MATERIALS AND METHODS

General

Unless otherwise stated, analytical/molecular biology grade reagents and solvents were purchased from Sigma-Aldrich (UK), Millipore Corporation (USA), Loba Chemie Pvt. Ltd. (India), AppliChem GmbH (Germany) and Qualigens Fine Chemicals Pvt. Ltd. (India). All glassware were sterilized and dried prior to use; consumable chemicals and plasticware were discarded according to institutional biosafety guidelines. All absorbance readings were taken using a UV-1600 PC UV/Vis Spectrophotometer (VWR International, USA). Microvolumetric readings were obtained using a NanoDrop™ 2000 Spectrophotometer (Thermo Fisher Scientific, USA). Microscopic images were taken using a Primo Star Upright Microscope (Carl Zeiss Microscopy, USA) with ZEN lite Digital Imaging Software. Lyophilization of samples was done using a Benchtop FDB-5502 Freeze Dryer (Operon Co. Ltd., Korea). For chromatographic separation, Easy-nLC 1000 HPLC system (Thermo Fisher Scientific, USA) was used in a 2-column configuration with a Thermo Acclaim PepMap C18 trap reversed-phase column (75 µm x 2 cm nanoviper, 3 µm particle size). All statistical analysis and computational simulation were done using an Intel® Core™ i5-6200U CPU @2.3-2.4 GHz with 16.0 GB 2400 MHz running on 64-bit Windows 10 Operating System.

Preparation of Samples

Collection of Fruit Materials

Fruits of 14 (~500g of each fresh sample) different fruit varieties preserved in germplasm were harvested from the Citrus Research Centre (CRC), Bangladesh Agricultural Research Institute (BARI) (http://crc.jaintiapur.sylhet.gov.bd) located at Jaintapur, Sylhet, Bangladesh in October 2021, and were identified by the scientific officers there. Hybrid origins for the collected samples are adapted from the Citrus Genome Database (https://www.citrusgenomedb.org) while local names were assigned upon consultation with the officers at CRC and the local sellers in Sylhet. One particular node to be noted is the naming of *Citrus reticulata blanco* which is contested as Chinese Mandarins and Rangpur in previous literature [1]–[3], naming provided by CRC was adopted in this manuscript, which recognizes it as a local variety. Notably, *Citrus nobilis* (Tangor/) is missing from the collected samples due to harvest time desynchronization.

Extraction and Quantification of Plant Peptides

Previous reports of citrus peptide isolation mostly relied on phytochemical setups using organic solvent fractionations that acquired peptides as byproducts rather than targeted isolates [4], [5]. While no optimized protocols for antioxidant or anti-inflammatory peptide isolation from plant samples have been established, a generalized procedure for separation of primarily antimicrobial peptides were adapted and modified for this study [6], [7].

Isolation of Peptide-Rich-Extracts (PREs) from Fruit Samples

Fruits were surface sterilized with 70% ethanol (EtOH) (2x) and dH_2O; pulps and peel were collected separately – seeds and membranes were removed. 25g of samples[1] ($n \geq 3$ for each fruit except Jara Lebu and Pummelo) was homogenized in a chilled mortar pestle with 1:2 (w/v) extraction buffer with the addition of liquid N_2. Homogenized tissue with the extraction buffer was incubated at 4C for 30 minutes, filtered using a microfiltration membrane (Whatman 7000-0002, 0.2µM) and then centrifuged at 12,000 RPM at 4C for 15 minutes. The supernatant was collected and stored at -20C.

Concentration/Purification of PREs

The extracted samples were first defatted using 95% n-Hexane according to previously used methods [7], [8]. Briefly, samples were mixed with n-Hexane at 1:5 ration (v/v) and incubated at 4C for 30 min followed by centrifugation at 10,000 RPM at 4C for 20 minutes; the supernatant was discarded.

The defatted samples were then subjected to chilled (-20C) organic solvent precipitation using 95% acetone (2x) [1:7 (v/v)], 100% Trichloroacetic Acid (TCA) [20:1 (v/v)] and 95% EtOH [1:3 (v/v)] according to previous reports[6]–[8] with slight modifications. The solvent-sample mixtures were incubated at 4C overnight (10 minutes for EtOH and TCA) followed by centrifugation at 6,000 RPM (10,000 RPM for TCA and EtOH) for 5 minutes at 4C and the pellet obtained was dried at room temperature and reconstituted to 15mL with 0.1M Phosphate Buffer Saline (PBS).

