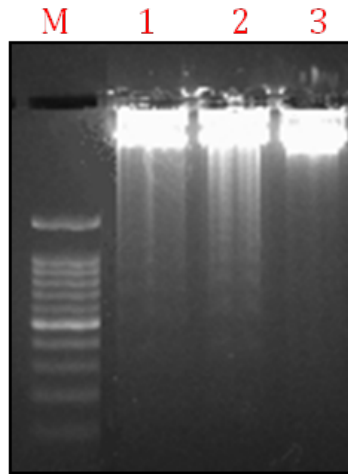


Figure 6: DNA fragmentation analysis of HeLa (H93N) cells following treatment with HI extract and camptothecin.

Agarose gel electrophoresis images showing DNA fragmentation patterns in different cell lines treated with hydroalcoholic extract (HI) and camptothecin (CPT), compared to untreated controls. DNA ladder (M) represents molecular weight markers. HeLa cells: Lane 1, CPT (4 μ M); Lane 2, HI-treated (30 μ g/mL); Lane 3, Control. Fragmentation patterns indicative of apoptotic DNA cleavage are observed in treated samples, characterized by ladder-like banding, whereas control samples exhibit intact genomic DNA.

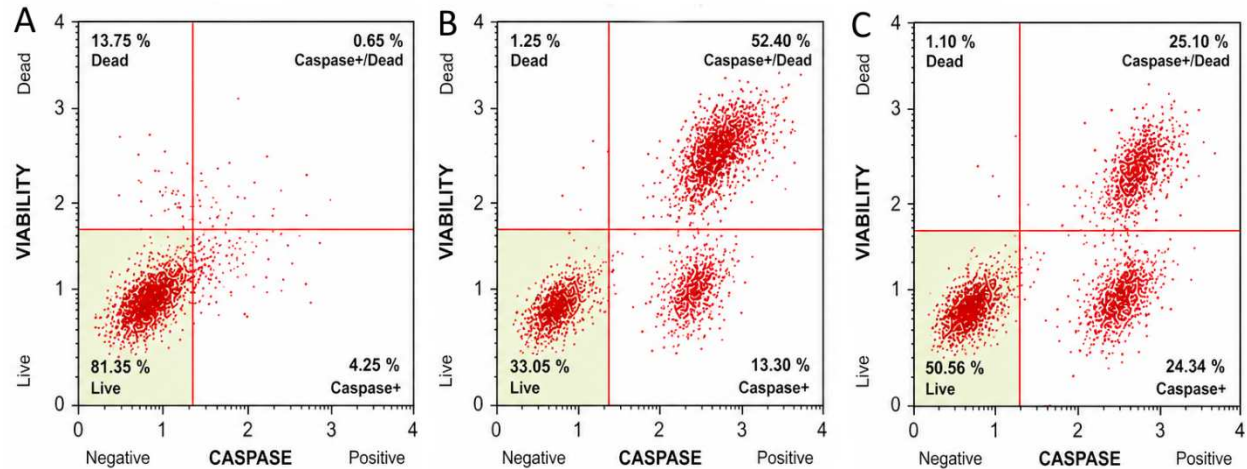


Figure 7: Multicaspase analysis of apoptosis induction in HeLa cells following HI extract treatment. Representative dot plots showing multicaspase activity in HeLa under (A) untreated control, (B) Camptothecin treated (3 μ M) and (C) hydroalcoholic extract-treated (HI, 60 μ g/mL) conditions. Cells were analyzed using a multicaspase assay to assess caspase activation and cell viability. Quadrant distribution represents different cell populations: live cells (Live), early apoptotic cells (Caspase+), dead cells or late apoptotic (Caspase+/Dead), and necrotic cells (Dead). The percentage of cells in each quadrant is indicated within the plots. An increase in caspase-positive populations is observed in HI-treated cells compared to control, indicating enhanced activation of apoptotic pathways.

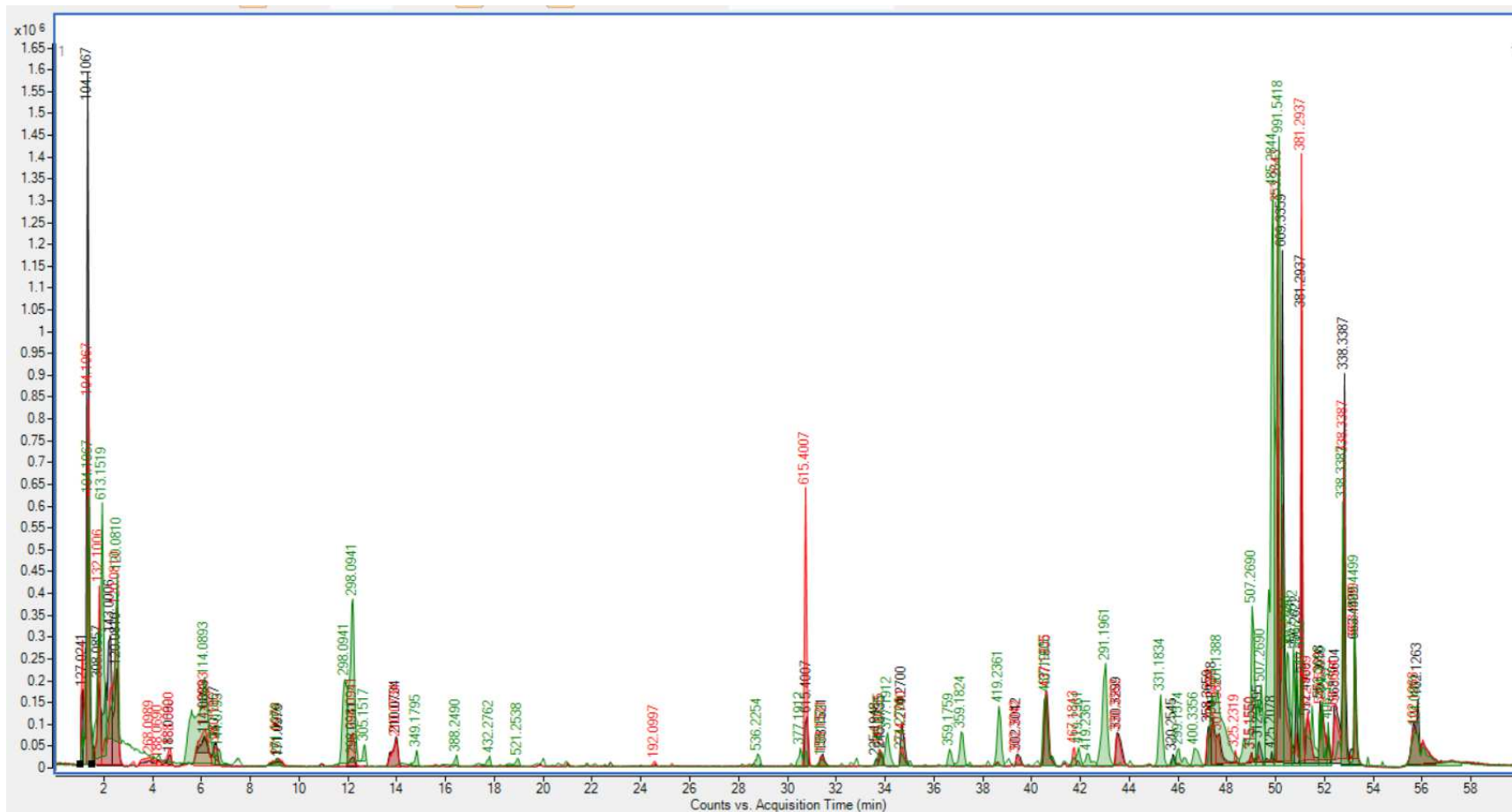


Figure 8: Representative LC–MS base peak chromatograms of protein digests from HeLa (H93N) cells under different treatment conditions. An overlap Base Peak Chromatogram of three samples: Control (Untreated) cells (Green), HI-treated cells (Red), and CPT-treated cells (Black). Chromatographic separation was performed using a reverse-phase C18 column, and mass spectra were acquired in positive ion mode over the specified m/z range. Peaks represent eluted peptide ions detected at different retention times, with variations in peak intensity and distribution reflecting differences in peptide abundance across experimental conditions. The overlaid chromatograms demonstrate treatment-dependent alterations in proteomic profiles.

