[2] MATERIALS AND METHODS
2.1. MATERIALS

All the chemicals, enzymes and other consumables with finest quality were purchased from specialized companies such as

**Sigma-Aldrich Chemical Co. (St Louis) USA:** 8-Anilinonaphthalene-1-sulfonic acid, agarose, anti-rabbit alkaline phosphatase (conjugate), blue dextran, bovine serum albumin, bromelain, chymotrypsin, Congo red, , cytochrome C, ficin, 2-mercaptoethanol, ovalbumin, papain, pepsin, PVDF membrane, sephacryl S 100-HR, soybean trypsin Inhibitor Thioflavin T, Baicalin hydrate. Baicilin, (-)-Gallocatechin, Glyoxal.

**Genel Pvt. Ltd. Bangalore, India:** Freund’s complete and incomplete adjuvants, Molecular weight markers, medium range (PMW-M).

**Sisco Research Lab (SRL. India):** Acetic acid, acrylamide, n-butanol, casein, Coomassie brilliant blue-R250, copper sulphate, L-cysteine, 5, 5’-Dithio-bis-2-nitrobenzoic acid (DTNB), Folin’s reagent, Hydrochloric acid (HCl), N, N’ methylene bis-acrylamide, poly ethylene glycol (PEG-1000 and PEG-4000), sodium azide, sodium carbonate, sodium chloride, sodium hydroxide, sodium potassium tartrate.

**SERVA Electrophoresis GmbH, USA:** Urea, thiourea, 3-[(3-Cholamidopropyl)-dimethylammonio]-1-propane sulfonate (CHAPS), servalyte, glycerol, tris-HCl, Dithiothreitol (DTT) and iodo-acetic acid (IAA) in a 2D-kit.

**Bio-Rad laboratories, USA:** Tween-20, PBS, secondary antibody in an ELISA-kit.

**Qualigens, India:** Ammonium persulphate, ammonium sulphate, bromophenol blue, disodium hydrogen phosphate, ethanol, ethylene diamine tetra-acetic acid (EDTA), glycerol, isopropanol, methanol, monosodium dihydro orthophosphate phosphate, sodium dodecyl sulphate, sodium potassium tartrate, sulphuric acid, TEMED, trichloroacetic acid (TCA), tris-(hydroxymethyl) aminomethane (Tris-base).

**Others:** Amiodarone, nitroglycerine.
2.2. METHODS

2.2.1. PURIFICATION AND CHARACTERIZATION OF BUFFALO HEART CYSTATIN (BHC)

Purification of BHC

The purification of BHC was done by the method of Hirado et al. (1981) with slight modifications. Buffalo heart whole mass (100 g) was brought fresh from slaughter house, thoroughly washed with distilled water and after removing the perineurium homogenized in homogenization buffer (50 mM sodium phosphate, 3 mM EDTA, 0.15 M NaCl, 2% butanol, pH 7.5) in a homogenizer and ultrasonication was done by an ultrasonic probe sonicator (model number DP-120) for 2 minutes after which it was centrifuged at 5000 rpm for 10-15 minutes at 4 °C in a Sigma cooling centrifuge (3K30 model, Germany).

Alkaline treatment

The supernatant was collected was subjected to alkaline treatment at pH 11.0 by 3 M sodium hydroxide (NaOH) and incubated for 30 min at 4°C to remove unwanted proteins. The precipitated proteins were removed by centrifugation at 8000 rpm for 30 min at 4°C in the cooling centrifuge. And after collecting the supernatant, the pH of the supernatant was brought back to 7.5 with glacial acetic acid.

Ammonium sulphate fractionation

The supernatant thus collected was made subjected to 30-70% ammonium sulfate saturation and the solution was again allowed to stand for 1-1.5 h at 4°C, the precipitate thus obtained after centrifugation under similar conditions as mentioned above, was dissolved in minimum amount of 50 mM sodium phosphate buffer pH 7.5. The fraction thus obtained was extensively dialyzed against several changes of the same buffer at 4°C containing 1% NaCl to remove ammonium sulphate.
Gel filtration chromatography

A sephacryl S 100-HR column was prepared as recommended by Peterson and Sober (1962). Pre-swollen gel suspended in ethanol was soaked in sufficient amount of double distilled water and washed at least thrice. The gel fines were removed by suspending the gel in two to four fold excess of 50 mM sodium phosphate buffer, pH 7.5 and gel was allowed to settle down. A glass column mounted on a sturdy vertical support and after fitting the glass-wool on its opening near the narrow opening which was fitted with rubber tubing. After clamping the rubber tubing the column was filled to one third of its length with operating buffer in order to check leaks and flush air bubbles from the dead space. The de-aerated gel slurry was poured with the help of glass rod in to the column with care to avoid generating air bubbles. The column was left standing overnight. Flow rate was increased gradually and after accomplishing a constant flow rate higher than that required for final elution, the column was adjusted to the required flow rate. The packed column was thoroughly washed with two bed volumes of operating buffer (50 mM sodium phosphate buffer, pH 7.5). In order to check uniform packing and to determine void volume of the column, 2% (w/v) solution of blue dextran in 50 mM sodium phosphate buffer (pH 7.5) was passed through the column. The volume of the blue dextran and protein solution applied was not more than 2-3% of the total bed volume. The dialyzed sample was subjected to gel filtration chromatography on Sephacryl S-100 HR column (70 x 2 cm) equilibrated with 50 mM sodium phosphate buffer pH 7.5. The flow rate of the column was 15 ml.h⁻¹. Fractions (5 ml) were collected and Papain inhibitory activity was checked in all fractions by Kunitz method (1947). Fractions with inhibitory activity were pooled, concentrated and kept at 4 °C for further experiments. Homogeneity was analyzed by 7.5% polyacrylamide gel electrophoresis (PAGE).