Quantification of Total Protein and Purity

Aliquots of 500µL reconstituted samples (as triplicates) were used to measure total protein quantity using the Bradford protocol [9] without modifications using 62.5 to 1500µg/mL Bovine Serum Albumin (BSA) standards. Parallelly, NanoDrop 280nm absorbances were

[1] From this point forward in this manuscript, pulp and peel of each fruit will be considered as separate samples.

collected for each sample using 1µL aliquots (as triplicates) for comparison and purity determination (nucleic acid contamination). Additionally total phenolic content of each purification step was quantified according to the improved Folin-Ciocalteu method[10] using gallic acid as standard in order to detect possible oxidation substrate noise.

in-vitro Intestinal Phase Digestion of Samples

Once the desired purification/concentration was obtained, the samples (10mg/mL) were subjected to static *in-vitro* intestinal phase digestion adapted from the INFOGEST protocol [11] with the supplantation of pancreatin with Trypsin. 1mL of trypsin solution was added (20mg/ml trypsin, 0.1mol/l sodium bicarbonate, 0.3 mol/L calcium chloride; preheated at 37C) to each tube; pH was fixed to 7.2 and kept at 37C overnight to mimic intestinal digestion of dietary proteins. 0.1% acetic acid was added to lower pH to 4.0-4.5 to inactivate the Trypsin, followed by centrifugation at 12,000 RPM for 5 minutes at 4C to remove the protease and the digested PREs (dPREs) in the supernatant were stored at -20C.

Detection of Low Molecular Weight Protein Fragments using SDS PAGE

SDS PAGE was performed using the Peters protocol [12] with modifications from Judd and Walker for low MW fragment/peptide detection [13], [14]. 1 mg sample was mixed with 1mL of solubilizing buffer and boiled for 5 minutes. 1X crosslinker resolving, spacer and stacking gels were prepared in a minigel casting tray (Gel dimensions: 10cm×8.5cm×1.5mm = 12.75 cc) and 15uL (1µg/µL) of sample mixtures and 10 µL molecular-mass markers were loaded onto wells. Gel electrophoresis was performed at 120 V at constant voltage for 3.5 hours. When the dye front reached the bottom of the gel, electrophoresis was terminated, and gel was placed in 200 mL of fixer/destainer solution and incubated for 30 minutes. 200 mL 0.1% CBB solution was added and incubate at 50°C for 60 minutes and left overnight in destainer. Subsequently, gel was placed in dH2O, and gently mixed until the background is completely clear and photographed using gelLITE Gel Documentation System (Cleaver Scientific Ltd, UK) under white light.

in-vitro Evaluation of Antioxidant Capacity of dPREs

A plethora of antioxidant peptides have been identified in the last few decades; while the mode of further evaluation varies – determination of antioxidant activity remains unchanged, majorly depending on radical scavenging, metal chelating/reducing, and cytoprotective capacity determination *in-vitro* [15]–[17]. In this study, a set of well-established procedures to determine the antioxidant capacity of the dPREs was used.

Free Radical Scavenging Assays

The dPREs were tested for their ability to scavenge stable free radical molecules using 2,2-diphenyl-1-picrylhydrazyl (DPPH) and hydrogen peroxide (H_2O_2) according to previous methods [18], [19] without change.

DPPH Scavenging Assay

1mL aliquots of treatment were mixed with 1mL 0.004% DPPH solution (reconstituted using 95% EtOH). Triplicates of 8 concentrations (3.125-400µg/mL; reconstituted with PBS) of standard (Quercetin) were used to determine the linear interval of quantification. The mixture was incubated at room temperature for 30 minutes under dark conditions and absorbances were taken at 517 nm to construct a standard curve.

Hydroxyl Scavenging Assay

For determination of hydroxyl free radical scavenging capacity, 800µL aliquots of treatment were mixed with 2mM 600µL of H_2O_2 solution. The mixture was incubated at room temperature for 10 minutes under dark conditions and absorbances were taken at 230 nm and a curve was generated similarly to DPPH.

Calculation and Interpretation

Based on the standard curves, concentrations of samples (reconstituted with PBS) were taken and the experiments were repeated accordingly for each sample (x3) and blank (PBS). For both assays, % of scavenging activity was calculated as:

$$\%SA = \frac{Absorbance_{Blank} - Absorbance_{Treatment}}{Absorbance_{Blank}} \times 100$$

Effective concentration (EC_{50}) for DPPH and H_2O_2 radical scavenging were derived from concentration vs %Scav plots and calculated as:

$$EC_{50} = \frac{50 - Intersept_{Conc.\ vs\ \%SA}}{Slope_{Conc.\ vs\ \%SA}}$$

Metal Chelating/Reducing Power Assays

The dPREs were examined for their capacity to chelate/reduce Ferric (Fe^{3+}) ions using previously established protocols for reducing power assay [20] and Ferric reducing ability of plasma (FRAP) assay [21] with slight modifications.

