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Proteomic Analysis of HepG2 Cells Reveals FAT10 and BAG2 Signaling Pathways Affected by a Protease Inhibitor from *Tinospora cordifolia* Stem Extract Among the Different Plant and Microbial Samples Analyzed

Short Title: *T. cordifolia* PI affects FAT10 Signaling

Bramhi Suresh Chougule, Kumar Gaurav, Mutthu Kumar, Nayana Mahadevaprasad, Nisarga Hk, Sreya Lakshmi Ponnada, Somasekhara Derangula, Varalakshmi Kilingar Nadumane
Department Of Biotechnology, School Of Sciences, Jain (deemed-to-be-university), Bangalore-560 027, Karnataka, India

Corresponding Author Information
Varalakshmi Kilingar Nadumane
https://orcid.org/0000-0001-9979-5007
kn.varalakshmi@jainuniversity.ac.in
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ABSTRACT
Objectives: Dysregulation of proteolysis underlies diseases like cancer. Protease inhibitors (PIs) regulate many biological functions and hence they have potential anticancer properties. With this background, the current study was focused to identify a PI from natural sources such as plants and microbes against trypsin (a protease), which was assayed against casein, using a UV Spectrophotometer based methodology.
Materials and Methods: PIs extracted from few plant and microbial samples were screened for their PI activity against trypsin. The PI from the most promising source in our study, *T. cordifolia* stem, was partially purified using Ammonium sulfate precipitation followed by dialysis. The PI activity of partially purified inhibitor was analyzed against chymotrypsin and collagenase enzymes, and the cytotoxic effect of the PI was checked on HepG2 (liver carcinoma) cells by MTT- [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide]-assay. LC-MS/MS based proteomic studies were performed on HepG2 cells to understand the signaling pathways affected by the PI in the liver cancer cell line.
Results: Among the samples tested the PI from *T. cordifolia* stem extract had the highest inhibitory activity (72%) against trypsin along with cytotoxicity to HepG2 cells. After partial purification by 80% ammonium sulfate precipitation the PI had increased inhibitory activity (83%) against trypsin and enhanced cytotoxicity (47%) to HepG2 cells. Proteomic analysis of the PI treated HepG2 cells revealed that BAG2 and FAT10 signaling pathways were affected, which might be causing inhibition of cancer cell proliferation.
Conclusion: PI from *T. cordifolia* stem has promising anticancer potential and hence can be taken up for further purification and characterization studies towards cancer drug development.
Key words: *T. cordifolia*, ammonium sulfate, trypsin, Protease Inhibitor, anticancer, BAG2 signaling.

INTRODUCTION

Proteases are enzymes that play a very essential and basic role in many biological events, such as apoptosis, necrosis, angiogenesis, wound healing, transcription, translation, immune responses, differentiation, senescence, etc., by a tightly regulated mechanism known as proteolysis. Dysregulation in the mechanism of proteolysis could lead to many underlying diseases such as cancer, cardiovascular disease, and neurological disorders. Regulation of proteolysis is therefore very crucial for the regular developmental activities of an organism.[1,2]
The functioning of proteases is being investigated for more than 3 decades for their potential role in cancer development. Protease inhibitors (PIs) inhibit specific classes of proteases by their ability to form a strong protease-PI complex, thereby maintaining the homeostatic balance necessary for preventing life-threatening diseases like cancer. With over 100 proteases already linked to various elements of tumor formation and its progression, there is a strong motive to utilize PIs in oncology.[3]
Surgery, chemotherapy, and radiotherapy are the three main cancer treatment techniques. Though these treatment modalities play an important role in controlling cancer to a certain extent, the associated side effects decrease the quality of life among patients.[4] As a result, scientists are attempting to identify alternative therapeutic strategies capable of suppressing cancer without causing additional morbidity.