2.2.2. COLRIMETRIC ANALYSES

Determination of protein concentration

Protein concentration was estimated by the method of Lowry et al. (1951). Aliquots of protein solution were taken in test tubes and final volume was made up to 1 ml with distilled water. 5 ml. of alkaline copper reagent (containing one part of 1%
(w/v) copper sulphate and 2% (w/v) sodium potassium tartarate in 1% (w/v) sodium hydroxide and sodium carbonate) was added and then after 10 min of incubation at room temperature, 0.5 ml. of 1N Folin’s reagent was added. The tubes were instantly vortexed. The color developed was read after 30 min at a wavelength of 660 nm against the reagent blank. A standard curve was prepared using bovine serum albumin (BSA) as standard and the concentration of purified protein was determined by this standard plot.

Assay of cystatin inhibitory activity

The inhibitory activity of cystatin was assessed by its ability to inhibit caseinolytic activity of papain by the method of Kunitz (1947). Papain was activated in the presence of 0.14 M l-cysteine and 0.047 M EDTA for 10 min. prior to incubation of papain with cystatin. The papain-cystatin complex was incubated for 30 min at 37 °C in 50 mM sodium phosphate buffer, pH 7.5. The enzyme (papain) inhibitor complex was further incubated with casein for 30 min at 37 °C and the reaction was stopped by addition of 10% TCA. Acid insoluble material was removed by centrifugation at 2500 rpm for 20 min. The supernatant was analyzed for acid soluble peptides with Folin’s reagent as described by Lowry et al. (1951). Ficin inhibition was also assayed by similar method. The inhibition of bromelain activity was measured according to the method of Murachi and Neurath (1960). The enzyme was activated at 37°C for 10 min in the presence of 0.14 M cysteine. Then the volume was made up to 1 ml by 50 mM sodium-phosphate buffer pH 7.5. 1 ml of 0.5 % casein was added and incubated for 30 min at 37°C. The reaction was stopped by addition of 1 ml of 10 % TCA. Acid insoluble material was removed by centrifugation at 2500 rpm for 15 min. The supernatant was analyzed for acid soluble peptides by the method of Lowry et al. (1951). For proteolytic activity of trypsin and chymotrypsin 0.1 M sodium phosphate buffer, pH 8.0 is used. Rest of the procedure is similar to that of other proteinases.

Carbohydrate estimation

Carbohydrate content was estimated by Dubois (1956) method. 2 ml. aliquots containing 10-70 μg of protein was pipetted in to a set of test tubes and 0.05 ml. of
80% phenol was added. This was followed by the addition of concentrated sulphuric acid. The tubes were allowed to stand for 10 min at 30°C. The color intensity was measured at 490 nm for the quantification of hexose content. Glucose was used as standard.

**Thiol group estimation**

The procedure described by Ellman (1959) was followed for estimating the thiol groups of the purified inhibitor. Sodium dodecyl sulphate (SDS) and β-mercaptoethanol (β-ME) induced appearance of free thiol group in the cystatin was followed by titration with DTNB reagent. Appropriate aliquots of 0.2 ml. containing 100 μg cystatin native, SDS and β-mercaptoethanol treated cystatin were mixed with 0.1 ml. of DTNB reagent (prepared by dissolving 40 mg DTNB in 100 ml of 0.05 M Tris-EDTA buffer, pH 8.0) in a total volume of 3.1 ml. The absorbance was read after 15 min at 412 nm. Free thiol concentration was calculated from the absorbance using molar extinction coefficient of 13,600 M⁻¹ cm⁻¹ for the released thionitrobenzoic acid. A standard plot was prepared using cysteine.

**Thermal stability of the inhibitor**

100 micrograms of the inhibitor in 50 mM sodium phosphate buffer (pH 7.5) was incubated at various temperatures in the range of 30-90 °C for 30 min. These samples were rapidly cooled in ice-cold water bath and checked for residual inhibitory activity against 100 μgs of papain. Inhibitory activity was assayed by the method of Kunitz (1947), as described earlier. Another 100 μg of the inhibitor at 70 °C was incubated for different time intervals, rapidly cooled and residual inhibitory activity measured against papain.

**pH stability**

100 μg. of BHC was incubated with buffers of different pH values such as 50 mM sodium acetate buffer (pH 3.0-6.0), 50 mM sodium phosphate buffer (pH 7.0-8.0), and tris-NaOH buffer (pH 10.0-12.0) for 30 min at 37 °C. This mixture was used for determination of remaining inhibitory activity against 100 μg of papain.
2.2.3. HOMOGENEITY AND MOLECULAR WEIGHT

Native PAGE (Polyacrylamide gel electrophoresis)

Electrophoresis was performed by the method of Laemmli (1970) using the slab gel apparatus manufactured by GeneI, India. Concentrated stock solution of 30% acrylamide containing 0.8% N, N' methylene bis-acrylamide and 1.5 M Tris, pH 8.8, were mixed in appropriate portion to give the desired concentration of gel. It was then poured in to the mold formed by the glass plates (8.5-10 cm) separated by 1.5 mm thick spacers. Bubbles and leak were avoided. A comb providing template for seven wells was inserted into the stacking gel solution before the polymerization began. The polymerization was complete in about 30 min after which the comb was removed and wells overlaid with running buffer. Routinely 7.5% and 10% gels were used. Samples containing 40-60 μg of protein were mixed with one fourth volume of 0.001% bromophenol blue as tracking dye. Electrophoresis was performed at 100V in the electrophoresis buffer containing 192 mM glycine and 25 mM Tris-HCl (pH 6.8) until the tracking dye reached the bottom of the gel.