Reducing Power Assay

585µL 0.2M phosphate buffer and 585µL 1% (w/v) potassium ferricyanide was added to 235µL aliquots of different concentrations (25-200µg/mL) of dPREs, standard (Quercetin) or blank (PBS). Mixtures were incubated at 50C for 20 minutes. 585µL 10% (v/v) TCA was added to the mixture to stop the reaction followed by centrifugation at 3,000 RPM for 10 minutes at 4C. 800µL of supernatant from each tube was mixed with 800µL PBS and 0 .1% (w/v) 160µL ferric chloride. Final absorbance was measured at 700 nm.

Calculation and Interpretation

% Reduction of Fe^{3+} was calculated as:

$$\%\text{Red} = \frac{Absorbance_{Treatment} - Absorbance_{Blank}}{Absorbance_{Treatment}} \times 100$$

Effective concentrations (EC_{50}) were derived from concentration vs %Scav plots and calculated as:

$$EC_{50} = \frac{50 - Intersept_{Conc.\ vs\ \%Red}}{Slope_{Conc.\ vs\ \%Red}}$$

FRAP Assay

Standard curve was constructed using iron [II] sulphate ($FeSO_4.7H_2O$; reconstituted with dH_2O) using a series of dilutions (62.5-2,000µg/mL). Triplicates of 600µL aliquots (200µg/mL) of treatment, standard (Quercetin; reconstituted with PBS) or blank (PBS) were mixed with 1,200µL FRAP reagent and incubated at 37C under dark condition for 30 minutes. The mixture tubes were cooled and absorbance was taken at 593nm.

Calculation and Interpretation

FRAP Value was measured using $FeSO_4$ standard curve to determine amount of released Fe^{2+} ions as:

$$FRAP\ Value = \frac{Absorbance_{Treatment} - Intersept_{Conc.\ vs\ Absorbance_{FeSO4}}}{Slope_{Conc.\ vs\ Absorbance_{FeSO4}}}$$

– expressed as mmol $FeSO_4.7H_2O$/100µL.

in-vitro Evaluation of Antihyperglycemic Capacity of dPREs

Antihyperglycemic or antidiabetic peptide research is a rich avenue for novel peptide characterization. Previous studies rely on major hyperglycemia inducing digestive enzymes which are targeted for identification of antihyperglycemic peptides [22]–[24]. In this study, two such enzymes, α-amylase and α-glucosidase were used to test for inhibition potentials of the dPREs.

α-Amylase Inhibition Assay

Standard curve was constructed using soluble starch (reconstituted with dH_2O) using a series of dilutions (62.5-2,000µg/mL). 200µL active lyophilized α-Amylase (Extra pure fungal α-amylase purchased from Loba Chemie, India) (4U/mL; reconstituted in PBS, pH 7.0) was added to 160µL of 200µg/mL sample peptides or standard (Metformin HCl; reconstituted with 0.1M PBS) and 0.1% (w/v) 400µL soluble starch solution. The reaction tubes were incubated for 15 minutes at 37C. Reaction was stopped with 10% TCA and absorbance was measured at 540nm.

α-Glucosidase Inhibition Assay

Previously described method for α-glucosidase inhibition assay [25] was taken as a template but chromogenic substrate p-nitrophenyl-α-D-glucopyranoside was substituted with soluble starch (0.1% w/v; reconstituted with dH_2O) and enzyme activity was inferred from substrate conversion.

Starch standard curve was constructed similar to α-amylase assay. 100µL standard (Quercetin) or sample peptides (200µg/mL) was added to 50µL yeast α-glucosidase (1U/mL; reconstituted in 0.1M PBS, pH 7.0) and incubated at 37C for 20 minutes. Reaction was stopped with 10% TCA and absorbance was measured at 540 nm.

Calculation and Interpretation

For both assays, inhibition of enzyme was calculated as:

$$\%\text{Inhibition} = \frac{Absorbance_{Blank} - Absorbance_{Treatment}}{Absorbance_{Blank}} \times 100$$

in-vitro Evaluation of Anti-thrombotic Activity of dPREs

Previously reported anti-thrombotic/anticoagulant peptides rely on clotting factor (serine proteases) inhibition activity of peptides that are primarily identified from prothrombin time[26]–[29]. This study, in addition to conventional hematological assays, included direct

inhibition of serine protease and platelet aggregation inhibition potentials as markers for anti-thrombotic activity of samples.