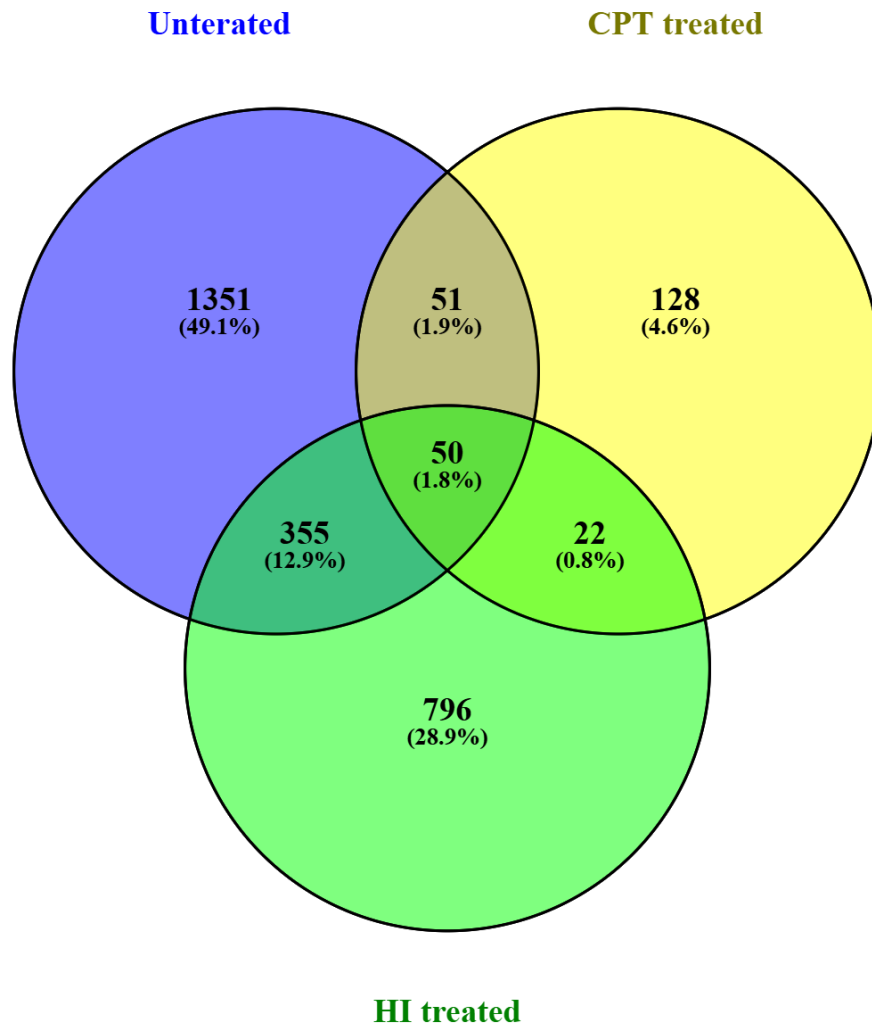


Figure 9: Overlap of identified proteins across untreated, HI-treated, and camptothecin-treated HeLa (H93N) cells. Venn diagram illustrating the distribution and overlap of identified proteins among three experimental groups: untreated control (HeLa H93N), hydroalcoholic extract-treated (HI-treated), and camptothecin-treated (CPT-treated) HeLa cells at passage 93 (H93N). The numbers within each section represent the count of proteins unique to or shared between the respective conditions, with percentages indicating their proportion relative to the total identified proteins. A total of 1351 proteins were uniquely identified in the untreated group, whereas 796 and 128 proteins were unique to the HI-treated and CPT-treated groups, respectively. Shared protein populations include 355 proteins common between untreated and HI-treated samples, 22 between HI-treated and CPT-treated samples, and 51 between untreated and CPT-treated samples. A core set of 50 proteins was common across all three conditions. These distributions indicate both treatment-specific and commonly expressed proteomic profiles in HeLa (H93N) cells under different experimental conditions.