SDS-PAGE

SDS-PAGE Sodium dodecyl sulphate polyacrylamide gel electrophoresis was essentially performed by the Tris-glycine system of Laemmli (1970) using slab gel electrophoresis apparatus. Concentrated stock solution of 30% acrylamide containing 0.8% N, N' methylene bis-acrylamide and 1.5 M Tris, pH 8.8 were mixed in appropriate proportions to give desired percentage of gel. Protein samples were prepared in solution containing 62.5 mM Tris-HCl pH 6.8, 10% (v/v) glycerol, 2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol and 0.001% (w/v) bromophenol blue. The samples were incubated at 100 ºC for 5 min. Electrophoresis was performed at 100 V till the tracking dye reached the bottom of the gel. Running buffer used during electrophoresis contained 1% SDS in addition to 192 mM glycine and 25 mM Tris-HCl (pH 6.8).
Materials and Methods

2D-Gel electrophoresis of purified BHC

Two-dimensional gel electrophoresis, abbreviated as 2-DE or 2D-electrophoresis, is a form of gel electrophoresis commonly used to analyze a mixtures of proteins and was first independently introduced by O'Farrell and Klose in 1975. 200 µg of protein sample was added to the rehydration buffer (7M urea, 2M thiourea, 4% w/v CHAPS, 66 mM DTT, 0.5% v/v servalyte, half mini protease inhibitor tablet) in which a IPG strip (3-10 pH, 7 cm) was dipped and left overnight at 4°C for rehydration. Rehydrated strip was gently washed and placed in a focusing tray and run for 6-8 hours on IEF100 First-dimension Isoelectric Focusing Unit, Hoefer, Inc. USA. After the isoelectric focusing IPG-strip was equilibrated with the equilibration buffer-I (2g DTT added in 10 ml of equilibration buffer (6M urea, 30% w/v glycerol, 2% w/v SDS, 50 mM tris-HCl (pH 8.8)) and equilibration buffer-II (2g. Iodo-acetic acid added in 10 ml of equilibration buffer) for 15 minutes each followed by second dimensional electrophoresis of IPG strip on 12.5% SDS-PAGE gel.

Staining

After electrophoresis the gels were stained with five gel volumes of coomassie brilliant blue R-250 in 50% methanol and 10% acetic acid for at least 4 hrs. For destaining, the gels were incubated with shaking in 5% methanol and 7.5% glacial acetic acid at room temperature.

Molecular weight determination

The molecular weight of purified goat liver thiol proteinase inhibitor was determined under native and denaturing (reducing and non-reducing) conditions by SDS-PAGE and gel filtration chromatography, respectively.

Molecular weight determination by SDS-PAGE

The molecular weight of cystatin under denaturing conditions was calculated from its mobility in SDS-PAGE was calculated by the procedure of Weber and Osborn (1969). BHC was allowed to run on SDS-PAGE along with the protein...
markers in the parallel lane. The mobilities of marker proteins determined under identical conditions were plotted against the logarithms of molecular weight. The standard proteins used were phosphorylase b (97.4 kDa), bovine serum albumin (68 kDa), ovalbumin (45 kDa), carbonic anhydrase (29.1 kDa), soybean trypsin inhibitor (20 kDa) and lysozyme (14.3 kDa). The analysis of data indicated a linear relationship between log M and relative mobility ($R_m$) and the plot was used for calculating the molecular weight of BHC.

**Molecular weight determination by gel filtration chromatography**

The molecular weight of native BHC was computed from its elution volume on a sephacryl S100-HR column (70 x 2 cm). The column was calibrated by determining the elution volume of some marker proteins of known molecular weight such as Cytochrome c (12 kDa), lysozyme (14 kDa), ovalbumin (44.5kDa), BSA (66 kDa). This data was analyzed according to the theoretical treatment by the method of Andrews (1964). The linear plot between $V_e/V_o$ and log M was used for calculating the molecular weight of liver cystatin where $V_e$ is the elution volume of the protein and $V_o$ is the void volume of the column determined using blue dextran.

**2.2.4. HYDRODYNAMIC PARAMETERS**

**Stokes radius**

Stokes radius ($r$) was determined by method of Andrews (1964) and Laurent-Killander (1964) using Sephacryl S100-HR column (70x2 cm) calibrated by determining the elution volume of several globular proteins with known stokes radii, such BSA (Stokes radii 35 Å); B, ovalbumin (Stokes radii 29 Å); C, lysozyme (Stokes radii 18 Å); D, Cytochrome c (Stokes radii 16.6 Å). Stokes radii of BHC was determined by plotting a graph of - log ($K_{av}$) $^{1/2}$ vs. stokes radius ($r$) of marker proteins:

$$K_{av} = (V_e - V_o) / (V_t - V_o)$$  \(1\)
Where, $V_e = \text{Elution volume}$, $V_o = \text{Void volume}$, $V_t = \text{Bed volume of the column}$.

**Frictional coefficient**

Frictional coefficient was determined by the equation

$$f = 6\pi \eta R$$

(2)

Where, $R = \text{Stokes radii}$, $\eta = \text{viscosity}$

**Diffusion coefficient**

Diffusion coefficient was determined by the equation:

$$D = \frac{K_b T}{f}$$

(3)

Where, $K_b = \text{Boltzmann’s constant}$, $T = \text{temperature in Kelvin}$, $f = \text{frictional coefficient}$.