Prothrombin Time (PTT) Assay

Freshly collected blood samples from healthy non-medicated non-smoker volunteers (n=9, male 6, female 3; aged 21-24) were stored K2-EDTA collection tube. Prothrombin time was determined as previously described [30] with minor changes incorporated.

Briefly, aliquots of the blood samples were centrifuged at 3,000 RPM for 15 minutes at 4C and the clear supernatant (platelet rich plasma) was again centrifuged for 10 minutes to collect and pool the platelet poor plasma (PPP). 200µL of the pooled PPP was mixed with 100µL of each sample (400µg/mL) in triplicates and incubated at 37C for 15 minutes. 300µL $CaCl_2$ was added into each reaction tube and time required for clot formation was measured with a stopwatch. Standard (Warfarin; reconstituted with 0.1M PBS) concentrations of 25-400µg/mL and blank (PBS) were used for comparison.

Serine Protease Inhibition Assay

Serine protease inhibition assay was performed according to the Kunitz protocol [31] with minor modifications. For replication purposes, 350µL aliquots of different concentrations (25-400µg/mL) of sample peptides, standard (Quercetin) or blank (PBS) were added to 350µL serine protease (Extra pure trypsin purchased from SciChem, UK; 0.1mg/mL in 0.1M PBS) solutions and incubated at 37C for 15 minutes.

700µL of preheated 1% casein (w/v in 0.1M PBS) was added to start the reaction and further incubated for 30 minutes at 37C. Finally, the reaction was stopped with 600µL 10% (w/v) TCA, centrifuged at 12,000 RPM for 15 minutes and absorbance was measured at 280 nm. Since peptides absorb light at 280 nm, additional blanks for each sample were prepared for accurate calculation.

Calculation and Interpretation

Inhibition of serine protease was calculated as:

$$\%\text{Inhibition} = \frac{Absorbance_{Treatment} - Absorbance_{Blank}}{Absorbance_{Treatment}} \times 100$$

Resting Platelet Aggregation Inhibition Assay

Measurement of resting platelet/thrombocyte aggregation was adapted from previous methods [32], [33] and modified for spectrophotometric quantitation of aggregation inhibition. Aliquots

of the collected blood samples described in the last assay was centrifuged at 3,000 RPM for 10 minutes at 4C to obtain platelet rich plasma (PRP). The PRP was diluted with Tyrode's buffer to correspond to 0.5 absorbance at 540 nm. Sample peptides (400µg/mL) were added to each tube and incubated for 10 minutes at 37C. Absorbance at 540 nm was taken after incubation and compared with blank (0.1M PBS) and positive control (Aspirin; reconstituted with 0.1M PBS) of concentrations 25-400µg/mL.

Calculation and Interpretation

Inhibition of platelet aggregation was calculated as:

$$\%\text{Inhibition} = \frac{Absorbance_{Blank} - Absorbance_{Treatment}}{Absorbance_{Blank}} \times 100$$

in-vitro Evaluation of Anti-inflammatory Potentials of dPREs

Inflammatory peptide design and discovery procedures incorporate a combined approach of computational, *in-vitro* and *in-vivo* testing methods [34]–[36]. In this study, the samples were interrogated for their capacity for inflammatory enzyme inhibition (15-LOX) and erythrocyte membrane stabilization.

15-Lipoxygenase Inhibition Assay

15-LOX inhibition assay was carried out for the sample peptides using lyophilized 15-LOX (Extra pure soybean lipoxidase purchased from Sigma Aldrich, US) according to the original method described by Wangensteen[37] with slight alterations.

50-200µg/mL concentrations of 25µL aliquots of peptides, standard (Quercetin) or blank (0.1M PBS) was mixed with 975µL 15-LOX solution (3,000 U/mL; reconstituted with 0.2M borate buffer; pH 9.0). An additional double blank was prepared without enzyme (25µL 0.1M PBS+975µL borate buffer) for baseline. 517.5µM LnA substrate solution (reconstituted with 0.2M borate buffer; pH 9.0) was introduced to each tube to start the reaction. Absorbance of solution was measured at 1-minute intervals for 10 minutes.