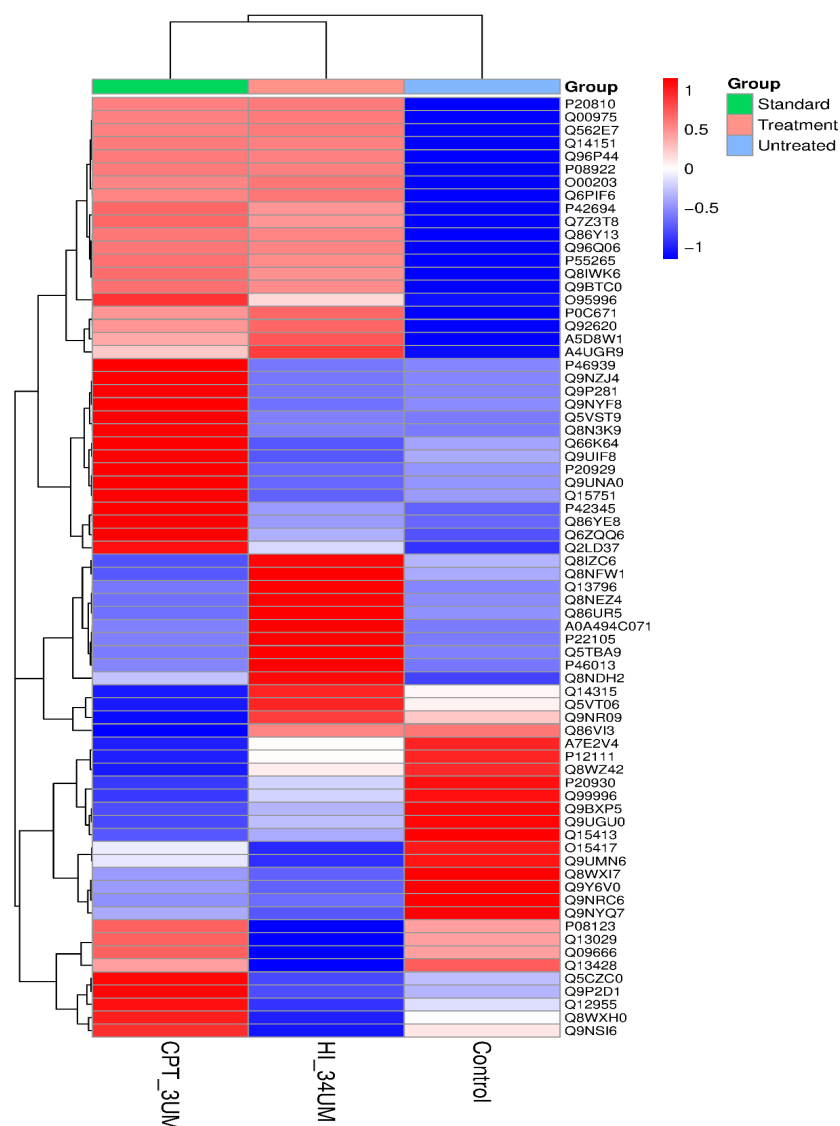


Figure 10: Heatmap of differentially expressed proteins in HeLa (passage No. 93) cells under treatment conditions. Hierarchical clustering heatmap illustrating the relative expression profiles of differentially expressed proteins in HeLa cells subjected to untreated control (Control), hydroalcoholic extract treatment (HI_34 μ M), and camptothecin treatment (CPT_3 μ M). Protein expression values were normalized and represented using a color scale, where red denotes upregulation and blue denotes downregulation relative to control. Unsupervised hierarchical clustering was performed using distance-based metrics to group proteins and samples according to similarity in expression patterns. Rows represent individual proteins (UniProt IDs), and columns represent experimental conditions. Dendrograms indicate clustering relationships among proteins and treatment groups. Distinct clustering patterns highlight treatment-specific alterations in the proteomic profile of HeLa cells.

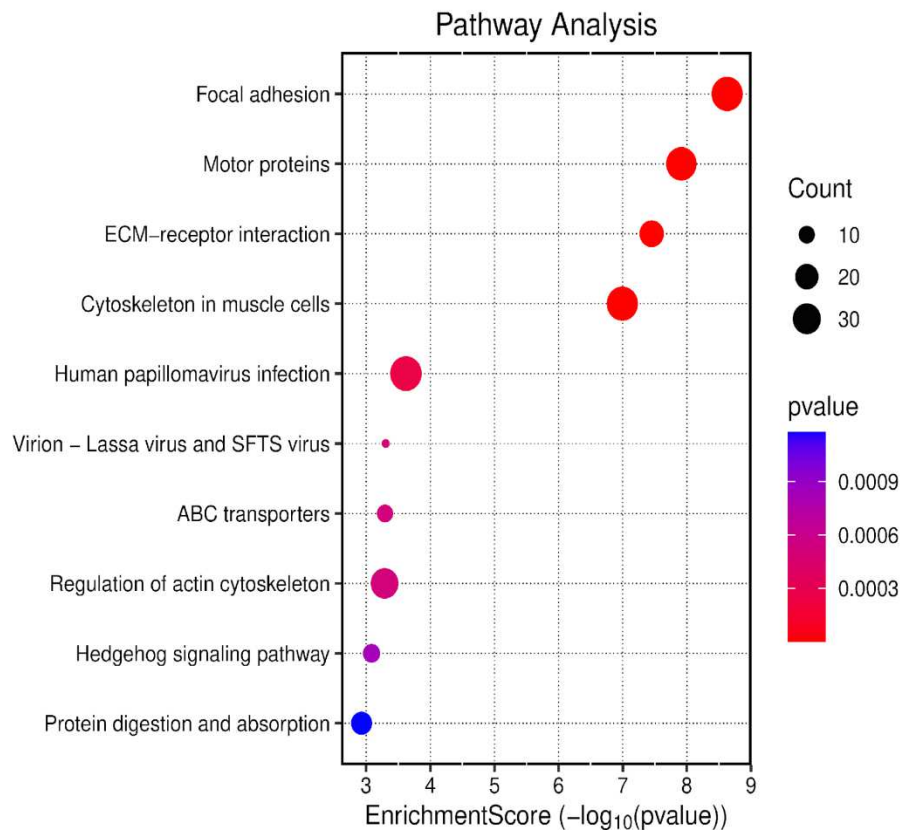


Figure 11: KEGG pathway enrichment analysis of proteins downregulated following HI treatment. Dot plot showing significantly enriched pathways among downregulated proteins. The enrichment score ($-\log_{10} \text{p-value}$) is represented by the x-axis, the number of proteins is indicated by dot size, and statistical significance is depicted by color (blue = lower significance, red = higher significance). Enriched pathways include Focal adhesion, Cytoskeleton in muscle cells, ECM-receptor interaction, and Motor proteins, highlighting suppression of structural and adhesion-associated networks.

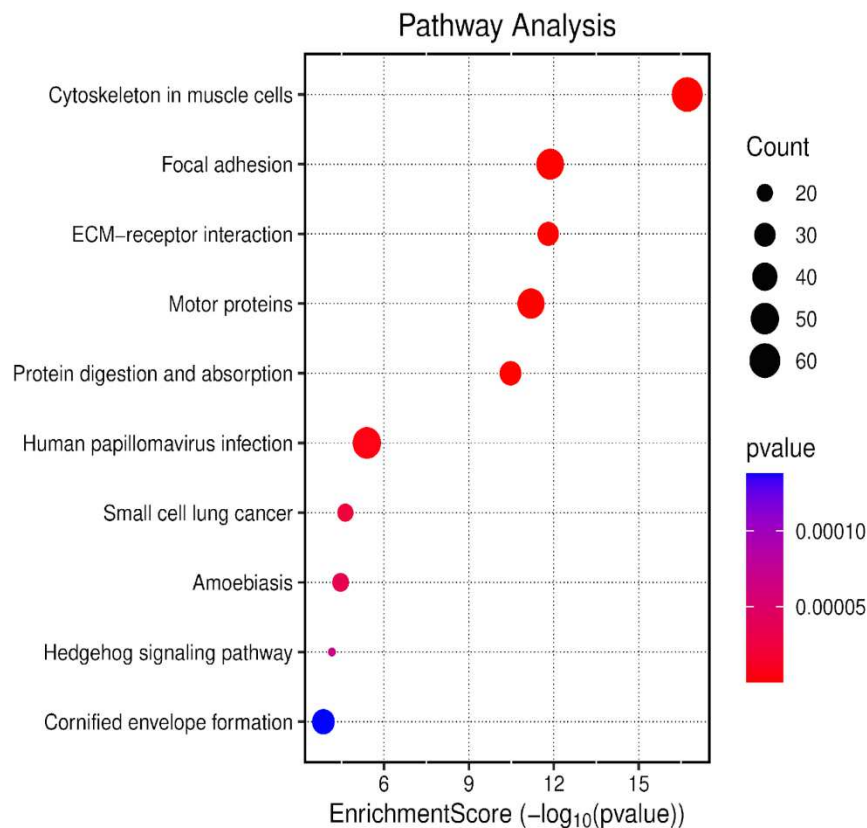


Figure 12: KEGG pathway enrichment analysis of proteins downregulated following CPT treatment. Dot plot showing significantly enriched pathways among downregulated proteins. The enrichment score ($-\log_{10} \text{p-value}$) is represented by the x-axis, the number of proteins is indicated by dot size, and statistical significance is depicted by color (blue = lower significance, red = higher significance). Enriched pathways include Cytoskeleton in muscle cells, Focal adhesion, ECM-receptor interaction, and Motor proteins, highlighting suppression of structural and adhesion-related pathways following CPT treatment.