**Dynamic light scattering (DLS)**

DLS is a technique that can be used to determine the size distribution profile of small particles in suspension or polymers in solution. DLS measurements were carried out using DynaPro-TC-04 dynamic light scattering equipment (Protein Solutions, Wyatt Technology, Santa Barbara, CA) equipped with a temperature-controlled micro sampler. Native BHC (1mg/ml) solution was spun at 10,000 rpm for 15 min. and filtered through 0.22 and 0.02 mm Whatman’s syringe filters directly into a 12 ml quartz cuvette. For each experiment, 20 measurements were taken. Mean hydrodynamic radius ($R_h$) and poly-dispersity were analyzed using Dynamics 6.10.0.10 software at optimized resolution. The $R_h$ was estimated on the basis of an
auto correlation analysis of scattered light intensity data based on translation diffusion coefficient by Stokes-Einstein relationship.

\[ R_h = \frac{K_b T}{6\pi \eta RD} \]  

(4)

Where, \( R_h \) is the hydrodynamic radius, \( K_b \) is Boltzmann constant, \( T \) is temperature, \( \eta \) is the viscosity of water and \( D \) is diffusion coefficient.

### 2.2.5. IMMUNOLOGICAL PROPERTIES

#### Generating antiserum

Antibodies against BHC were raised by injecting 300 µg of purified BHC in Fruend’s complete adjuvant subcutaneously into healthy male albino rabbit. Booster dose were given repeatedly every week in Fruend’s incomplete adjuvant and the rabbit was bled every second week. The blood collected was allowed to coagulate at room temperature for 3h. The antisera were decomplexed at 57 °C for 30 min and stored at -20 °C in small aliquots.

#### Immunodiffusion

Immunodiffusion experiment was performed by the method of Ouchterlony (1961). 1%(w/v) agarose in normal saline containing 0.2% sodium azide was poured on glass petridish and allowed to solidify at room temperature. 20 µl of suitably diluted antiserum and 50 µg of antigen (BHC, chickpea cystatin and goat brain cystatin) prepared in normal saline were added in different wells. The reaction was allowed to proceed for 24-48 h in a moist chamber at room temperature. The chick pea (Bhat et al, 2014) and goat brain cystatin (Bhat et al., 2015) for immunodiffusion studies were also purified in our laboratory.
Direct binding ELISA

Direct binding ELISA was performed according to Voller et al. (Ninety-six wells of microtitre plate were coated with 100 ml of coating buffer (15 mM Na₂CO₃+ 35 mM NaHCO₃, pH 9.4) containing 10 µg of antigen and left overnight at 4 °C. The plate was washed thrice with phosphate buffered saline-tween 20 buffer (PBST-20 mM, pH 7.4). The unoccupied sites in each well were saturated with 200 µl of 1% (w/v) BSA in phosphate buffer for 5-6 h at room temperature and plates were washed twice with PBST. The test and the control wells were then loaded with 100 µl of serially diluted antiserum (1° antibody) and incubated for 2-4 h at room temperature or left overnight at 4 °C. 100 µl of appropriately diluted conjugates of anti-rabbit alkaline phosphatase (2° antibody) is coated in each well and kept for 2 h at room temperature. After regular washing with PBST and distilled water, the substrate orthophenyl-diamine solution (5 mg/100 ml in bicarbonate buffer, pH 9.5, and 50 mM containing 0.02% sodium azide) was added in each well and incubated for 30-45 min at room temperature. The reaction was stopped by addition of 100 µl of conc. H₂SO₄ in each well. The absorbance of each well was monitored at 492 nm on a Qualigens ELISA reader.

2.2.6. KINETICS OF INHIBITION

Stoichiometry of cystatin papain complex

The inhibitory activity of cystatin was assessed by its ability to inhibit caseinolytic activity of papain (Kunitz, 1947) and Lowry’s method (1951) was used for protein estimation. The concentration of the inhibitor was fixed at 0.06 µM and the concentration of papain was varied from 0.02-0.12 µM. Papain was activated by incubation with 0.14 M cysteine and 0.047 M EDTA for 10 min at 37°C. Identical experiments were carried out for BHC with the other proteinases, ficin and bromelain using casein as substrate (Kunitz 1947; Murachi and Neurath, 1960). The determination of binding stoichiometry was monitored by changes in the fluorescence emission intensity accompanying the interactions (Bjork et al., 1989).
**Materials and Methods**

**Determination of inhibition constant ($K_i$)**

$K_i$ determinations were carried out by lowering the enzymes and inhibitor concentrations to obtain a nonlinearity of dose-response curves. Papain, ficin and bromelain were used at a concentration of 0.06 µM to react with inhibitor in varying concentrations from 0.06 to 0.30 µM. Residual activity was measured by the method of Kunitz (1947) using casein as substrate. Four different substrate concentrations were used: 0.5 $K_m$, 1 $K_m$, 1.5 $K_m$ and 3 $K_m$ with $K_m$=2.4. The results were analyzed by the procedure of Krupka and Laidler (1959) and Handerson (1972).

\[
\frac{[I]_o}{V_i} = K_i \left( 1 + \frac{[S]}{K_m} \right) \frac{V_i}{V_o} + [E]_o
\]  

(5)

Where, $[I]_o$ = Initial substrate concentration,  
$[E]_o$ = Initial enzyme concentration  
$K_i$ = Inhibition constant  
$[S]_o$ = Initial substrate concentration  
$V_i$ = Reaction rate in presence of inhibitor  
$V_o$ = Reaction rate in absence of inhibitor  
$K_m$ = 2.4 mM for casein (predetermined)  
Slope of this plot gives $K_i$ (app.)

\[
K_i^{(app.)} = K_i \left( 1 + \frac{[S]_o}{K_m} \right)
\]  

(6)

Where, $K_i^{(app.)}$ = Apparent inhibition constant  
True $K_i$ was obtained from re-plot of $K_i^{(app.)}$ vs. $[S]_o$
Determination of dissociation rate constants (K⁻₁)

For K⁻₁ determination, the conditions for maximal association between protease (4 µM) and inhibitor (4 µM) was achieved before the reaction was shifted towards dissociation by adding excess substrate. Excess substrate (6% casein) was added to the enzyme-inhibitor [EI] complex for different time periods and assayed for enzymatic activity. Dissociation of [E] complex obeys first order kinetics. Thus, integrated form of the dissociation rate equation is given by

$$\ln \frac{[EI]}{[EI]_0} = K_{-1} t$$  \hspace{1cm} (7)

Where [EI] was the concentration of enzyme inhibitor complex at different time interval, [EI]₀ was their complex concentration initially. Slope of of log [EI]/ [EI]₀ vs. t gives the dissociation constant.