Calculation and Interpretation

Absorbance vs time plot for each sample was prepared (ΔAbsorbance/ΔTime) and the slope was measured for enzyme inhibition as follows:

$$\%Inhibition = \frac{Slope\left(\frac{\Delta Absorbance}{\Delta Time}\right)_{Blank} - Slope\left(\frac{\Delta Absorbance}{\Delta Time}\right)_{Treatment}}{Slope\left(\frac{\Delta Absorbance}{\Delta Time}\right)_{Blank}}$$

Erythrocyte Membrane Stabilization Assay

Membrane stabilization assays were performed as reported in previous studies (Gandhidasan et al., 1991). Freshly collected blood samples from healthy volunteers were prepared as described in **3.4.2,** centrifuged at 3,000 RPM for 10 minutes at 4C and the pellet (erythrocyte layer) was collected for this assay. The erythrocyte pellets were pooled and washed with 0.9% sodium chloride and subsequently centrifuged at 3,000 RPM for 5 minutes (3x). The erythrocytes were then reconstituted to 10% (v/v) isotonic suspension using 0.1M PBS and 180µL aliquots were incubated with 900µL of 25-400µg/mL solutions of dPREs or standard (Aspirin; reconstituted with 0.1M PBS); dH_2O was used as blank. Afterwards, lytic stress was induced using two different stimulants (heat and hypotonicity) as described herewith

Heat-induced Hemolysis

Quadruplicates of reaction mixtures were taken and one of the tubes were stored in 4C (Unheated) while the other tubes were heat-induced at 54C for 20 minutes with intermittent inversion. After incubation, unlysed cells were settled by centrifugation at 5,000 RPM for 5 minutes at 4C; subsequently absorbances of all mixtures were taken at 560 nm.

Calculation and Interpretation

Inhibition of heat-induced hemolysis was calculated as:

$$\%\text{Inhibition} = \left(1 - \frac{Absorbance_{Treatment} - Absorbance_{Unheated}}{Absorbance_{Blank} - Absorbance_{Unheated}}\right) \times 100$$

Hypotonicity-induced Hemolysis

Quadruplicates of reaction mixtures were taken with the supplantation of 0.1M PBS isotonic buffer with hypotonic buffer in three of the tubes. All tubes were incubated at 37C for 20 minutes and performed similarly to heat-induced hemolysis.

Calculation and Interpretation

Inhibition of hypotonicity-induced hemolysis was calculated as:

$$\%\text{Inhibition} = \left(1 - \frac{Absorbance_{Treatment} - Absorbance_{Isotonic}}{Absorbance_{Blank} - Absorbance_{Isotonic}}\right) \times 100$$

Shotgun Proteomics Approach for Identification of Peptides

For peptide identification from the mixed samples, selected peptide isolates were bulk extracted and purified as mentioned in previous sections (500mg final product suspended in 300mL 0.1M PBS). They were subsequently lyophilized at -55C under vacuum for 28 hours with 0.1M glucose and 0.1M sodium ascorbate as added cryoprotectants. The dried samples were reconstituted and validated for retained bioactivity.

Separation was achieved at 300nL/min using 0.1% (v/v) formic acid in acetonitrile as mobile phase for elution with a 75µm x 25cm PepMap RSLC C18 Easy-Spray Column at 35C. Peptide elution was performed with a 3–10% acetonitrile gradient for 10 min followed by 10–38% acetonitrile gradient for 47 min. The total acquisition time was 70 min. The eluted peptides were introduced to the mass spectrometer via nano-ESI and analyzed using the Q-Exactive Plus (Thermo Fisher Scientific). Full MS scans were acquired in the Orbitrap mass analyzer over the range m/z 50–1,000.

The raw files generated were analyzed on Proteome Discoverer 2.1 using SEQUEST HT as a search engine against a composite database constructed from PlantPepDB [39], Citrus Genome Database (https://www.citrusgenomedb.org) and GenBank non-redundant protein database limited by *Citrus* (NCBI: txid2706). MS1 was selected as precursor for higher order MS spectra, and lower and upper retention time (RT) limit was set to 0-60. The scan selected 1 to 2 charged state ions in positive mode with a minimum and maximum precursor mass of 100Da and 3,000Da. A full scan in positive mode polarity was collected and the peaks were filtered with a signal to noise (S/N) threshold of 1.3 and peak intensities were normalized. The parameters were set for the filtration of relevant spectra where the minimum and maximum peptide length were set at 2 and 20, respectively, and the digestion cleavage parameter was set to trypsin cleavage. No post-translational modifications (PTMs) were predicted to detect for. The peptide spectra match with maximum Δmass of 15 ppm were grouped, validated and visualized.

Tertiary Structure and Pharmacokinetic Parameters of Identified Peptides

Prediction of 3D Structure from Peptide Sequences

Linear sequences obtained from LC-MS spectra were used to predict the tertiary structure of individual peptides using the PEPstrMOD server (for sequences ≥ 7); no PTMs were selected for; 100 ps simulations were conducted in vacuum and best model topology were selected as

predicted tertiary structures. Peptide sequences <7 was generated using Discovery Studio 2021 Client[40]. Structures for FDA approved therapeutic peptides were collected from THPdb database [41] based on "Immunological", "Cardiac" and "Hematological" disease area parameters.