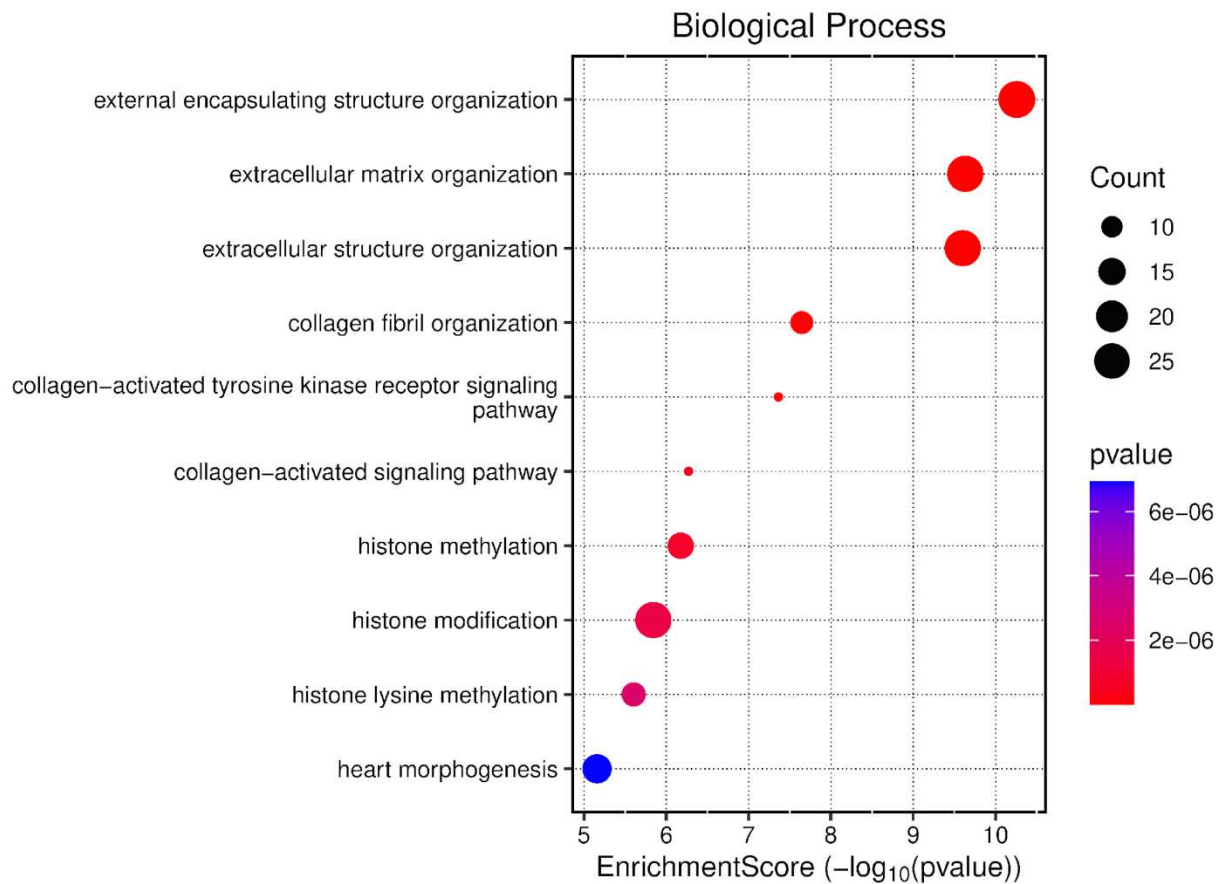


Figure 13: Gene Ontology (GO) biological process enrichment analysis of proteins downregulated following HI treatment. Dot plot showing significantly enriched biological processes among downregulated proteins. The x-axis depicts the enrichment score ($-\log_{10} \text{p-value}$), while the y-axis lists enriched biological processes. Dot size indicates the number of proteins associated with each term, and color represents statistical significance (red = higher significance, blue = lower significance). Enriched processes include microtubule-based movement, regulation of small GTPase-mediated signal transduction, and cell-substrate adhesion, highlighting suppression of cytoskeletal dynamics, adhesion, and intracellular organization following HI treatment.

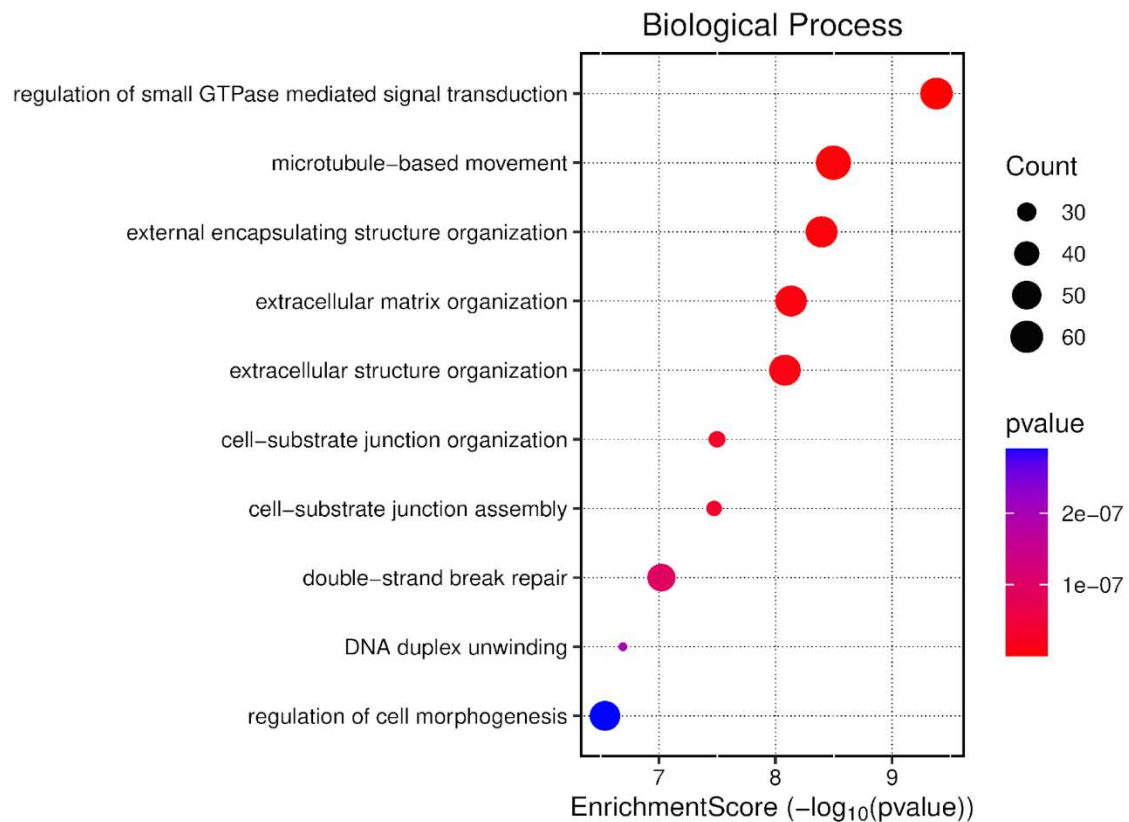


Figure 14: Gene Ontology (GO) biological process enrichment analysis of proteins downregulated following CPT treatment. Dot plot showing significantly enriched biological processes among downregulated proteins. The x-axis displays enrichment score ($-\log_{10} \text{p-value}$), dot size represents the number of proteins, and color reflects statistical significance (red = higher significance, blue = lower significance). Enriched processes include extracellular matrix organization, external encapsulating structure organization, microtubule-based movement, and regulation of small GTPase-mediated signal transduction, highlighting suppression of cytoskeletal dynamics, adhesion, and DNA repair processes.