Determination of association rate constants

(K⁺₁) Dissociation rate constant is also related to inhibition constant by the equation.

$$K_i = \frac{K_{-1}}{K_{+1}}$$  \hspace{1cm} (8)

Where, Kᵢ is inhibition, K⁻₁ is dissociation and K⁺₁ is association rate constants respectively.
**Determination of half-life ($t_{1/2}$)**

$t_{1/2}$ of the complex was calculated by rearranging the equation (7) as follows

$$t_{1/2} = \frac{0.693}{K_{-1}}$$  \hspace{1cm} (9)

Where, $K_{-1}$ is the dissociation rate constant.

**Determination of IC$_{50}$**

IC$_{50}$ of the BHC with various cysteine proteases was calculated by the following equation

$$K_i = \frac{IC_{50}}{1 + \frac{[S]}{K_m}}$$  \hspace{1cm} (10)

Where, $K_i$ is inhibition constant of BHC with respective proteases, $K_m$ is the rate constant for casein (predetermined) and $[S]$ is substrate concentration. Results were analyzed by the procedure reported earlier (Rashid et al., 2004).
2.2.7. SPECTRAL ANALYSIS

The nature of interaction of thiol proteinases with the isolated inhibitor was observed through the spectroscopic changes that accompanied the binding mode of thiol proteinase papain with BHC in their specific stoichiometric ratio. These binding interactions were followed by UV–absorption difference and fluorescence emission spectroscopies.

**Fluorescence spectroscopy**

The fluorescence spectra of cystatin, papain and cystatin-papain (1:1 concentration ratio) complex were recorded at room temperature (25 °C) in the range of 300-400 nm upon excitation at 280 nm on Shimadzu Spectrofluorometer equipped with a data recorder DR-3. Protein concentration used was 5 µM and slit width for both excitation and emission was 5 nm and the path length was 1 cm appropriate controls were run and corrections made wherever necessary.

**Absorption difference spectra**

An ultraviolet absorption difference spectrum was measured for BHC along with activated papain with a molar ratio of 1:1 at room temperature Spectra were recorded by measuring the absorption between 200-300 nm on a Shimadzu UV mini-vis spectrophotometer UV-1700 using a cuvette of 1 cm path length. Appropriate controls of the solvent were run and corrections were made wherever necessary.

**Circular dichroism (CD) spectra measurements**

Far UV-CD CD-measurement was performed on JASCO spectropolarimeter (J-815). The instrument was calibrated with d-10-camphorsulfonic acid. All measurements were taken at 25°C with a thermostatically controlled cell holder attached to a peltier with multitech water circulator. The concentration of inhibitor for far-UV-CD analysis was 12.5 µM and binding study with papin was done by taking papain and inhibitor in 1:1 ratio. For near-UV-CD analysis of inhibitor the concentration was 50 µM. The path lengths used were 0.1 cm for far-UV-CD analysis and 1 cm for near-UV-CD analysis. Spectra were collected with 100 nm/min scan
Materials and Methods

Speed and response time of 1 s. Each spectrum was average of two scans. The spectra obtained were normalized by subtracting the baseline recorded for each condition. Near UV-CD spectra was taken between 190-250 nm and for near UV-CD the spectral range was 250-350 nm. To calculate the $\alpha$-helical content the far UV-CD data obtained were expressed as mean residue ellipticity (MRE) in degrees·cm$^2$·dmol$^{-1}$ which is defined as

$$\text{MRE} = \frac{\theta_{\text{obs. (mdeg)}}}{10 \times n \times l \times C_p}$$  \hspace{1cm} (11)

Where, where “$\theta_{\text{obs.}}$” is the CD in millidegrees (mdeg), “n” is the number of amino acid residues, “l” is the pathlength of the cell (0.1-mm for far-UV CD) and “$C_p$” is the molar concentration of protein.

And the $\alpha$-helical content of BHC was calculated from the MRE value at 222 nm using the following equation as described by Chen et al. (1972)

$$\% \text{ helix} = \left\{ \frac{- (\text{MRE}_{222} - 2340)}{30300} \right\} \times 100$$ \hspace{1cm} (12)

Where, MRE$_{222}$ is mean residual ellipticity at 222 nm.
2.2.8. MACROMOLECULAR CROWDING AND BHC STABILITY

Sample preparation protocol

Three set of experiments using BSA, PEG-1000 and PEG-4000 as crowding agents, were performed separately in which a fixed concentration of cystatin (0.2 mg/ml) was treated with increasing concentration (10-80 mg/ml) of respective crowding agents and kept for incubation at 37 °C and aliquots were checked at different time intervals for structural and functional changes. Solutions were shaken slowly on a stirrer for proper mixing.

Papain inhibition activity of the treated samples

Papain inhibitory activity was checked by Kunitz method (1947). 100 µg of cystatin was checked for its inhibitory activity which was assessed by its ability to inhibit caseinolytic activity of papain. The activity of untreated BHC was taken as 100% for reference.

Intrinsic fluorescence

Intrinsic fluorescence is used to study the conformational and structural change in the proteins and peptides (Longworth, 1983). Fluorometric analysis was done by RF-1501 spectrofluorometer (Shimadzu Co. Japan). Intrinsic fluorescence of BHC was recorded with an excitation wave length of 280 nm at which both tryptophan and tyrosine residues gets excited and emit energy in the range of 300-400 nm. Protein concentration of 0.2 mg/ml was fixed for these measurements.