Prediction of Pharmacokinetic Properties of Peptides

Hydropathicity, molecular weight and theoretical iso-electric point (pI) of the detected peptides were determined using grand average of hydropathy (GRAVY) calculator server (http://www.gravy-calculator.de). Additionally, peptide *in-vivo* half-life values, instability index and cellular penetration capacities were estimated using linear sequences via the ExPASy ProtParam tool [42] and CPPred-RF server [43].

Prediction of Toxicity Profiles of Peptides

Allergenicity profiles of the peptides were predicted using AllerTOP v2.0 (https://www.ddg-pharmfac.net/AllerTOP) server based on sequence auto cross covariance transformation mining.

Sequences were also used to predict for toxic peptides using the ToxinPred database [44]; support vector machine (svm) algorithm was set to SwissProt method, E-value=10, svm threshold was set to 0.1.

Furthermore, peptide structure files were used to generate input files (Canonical SMILES) for prediction of peptide organotoxicity, carcinogenicity and cytotoxicity probabilities using the Protox-ii server [45].

Molecular Docking of Peptides with Proteins of Interest

Ligand Structure Preparation

3D ligand structures of sample peptides, therapeutic peptides and drug compounds were optimized through minimization and polar protonation (pH = 7.4) according to established protocols [46] using Biovia Discovery Studio Modeling Environment 3.5 [40].

Receptor Crystal Structure Acquisition and Validation

3D crystal structures of inhibitor-bound open conformations of the target receptors from the RCSB-PDB database. The structures were optimized using Biovia Discovery Studio Modeling Environment 3.5. The crystals were titrated and protonated at pH = 7.4, co-crystal water, non-cofactor ligand molecules were removed and the appropriate binding cavities were determined using the DeepSite [47] server (Score≥9.0). All crystal structures were subjected to the

PROCHECK algorithm [48] for stereochemical quality assessment in order to ensure accurate docking pose predictions. Estimations of the whole-model reliability of the 3D structures was performed by evaluating the QMEAN Z-score using the ProSA server [49].

Docking Simulation

Fourier transform (FFT) based docking using HEX 8.0.0 [50] standalone software for the peptides was employed, followed by flexible refinement using FlexPepDock module of Rosetta modeling package [51]. For HEX 8.0.0, a receptor box of 25Åx25Åx25Å was considered near the binding site of each target protein and post-docking OPLS forcefield minimization was carried out.

Structure-based flexible molecular docking of the ligands into the receptor binding cavities using the DockThor Portal [52]. Monomeric subunits of the proteins were used as receptor files and grids were manually defined around the binding cavity for running the docking simulations. DMRTS method was employed for the simulation with 100,000 evaluations per run with an initial population of 750 and 25 runs per ligand.

In addition to peptides and drug molecules, 20 decoy molecules per complex were generated using the DUD-E database [53] to assess the specificity of the docking protocol. The scoring function used to score the docked poses of the is based on the MMFF94S force field and is expressed as:

$$\Delta G = \Delta E\ (vdW) + \Delta E\ (Electrostatic) + \Delta E\ (Intramolecular) + \Delta E\ (Tortional)$$

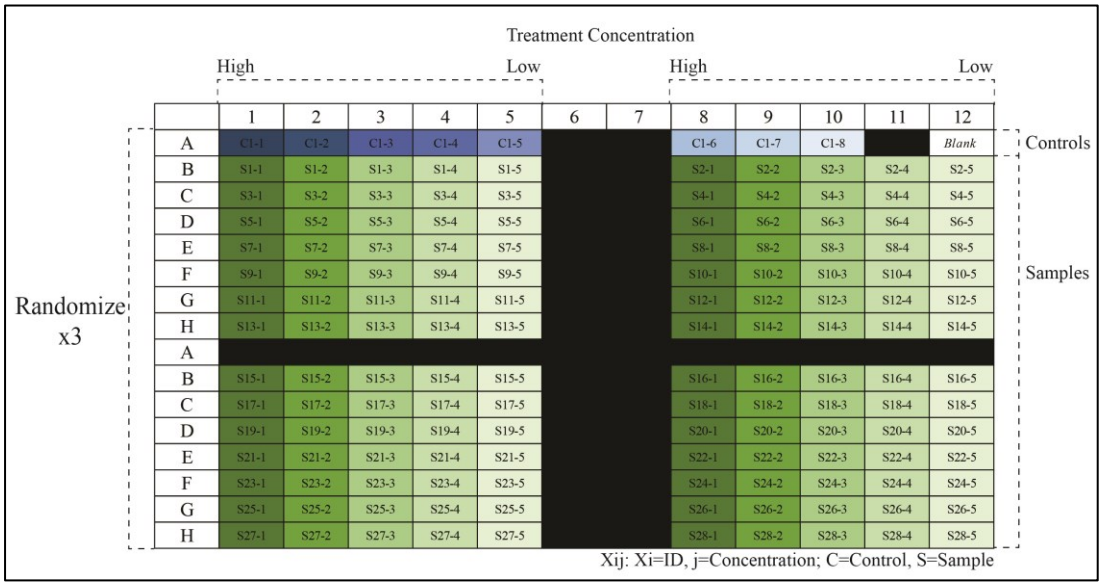
KEGG Pathway Interaction Probability Analysis