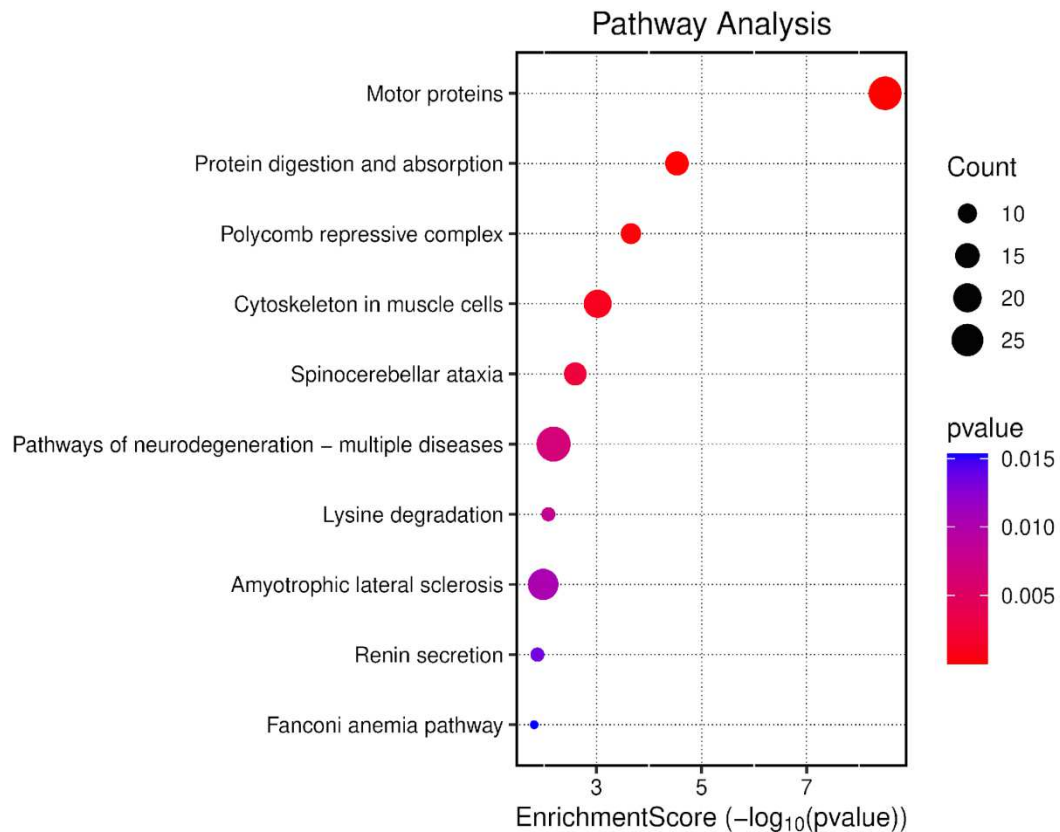


Figure 15: KEGG pathway enrichment analysis of proteins upregulated following HI treatment. Dot plot showing significantly enriched pathways among upregulated proteins. The enrichment score ($-\log_{10} \text{p-value}$) is represented by the x-axis, the number of proteins is indicated by dot size, and statistical significance is depicted by color (blue = lower significance, red = higher significance). Enriched pathways include Motor proteins, Protein digestion and absorption, Cytoskeleton in muscle cells, and Polycomb repressive complex.

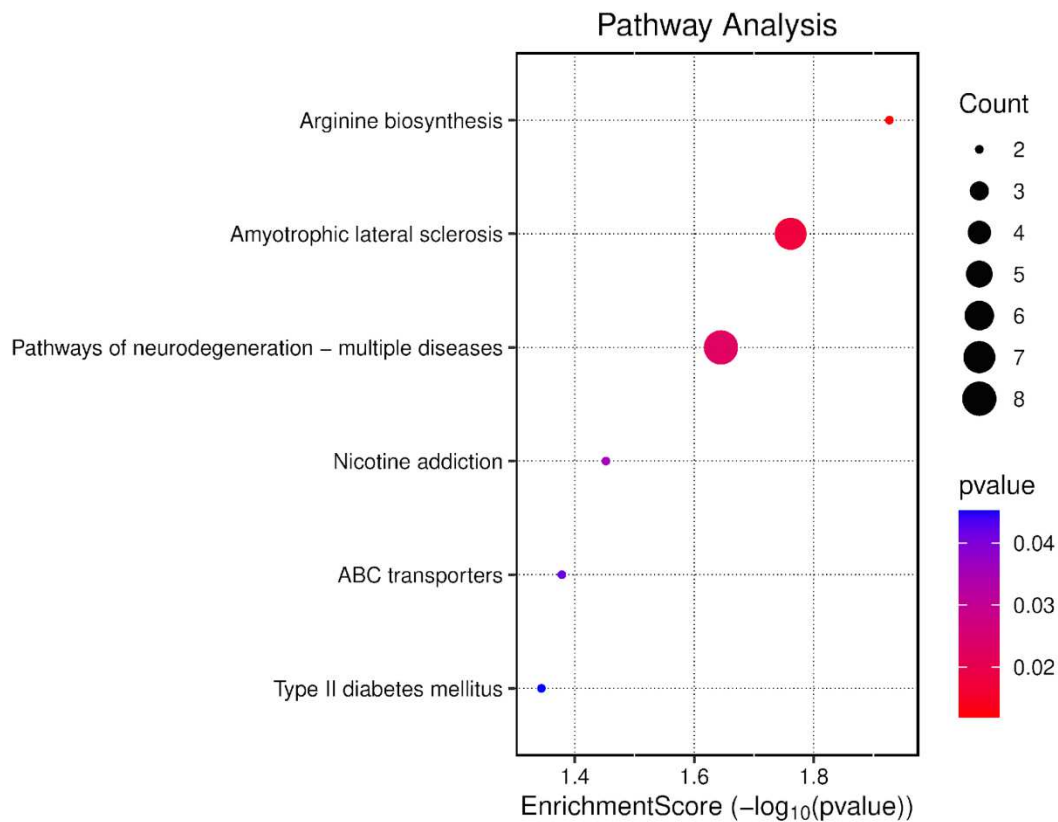


Figure 16: KEGG pathway enrichment analysis of proteins upregulated following CPT treatment. Dot plot showing significantly enriched pathways among upregulated proteins. The enrichment score ($-\log_{10} \text{p-value}$) is represented by the x-axis, the number of proteins is indicated by dot size, and statistical significance is depicted by color (blue = lower significance, red = higher significance). Enriched pathways include Amyotrophic lateral sclerosis, Pathways of neurodegeneration, and Arginine biosynthesis, indicating activation of stress-associated and metabolic pathways.