Far UV- CD spectra measurements

CD-measurements were performed on JASCO spectropolarimeter (J-815). Far UV-CD (Ultraviolet Circular Dichroism) spectroscopy is used to assess the secondary structure of proteins such as α-helix, β-sheet and random coil. The instrument was calibrated with D-10-camphorsulfonic acid. All measurements were taken at 25°C with a thermostatically controlled cell holder attached to a peltier with multitech water
Materials and Methods

circulator. 0.2 mg protein was used to take far-UV CD spectra (190-250 nm) and the spectra were taken in cuvette of 0.1 cm path length along with 100 nm/min scan speed and response time of 1 s. Each spectrum was average of two scans. The spectra obtained were normalized by subtracting the baseline recorded for each condition.

ANS fluorescence

ANS is a naphthalene dye that upon binding to the hydrophobic regions of proteins shows a dramatic increase in its fluorescence. ANS was prepared in 50 mM sodium-phosphate buffer, pH 7.5, and appropriate controls/blanks were also included in the assay. Fluorescence was measured by using an excitation wavelength of 380 nm and emission was recorded between 300-400 nm. Slit width for both excitation and emission was 5 nm and path length was 1 cm. ANS concentration was taken 100 molar excess of protein concentration as proposed by Matulis et al. (1999).

Thioflavin T (Th T) fluorescence

ThT is an azo-dye which specifically binds to protein aggregates. It was employed to monitor the extent of aggregation of BHC in presence of the various crowding agents. ThT was prepared in 50 mM sodium phosphate buffer and Protein: ThT molar ratio was taken as 1:5. ThT-fluorescence was recorded by exciting the samples at 440 nm and emission was recorded between 450-600 nm (Naiki et al., 1990). Slit width for both excitation and emission was 5 nm and path length was 1 cm.

Congo red (CR) binding assay

Spectrophotometric analysis of CR and BHC amyloid binding was performed by double beam Shimadzu UV-1700 (Japan) Spectrophotometer. Similar to ThT fluorescence assay, Congo red binding assay detects the amyloid fibers (Steenstra 2001; Stathopulos et al., 2004) and thus was performed to study the amyloidogenesis of the BHC treated with increasing concentrations of the various crowding agents. Proteins and dye ratio was 1:8. After 20-30 minutes of incubation at 37 °C, the CR
absorption spectra were recorded between 400 and 600 nm with a path length of 1.0 cm.

**Scanning electron microscopy (SEM)**

200 μl of sample of protein solution was placed on a cover slip and dried. Cover slip was then glued to aluminum stub with adhesive tape and kept at room temperature for 30 minutes. After that samples were directly sputtered with gold/palladium in autofine sputter coater (JEOL, Japan, and JFC-1600) and examined using a scanning electron microscope, JSM-6510 LV (JEOL, Japan) at University Sophisticated Instruments Facility (USIF), Aligarh Muslim University, and Aligarh, India. No carapace shrinkage occurred during the whole process of SEM studies.

**2.2.9. INTERACTION BHC WITH ZnO AND Fe₂O₃ NANOPARTICLES**

**Synthesis of ZnO**

ZnO nanoparticles were synthesized by a gel-combustion route. In typical synthesis process, the required molar ratios of zinc nitrate and citric acid were completely dissolved in a beaker to obtain a 50 ml aqueous solution. The solutions were mixed and homogenized on magnetic stirrer at room temperature, keeping citric acid to metal cation ratio unity. The solution was heated to about 150 °C on the magnetic stirrer in order to mix the solution uniformly and was evaporated under constant stirring until it converts into gel form. The product was ignited on persistent heating, giving voluminous and fluffy product on combustion. The resultant material was heated at about 600 °C followed by grinding to fine powder.

**Synthesis of Fe₂O₃**

The Iron oxide nanoparticles were prepared using a controlled co-precipitation method. FeCl₃·6H₂O (6.794 g, 25 mmol), FeCl₂·4H₂O (2.479 g, 12.51 mmol), and 1 mL of 38% (v/v) HCl were dissolved in 24 mL of HPLC ultrapure water under vigorous stirring. The co-precipitation of Iron oxide nanoparticles was achieved by
Materials and Methods

Adding the Iron solution to 150 mL of 2M KOH (under stirring at 1000 rpm), which was preheated to 75 °C. The reaction was carried out for 1 hour under the nitrogen. These particles were collected by sedimentation with the help of an external magnet and washed several times with HPLC ultrapure water until a stable ferro-fluid was obtained. The precipitate thus formed was isolated by centrifugation. The nanoparticles were further washed with HPLC ultrapure water followed by acetone and annealed in a furnace for 7 hours at 700 °C.

CHARACTERIZATION OF NANOPARTICLE

X-RAY diffraction studies

The crystallinity was determined by XRD powder diffraction. XRD diffractograms of nanoparticles were recorded on a MiniFlexTM II bench top XRD system (Rigaku Corporation, Tokyo, Japan) operating at 40 kV. Dried ZnO particles were deposited as a randomly oriented powder into a plexiglass sample container, and the XRD patterns were recorded between 5° and 50° angles, with speed of 5.0 deg/min.

Fourier transformed infrared spectroscopy (FTIR)

FTIR analysis of Nanoparticles was performed using Spectro lab Interspace 2020 spectrometer. A disk of 1:3 ratio of KBr was prepared with a mixture of dried nanoparticles and then examined under IR Spectrometer. Infrared spectra were recorded in the transmission mode in region of 400 to 4000 cm⁻¹.