In order to determine KEGG network perturbations by peptides and control molecules, deep self-normalizing neural-network (DSNNN) based metabolic pathway interaction probability predictions were simulated using the PathwayMap tool [54]. Canonical SMILES of peptides and controls (retrieved from PubChem database; https://pubchem.ncbi.nlm.nih.gov) were used as input and the interaction probabilities of the molecules to KEGG pathways were retrieved.

Experimental Design and Statistical Analysis

Unless otherwise stated, all experiments were conducted in triplicates, using non-treated buffer systems or decoys as negative controls. All *in-vitro* experiments were carried out in 2mL Eppendorf tubes and assigned in a randomized set layout.

in-silico studies used tool/server built-in statistical tools for statistical validation (**p < 0.005; *p < 0.05) of results against test databases. All values of dependent variables were subjected to independent samples t-test (**p < 0.005; *p < 0.05) to determine their level of significance. Pearson correlation coefficient value was employed for intervariable correlation and evaluated using 2-tailed paired t-test.



	1	2	3	4	5	6	7	8	9	10	11	12
A	C1-1	C1-2	C1-3	C1-4	C1-5			C1-6	C1-7	C1-8		*Blank*
B	S1-1	S1-2	S1-3	S1-4	S1-5			S2-1	S2-2	S2-3	S2-4	S2-5
C	S3-1	S3-2	S3-3	S3-4	S3-5			S4-1	S4-2	S4-3	S4-4	S4-5
D	S5-1	S5-2	S5-3	S5-4	S5-5			S6-1	S6-2	S6-3	S6-4	S6-5
E	S7-1	S7-2	S7-3	S7-4	S7-5			S8-1	S8-2	S8-3	S8-4	S8-5
F	S9-1	S9-2	S9-3	S9-4	S9-5			S10-1	S10-2	S10-3	S10-4	S10-5
G	S11-1	S11-2	S11-3	S11-4	S11-5			S12-1	S12-2	S12-3	S12-4	S12-5
H	S13-1	S13-2	S13-3	S13-4	S13-5			S14-1	S14-2	S14-3	S14-4	S14-5
A												
B	S15-1	S15-2	S15-3	S15-4	S15-5			S16-1	S16-2	S16-3	S16-4	S16-5
C	S17-1	S17-2	S17-3	S17-4	S17-5			S18-1	S18-2	S18-3	S18-4	S18-5
D	S19-1	S19-2	S19-3	S19-4	S19-5			S20-1	S20-2	S20-3	S20-4	S20-5
E	S21-1	S21-2	S21-3	S21-4	S21-5			S22-1	S22-2	S22-3	S22-4	S22-5
F	S23-1	S23-2	S23-3	S23-4	S23-5			S24-1	S24-2	S24-3	S24-4	S24-5
G	S25-1	S25-2	S25-3	S25-4	S25-5			S26-1	S26-2	S26-3	S26-4	S26-5
H	S27-1	S27-2	S27-3	S27-4	S27-5			S28-1	S28-2	S28-3	S28-4	S28-5

Figure **Experiment Layout for *in-vitro* Assay Systems**. Treatment varied systems (Eppendorf tubes) were placed in separate rows while concentrations were gradiated in columns. The columns and rows were assigned randomly sorted for replication of the experiment replicates (x3).