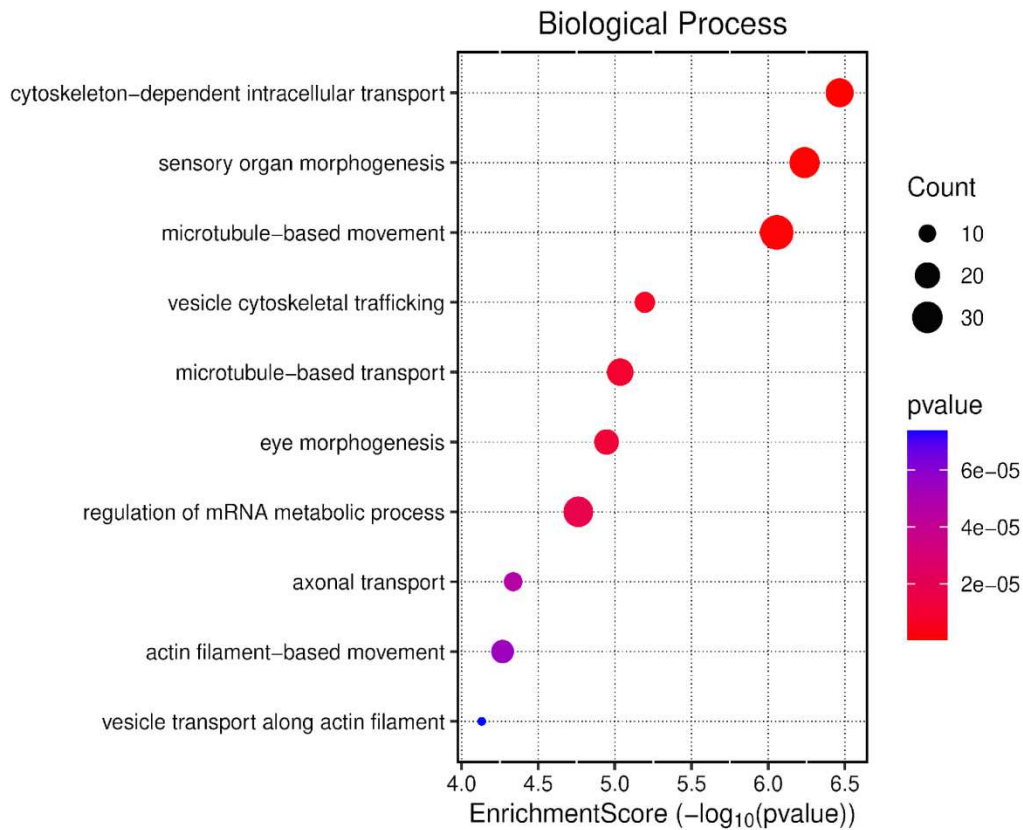


Figure 17: Gene Ontology (GO) biological process enrichment analysis of proteins upregulated following HI treatment. Dot plot showing significantly enriched biological processes among upregulated proteins. The x-axis depicts enrichment score ($-\log_{10} \text{p-value}$), dot size displays the number of proteins, and color reflects statistical significance (red = higher significance, blue = lower significance). Enriched processes include cytoskeleton-dependent intracellular transport, microtubule-based movement, and vesicle trafficking, highlighting activation of intracellular transport and cytoskeletal dynamics following HI treatment.

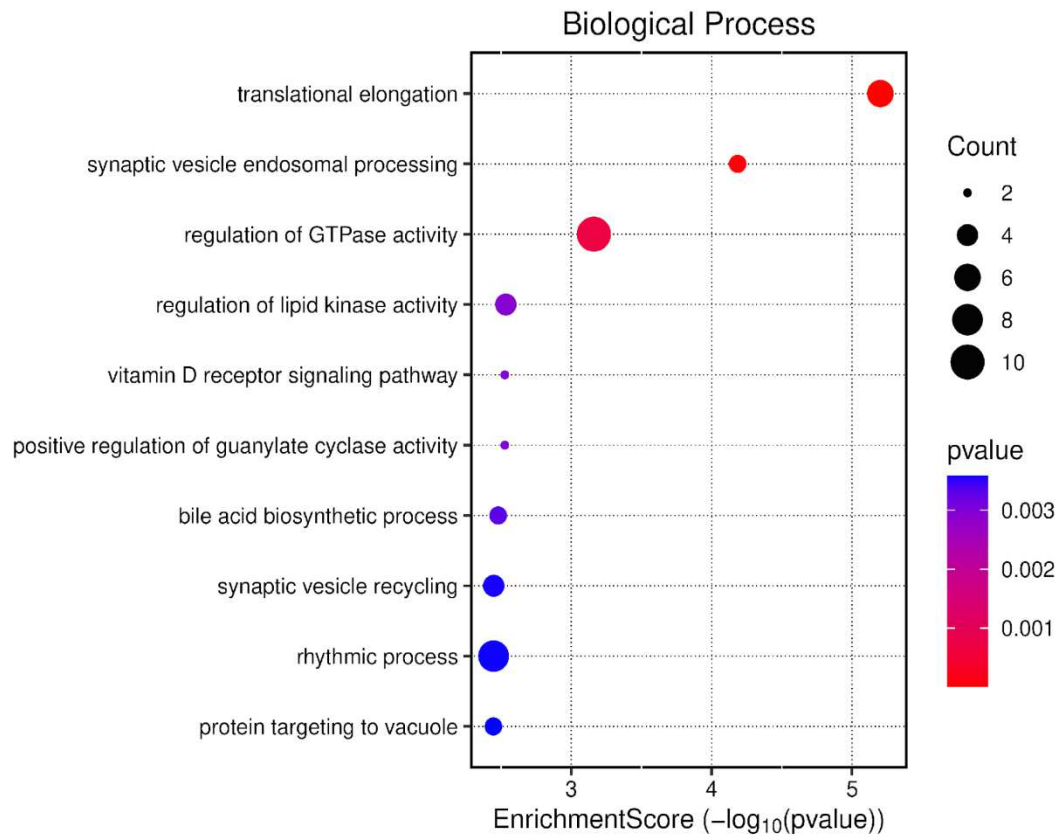


Figure 18: Gene Ontology (GO) biological process enrichment analysis of proteins upregulated following CPT treatment. Dot plot showing significantly enriched biological processes among upregulated proteins. The x-axis represents enrichment score ($-\log_{10} \text{ p-value}$), dot size indicates the number of proteins associated with each process, and color reflects statistical significance (red = higher significance, blue = lower significance). Enriched processes include translational elongation, regulation of GTPase activity, and vesicle-related processes.