Scanning electron microscopy (SEM)

Scanning electron micrographs of nanoparticles were taken by a JEOL (JSM840) scanning electron microscope at a voltage of 15 kV to study the morphological behavior of the nanoparticles. The elemental analysis was determined using the Oxford Instruments INCAx-sight energy dispersive X-ray (EDAX) spectrometer equipped SEM. Specimen were prepared by ultrasonic stirring of aqueous dispersion, dropping some of the solution onto a glass slide, and get it air dried. Then these specimens were sputter coated with a thin gold/palladium layer of
about 3 nm thick in vacuum in autofine sputter coater (JEOL, Japan, and JFC-1600) and examined under SEM.

**Transmission electron microscopy (TEM)**

The morphological analysis such as average particle size, size distribution and shape were examined using a Philips Morgagni 268-D transmission electron microscope, TEM, FEI (USA) at a voltage of 120 kV. The aqueous dispersion of the particles was drop cast onto the carbon coated copper grid and the grid was air dried at room temperature before loading into the transmission electron microscope.

**Cystatin nanoparticle interaction (SAMPLING)**

All samples were prepared in 50 mM sodium phosphate buffer (pH 7.5). As nanoparticles have a tendency to form aggregates in solution, the colloidal suspension was sonicated extensively before use (Chatterjee et al. 2010). 100 μM concentration of cystatin was prepared as a stock from which various experiments were done after suitable dilution independently. Stock solution was mixed with varying concentrations of nanoparticles (100-500 μM) and incubated at 37 °C.

**Assay of cysteine protease inhibitor activity**

The inhibitory activity of cystatin was assessed by its ability to inhibit caseinolytic activity of papain by the method of Kunitz (1947) as described earlier and the acid soluble peptides were determined with Folin’s reagent as described by Lowry et al. (1951).

**Intrinsic fluorescence**

Fluorometric analysis was done by RF-1501 spectrofluorometer (Shimadzu Co. Japan). Intrinsic Fluorescence of cystatin was recorded in the range of 300-400 nm with an excitation wave length of 280 nm at which both tryptophan and tyrosine residues gets excited and emit energy (Longworth, 1983), slit with was 5 nm for both excitation and emission and the path length was 1 cm. Protein concentration of 5 μM was used to take the fluorescence spectra.
UV spectrophotometric analysis

Spectrophotometric analysis of was performed by double beam Shimadzu UV-1700 (Japan) spectrophotometer. The absorption spectra were recorded between 195-350 nm with a path length of 1 cm.

Isothermal titration calorimetry (ITC)

Isothermal titration calorimetric measurements were carried out on a VP-ITC titration microcalorimetry system (MicroCal, Northampton, MA). Solutions were filtered and degassed using a Thermovac. The sample cell was filled with 10 µM BHC at the pH 7.5 and the reference cell with respective buffer (50 mM sodium-phosphate buffer pH 7.5). 100 µM nanoparticles were introduced into a syringe of 250 µl volume, ensuring the removal of any trapped air bubbles. The titration experiments consisted of 29 injections of 10 µL each of duration 20 s with a 2 s filter period and 180 s spacing between each injection. The stirring speed was 307 rpm. The analog input range was ±21.25 V and the reference power was set at 20 µcal s⁻¹.

Circular dichroism (CD) study

CD spectra of protein samples were recorded on a J-810 Jasco CD spectropolarimeter calibrated with ammonium D-10-camphorsulfonate. Path length of 0.1 cm was used for far UV-CD scanning between 190-250 nm. Each spectrum was the average of 3 scans (Greenfield et al., 2007). Protein concentration for the scan was 12.5 µM.

FTIR analysis

FTIR spectra of the native cystatin and cystatin treated with nanoparticles were recorded by INTERSPEC 2020 model. The samples were injected by Hamiet 100 ll syringe in ATR box. Proceeding to loading, the syringe was first washed by acetone followed by distilled water. FTIR absorption spectra of all the proteins samples were measured on the same samples of 1,500–1,800 cm⁻¹ spectral region. FTIR analysis was done to monitor the interaction of the cystatin with nanoparticles. Original spectra of native BHC along with BHC co-incubated with nanoparticles at
37°C were taken with a fixed concentration of cystatin (60 µM) and an increasing concentration of nanoparticles (60-300 µM) with a resolution of 4 cm$^{-1}$ and 128 scans. The changes in peak frequency and intensity were then assigned to conformational changes within the protein.

**TEM analysis of BHC-NPs interaction**

The physical evidence of BHC-NPs interaction was analyzed using JEM 2100 transmission electron microscope (Jeol Japan), at a voltage of 120 kV.

### 2.2.10. INTERACTION OF CARDIOVASCULAR DRUGS (AMIODARONE AND NITROGLYCERINE) WITH BHC

#### Sample preparation

Samples were prepared in 50 mM sodium phosphate buffer (pH 7.5). In two independent sets of experiments (one for each drug) 100 mM stock of cystatin was incubated with increasing concentrations of drugs (10 µM to 100 µM) in 50mM, in a final reaction volume of 2 ml at 37 ºC for 2 hours. Aliquots were taken from the stock to perform further experiments.

#### Fluorescence measurements

Intrinsic fluorescence spectra were taken on a spectrofluorimeter model RF-5301PC (Shimadzu, Japan) equipped with a 150W xenon lamp at different temperatures 298K, 308K and 318K. The fluorescence was recorded in wavelength region 300-400 nm after exciting the protein at 280 nm. The slits were set at 5 nm for excitation and emission. The path length of the sample was 1 cm.

#### Stern-Volmer analysis

The fluorescence quenching data was analyzed by the Stern-Volmer equation

$$\frac{F_0}{F} = 1 + K_{sv} [Q] \quad (13)$$
Where, \( F_0 \) and \( F \) are the steady-state fluorescence intensities in the absence and presence of quencher, respectively, \( K_{sv} \) the Stern-Volmer quenching constant and \([Q]\) is the concentration of the quencher.