Table Peptide Extraction Buffer Composition

Serial	Component	Final Concentration
01	Potassium phosphate (dibasic)	5% (v/v)
02	Sodium EDTA	0.5 M
03	Triton X-100	1% (v/v)
04	Glycerol ≥99.5%	80% (v/v)
05	β-mercaptoethanol	5% (v/v)
06	pH 7.5-7.8 (using 0.1M HCl and NaOH Pellets)	

Table 0.1M Phosphate Buffer Saline Composition

Serial	Component	Final Concentration
01	Sodium chloride	0.137 M
02	Potassium chloride	0.0027 M
03	Disodium hydrogen phosphate dihydrate	0.001M
04	Potassium dihydrogen phosphate	0.000176 M
05	pH 7.4 (using 0.1M HCl and NaOH Pellets)	

Table 0.2 M Phosphate Buffer Composition

Serial	Component	Final Concentration
01	Disodium hydrogen phosphate dihydrate	0.008 M
02	Monobasic sodium phosphate	0.016 M
03	pH 6.6 (using 0.1M HCl and NaOH Pellets)	

Table FRAP Reagent Composition (60mL)

Acetate Buffer		
Serial	Component	Final Concentration
01	Sodium acetate	0.31% (w/v)
02	Glacial acetic acid	1.6% (v/v)
03	pH 3.6 (using 0.1M HCl and NaOH Pellets)	
TPTZ Solution		
Serial	Component	Final Concentration
01	2, 4, 6-tripyridyl-s- triazine (TPTZ)	0.009 M
02	HCl (37%)	0.004 M
03	Kept at 50C for 30 minutes	
FRAP Reagent		
Serial	Component	Final Volume
i	Acetate Buffer	50mL
ii	Preheated TPTZ Solution	5mL
iii	dH_2O	5mL
Total Volume		60mL

Table Tyrode's Solution Composition

Serial	Component	Final Concentration
01	Sodium chloride	0.093M
02	Potassium chloride	0.005M
03	Magnesium sulphate	0.001M
04	Sodium bicarbonate	0.02M
05	Sodium biphosphate	0.001M
06	Glucose anhydrous	0.01M
07	Sodium acetate	0.02M
08	pH 7.4 (using 0.1M HCl and NaOH Pellets)	

Table Hypotonic Suspension Buffer Composition

Serial	Component	Final Concentration
01	Sodium chloride	0.0013 M
02	Potassium chloride	0.0071M
03	pH 7.4 (using 0.1M HCl and NaOH Pellets)	

SDS PAGE PROTOCOL

Reagents and Buffer Preparation

- Separating/spacer gel acrylamide (1X crosslinker for ≥10 kDa band separation)
- 48 g acrylamide, 3.0 g N,N'-methylene-bis-acrylamide.
- Bring to 100 mL, and then filter through qualitative paper to remove cloudiness
- Separating/spacer gel buffer
- 3 M tris-base, 0.3% sodium dodecyl sulfate. Bring to pH 8.9 with HCl.
- Stacking gel acrylamide
- 30 g acrylamide, 0.8 g N,N'-methylene-bis-acrylamide.
- Bring to 100 mL, and then filter through qualitative paper to remove cloudiness.
- Stacking gel buffer
- 1 M Tris-HCl, pH 6.8.
- Cathode (top) running buffer (10X stock)
- 1 M Tris-base, 1 M tricine, 1% SDS. Dilute 1:10 immediately before use. pH about 8.25.
- Anode (bottom) buffer (10X stock)
- 2 M Tris-base. Bring to pH 8.9 with HCl. Dilute 1:10 immediately before use.
- Sample solubilization buffer
- 2 mL 10% SDS (w/v) in dH2O, 1.0 mL 100% glycerol, 0.625 mL 1 M Tris-base, 0.5% (v/v) β-mercaptoethanol, pH 6.8, 6 mL dH2O, 0.1% (w/v) bromophenol blue to color.
- Fixer/destainer
- 25% isopropanol, 7% glacial acetic acid in dH2O (v/v/v)
- 0.2 M Na-EDTA.
- 10% ammonium persulfate (make fresh as required).
- TEMED.
- 1% Coomassie brilliant blue (CBB) (w/v) in fixer/destainer.
- Molecular-mass markers
- Qualitative Filter Paper.
- Distilled water (dH2O).

GEL PREPARATION (prepare before use)

Separating Gel Recipe

- 6.7 mL water
- 10 mL separating/spacer gel buffer
- 10 mL separating/spacer gel acrylamide
- 3.2 mL glycerol
- 10 µL TEMED
- 100 µL 10% ammonium persulfate.

Spacer Gel Recipe (optional)

- 6.9 mL water
- 5.0 mL separating/spacer gel buffer
- 5 µL TEMED
- 50 µL 10% ammonium persulfate.

Stacking Gel Recipe

- 10.3 mL water
- 1.9 mL stacking gel buffer
- 2.5 mL stacking gel acrylamide
- 150 µL EDTA
- 7.5 µL TEMED
- 150 µL 10% ammonium persulfate.

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