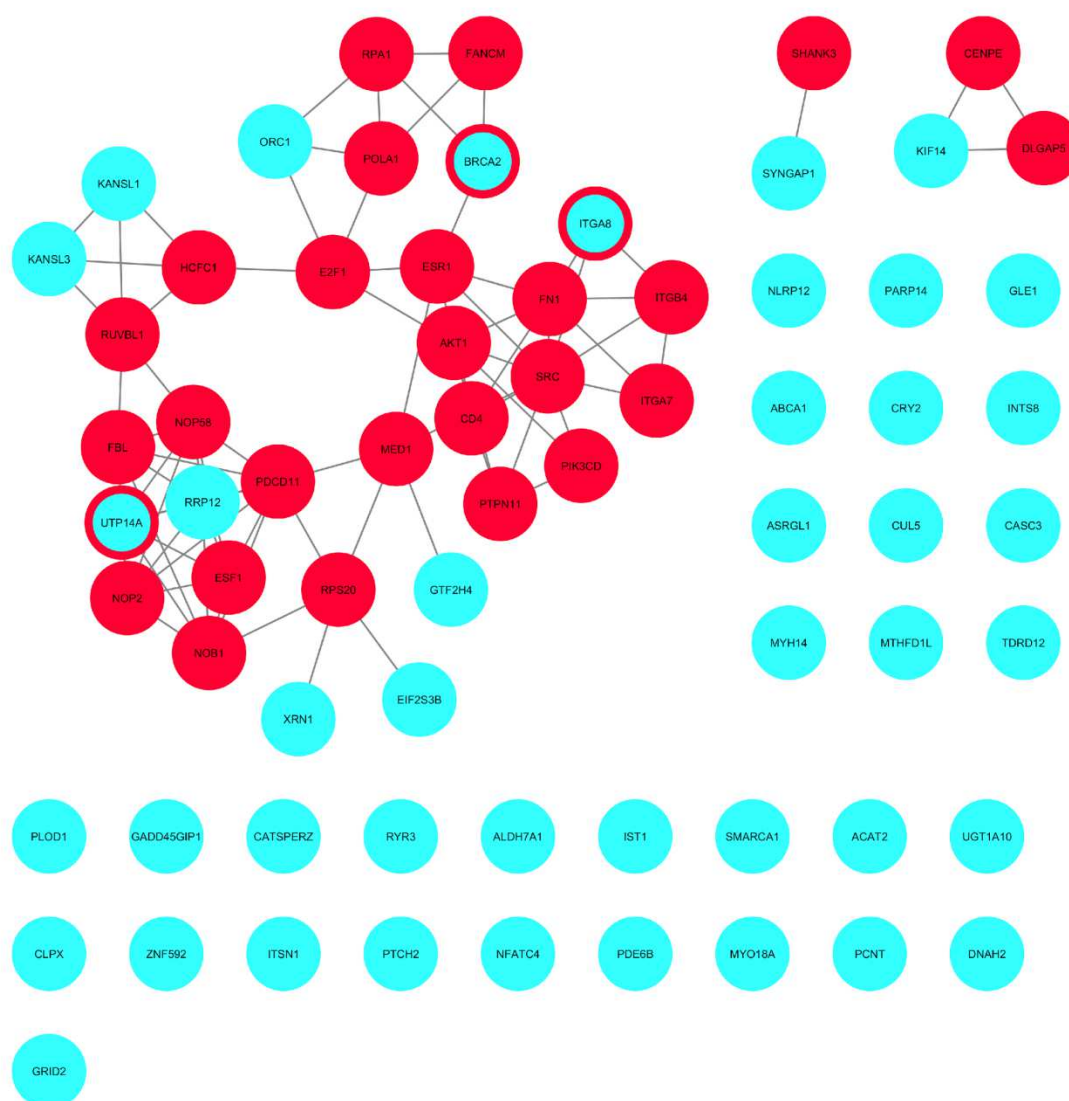


Figure 19: PPI network of proteins downregulated following HI treatment. Protein–protein interaction (PPI) network derived using STRING and pictured in Cytoscape. All the hub proteins (high degree centrality) are shown in red, seed proteins in cyan, and proteins exhibiting both hub and seed characteristics are represented with a red border and cyan core. The network highlights key interaction modules associated with cytoskeletal organization, cell adhesion, and signalling pathways.

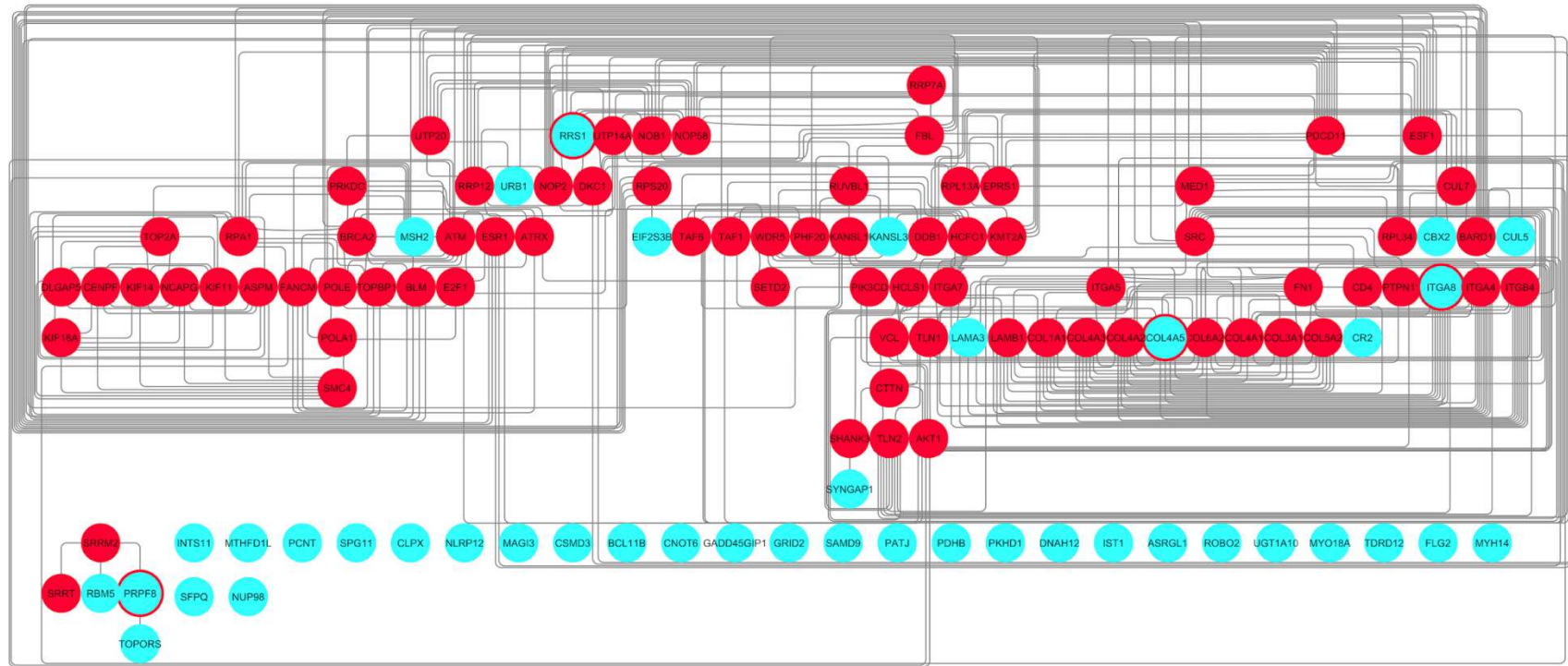


Figure 20: PPI network of proteins downregulated following CPT treatment. Protein–protein interaction (PPI) network derived using STRING and pictured in Cytoscape. Hub proteins are shown in red, seed proteins in cyan, and proteins exhibiting both characteristics are highlighted with a red border and cyan core. The network demonstrates densely interconnected modules associated with DNA replication and repair, ribosome biogenesis, epigenetic regulation, and extracellular matrix organization.