**Determination of binding constant (K) and number of binding sites (n)**

When small molecules bind independently to a set of equivalent sites on a macromolecule, the equilibrium between free and bound molecules is given by the equation (Feng et al., 1998; Goa et al., 2004).

\[
\log \frac{F_0 - F}{F} = \log K + n \log [Q] \tag{14}
\]

Where \( K \) and \( n \) are the binding constant and the number of binding sites, respectively.

**The thermodynamic parameters**

The thermodynamic parameters were calculated in order to elucidate the interaction between the drug and BSA, which was determined from the Van’t Hoff equation:

\[
\ln K_b = -\left(\frac{\Delta H}{RT}\right) + \left(\frac{\Delta S}{R}\right) \tag{15}
\]

where \( \Delta S \) is the entropy change, \( \Delta H \) is the enthalpy change, \( R \) is the universal gas constant, and \( K_b \) is the constant which is analogous to the Stern-Volmer quenching constants \( K_{sv} \) at the corresponding temperature. The enthalpy change (\( \Delta H \)) and the entropy change (\( \Delta S \)) can be determined from the slope and intercept of the fitted curve of \( \ln K_b \) against \( 1/T \), respectively. The free energy, \( \Delta G \), can be estimated from the following relationship:

\[
\Delta G = \Delta H - T\Delta S \tag{16}
\]
Materials and Methods

UV measurements

The UV measurements of BHC in the presence and absence of drugs were recorded in the range of 195-350 nm and the inhibitor BHC. Absorption spectra were recorded on a double beam Shimadzu UV-vis spectrophotometer UV-1700 using a cuvette of 1 cm path length.

Activity measurements

The inhibitory activity of BLC was assessed by its ability to inhibit caesinolytic activity of papain by the method of Kunitz (1947). The inhibitor was incubated with increasing concentration of drugs for different time intervals at 37 °C before the activity was measured.

CD analysis

CD spectra of protein samples were recorded on a J-810 Jasco CD spectropolarimeter as explained earlier. Protein concentration for the scan was 12.5 µM. The results were expressed as the mean residue ellipticity (MRE in cm². dmol⁻¹).

2.2.11. BHC-GLYOXAL INTERACTION: EFFECT OF POLYPHENOLS

SAMPLE PREPARATION

Aggregation of BHC in the presence of glyoxal

BHC (15 µM) was incubated with varying concentrations of glyoxal (10, 20, 30, 40, 50, 60, 70 and 80 µM) in 50 mM sodium phosphate buffer at 37 °C. Before incubation samples were passed through a 0.2 µm filter to remove dust or traces of aggregated material.
Anti-aggregation presence of polyphenols (baicalin and gallocatechin)

To study the effects of baicalin and gallocatechin on fibrillation of BHC prior to induction of fibril formation two sets of samples with varying concentrations (10-100 µM) of baicalin and gallocatechin (10-100 µM) were added to the reaction mixture (15 µM BHC incubated with glyoxal).

ANS fluorescence

ANS is a compound used for probing the available hydrophobic domains in proteins. Fluorescence study was done using Shimadzu RF-5301 spectrofluorometer (Tokyo, Japan). Its binding was measured by exciting the samples at 380 nm and emission recorded from 400 to 600 nm. Typically, ANS concentration was 50-fold to that of the protein concentration which was around 1.5 µM.

FAR UV-CD measurement

Circular dichroism (CD) measurements were carried out on a JASCO spectropolarimeter (J-816). The instrument was calibrated with D-10-camphorsulfonic acid. All the CD measurements were carried out at 25 °C with a thermostatically controlled cell holder attached to a Neslab RTE-110 water bath with an accuracy of ±0.1 °C. Spectra were collected with a scan speed of 100 nm/min and a response time of 2s. Each spectrum was average of three scans. Far-UV CD spectra measurements were carried at a protein concentration of 0.2 mg in the range of 190-250 nm range in a cell of 0.1 cm path length.

FTIR analysis

FTIR analysis was done by INTERSPEC 2020 model. The samples were injected by Hamiet 100 ll syringe in ATR box. Proceeding to loading, the syringe was first washed by acetone followed by distilled water. Spectra of the native as well as treated BHC were measured on between 1500-1800 cm⁻¹ spectral region. The changes in peak frequency and intensity were then assigned to conformational changes within the protein.
Materials and Methods

Th T assay

Th T fluorescence of the samples were measured using a Shimadzu RF 5301 spectrofluorophotometer (Tokyo, Japan). Aliquots, withdrawn from the sets of BHC fibrillar solutions with and without polyphenols, and were diluted using 50 mM sodium phosphate buffer to achieve final protein concentration of 1.5 µM. 1: 5 ratio of protein and Th T was mixed with the solution. Sample was excited at 440 nm and emission was taken in the range of 450-700 nm. Excitation and emission slit widths were 5 nm.

Congo red assay

Congo red dye binding assay was used for probing the amyloid aggregates in presence of glyoxal in presence/ absence of baicalin and gallocatechin using UV-spectrophotometer (UV-VIS 1700 Shi-madzu, Japan). Protein concentration was kept 5 µM and protein: dye concentration was 1:8. Absorption was taken in the range of 400-700 nm. Path length was 1 cm.

Transmission electron microscopy (TEM)

Fibril like aggregated structure of BHC and treated samples with polyphenols were assessed by applying 10 µl of sample on a carbon coated copper grid and left to adsorb for 1 min. After excess fluids were removed from the grid surface, the grid was washed with distilled water and stained with 0.3% aqueous uranyl acetate. Excess stain was removed and the samples were dried at room temperature. Specimen prepared were then observed using JEM 2100 transmission electron microscope (Jeol, Japan).