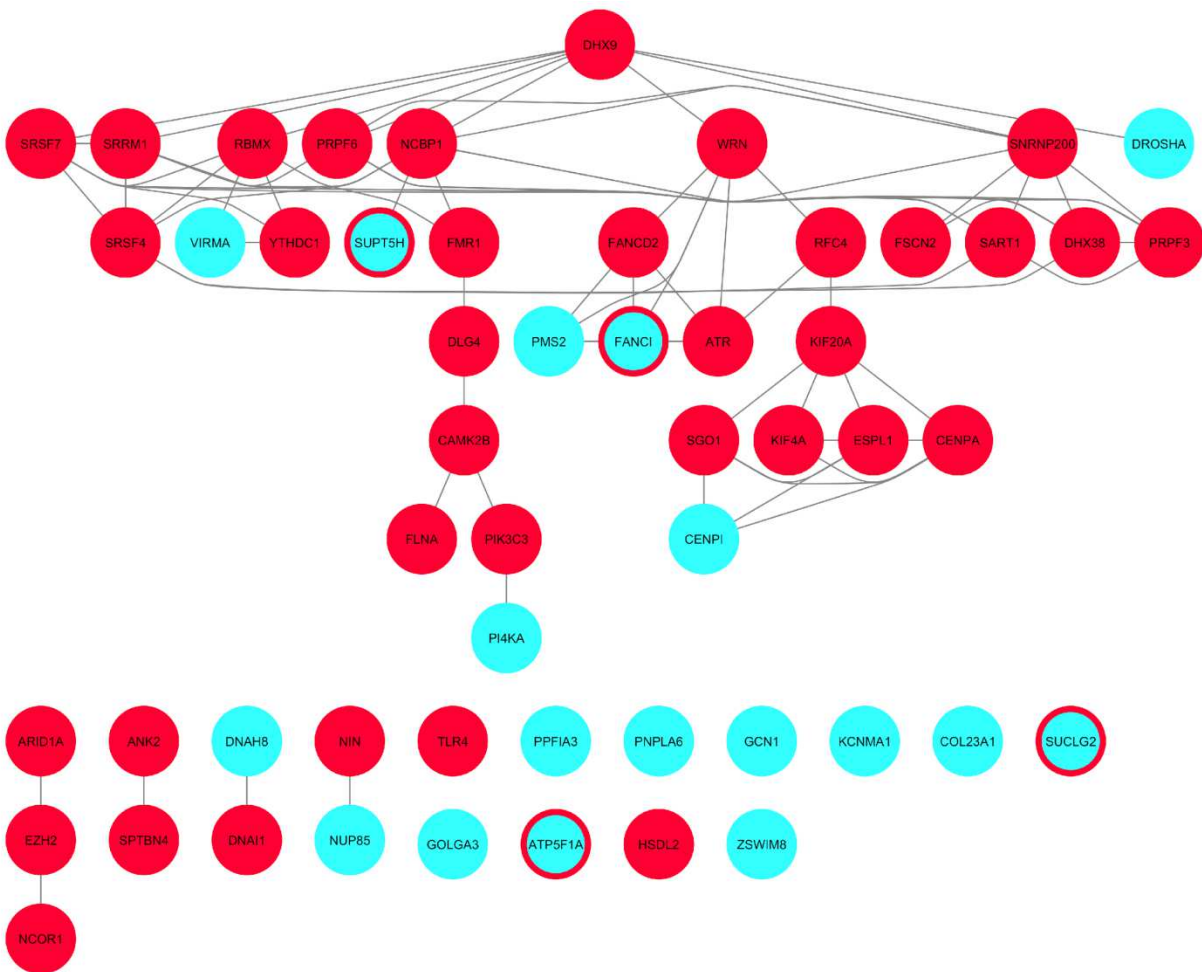


Figure 21: PPI network of proteins upregulated following HI treatment. Protein–protein interaction (PPI) network derived using STRING and pictured in Cytoscape. Hub proteins are shown in red, seed proteins in cyan, and proteins with both hub and seed properties are indicated by a red border with cyan core. The network reveals interaction clusters related to RNA processing, DNA repair, and cell cycle regulation.

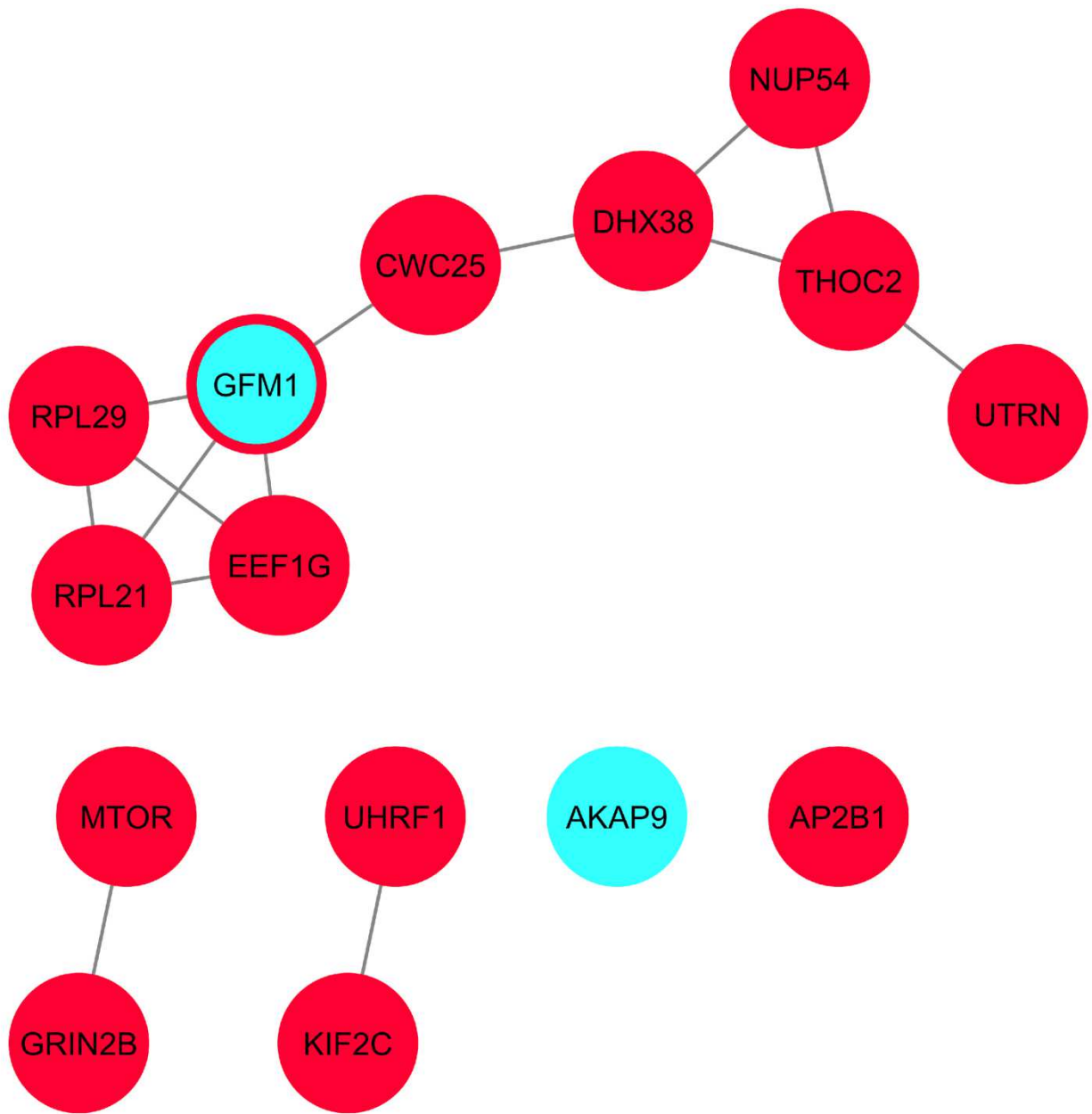


Figure 22: PPI network of proteins upregulated following CPT treatment. Protein–protein interaction (PPI) network derived using STRING and pictured in Cytoscape. Hub proteins are shown in red, seed proteins in cyan, and proteins with both hub and seed features are indicated by a red border with cyan core. The network highlights interaction modules associated with translation, RNA processing, and signalling pathways.

Supplementary Files

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

- [Table030526.docx](#)
- [SupplimentaryTable080526.docx](#)