The use of alternative cancer therapies from medicinal plants is gaining greater significance due to the existence of a variety of anti-tumor compounds in plants such as *Tinospora cordifolia* (Willd.) Hook. f. and Thoms.[5] *T. cordifolia*, belonging to family Menispermaceae, can be found all over tropical regions like India and China, growing at altitudes up to 300 metres. It is also referred to as Guduchi or Giloy and was called 'Amritha' or 'heavenly elixir' in Hindu Vedic times. Guduchi is a veterinary folk medicine and Ayurvedic staple. Its stems and roots are anciently used as therapeutics due to their antispasmodic, general tonic, anti-arthritic, anti-diabetic, anti-inflammatory, anti-allergic, and anti-inflammatory properties.[6]

Not only plants but also some microorganisms have similar effects among natural resources. Recently, actinobacteria have also been identified as producers of enzyme inhibitors. A cysteine protease inhibitor (CPI-2081) from *Streptomyces* sp. 2081 exhibited potent inhibition against papain along with significant inhibition of tumor cell migration.[7] Thus, studies about the protease landscape connected to cancer have resulted in the emergence of better clinical technologies for diagnosis and therapy that use pathologic patterns of proteolytic activity.

Though much work has been carried out in the area of PIs for HIV and other viral diseases, their utility towards cancer therapy has not been explored extensively. Hence analyzing the ability of PIs for cancer treatment appears as a very promising approach. The current study is aimed at isolating compounds from medicinal plants, and screening them for their PI activity against trypsin and chymotrypsin, along with checking for their cytotoxicity on the HepG2 (liver cancer) cell line.

MATERIALS AND METHODS

T. cordifolia stem and leaves, *Cascabela thevetia* stem and leaves and methi seeds (*Trigonella foenum-graecum* L.), were collected from Lalbagh in Bangalore during January 2022, and were identified (by Dr. Rama Rao, Research officer, Central Council for Research in Ayurvedic Sciences, Bangalore, India). The herbarium specimens were kept at the herbarium of Jain University (with specimen numbers *T. cordifolia*-JUH96; *C. thevetia*- JUH97; *Trigonella foenum-graecum* -JUH98). Pure cultures of microbes like *Acinetobacter vinetianus*, *Streptomyces* sp., maintained in our lab were used in the study. Chemicals included, Trypsin EDTA, tris HCl, chymotrypsin, collagenase, phosphate buffer saline (PBS), casein, ammonium sulfate, TCA, sodium bicarbonate, EDTA (1 mM), diphenyltetrazolium bromide and dimethyl sulfoxide (DMSO). All analytical grade chemicals were used in this work and were procured from Himedia, India.

Extraction of the PI

All plant materials were separately dried at room temperature, the stem, leaves and seeds were separately ground using a blender. 10 g of stem/leaf/seed powder was mixed in 100 mL of PBS (0.1 M) having a pH of 7.2, and incubated at room temperature for 30 min. Then the mixture was centrifuged to remove all the insoluble material at 6000× g at 4°C for 10 min. The clear supernatant was stored at 4°C for further analysis. Microorganisms were cultured in Nutrient broth for 48 h, centrifuged at 6000 x g at 4°C for 10 min. The clear supernatant was stored at 4°C for further analysis. PI activity was determined for all these supernatants using trypsin and chymotrypsin as the proteases.

Assay of PI activity

As per the methodology given by Kunitz with minor modifications, the PI activity against the proteases, trypsin, chymotrypsin and collagenase was assessed.[8] Casein was used as the substrate, whose hydrolysis produces amino acids like tyrosine. The absorbance of the amino acids released was measured at 280 nm. The inhibitory activity was assessed as the residual proteolytic activity in the presence of the inhibitor and expressed as a percentage of the proteolytic activity of the uninhibited control.[9] One unit of enzyme activity is the amount of the enzyme that releases 1 μmole/min/mL of tyrosine under the assay conditions. One unit of PI activity unit (PIU) was defined as the quantity of inhibitor that reduced the absorbance (at 280 nm) by one unit of TCA soluble casein hydrolysis product, due to the enzyme action per minute under the assay conditions. The PI activity was calculated and expressed as a percentage of the activity of uninhibited protease.[10]

Tyrosine standard

A standard graph of tyrosine was prepared at a final concentration of 200 μg/mL, which was used to quantify the amino acid liberated by the proteases.

Effects of pH, temperature, and metal ions on the PI activity of *T. cordifolia*

To analyze the effect of pH on the PI activity of the selected plant extract (*T. cordifolia* stem), the plant sample was extracted in buffers of various pH (pH 3, pH 5, pH 7, pH 9, and pH 11). All these extracts were analyzed on Trypsin for PI activity. The samples extracted with distilled water (DW) and PBS were diluted using DW and PBS respectively (in the ratio 1:5 and 1:10). To check the effect of temperature, the extracts were incubated at different temperatures (25°,50°,75°, and 37°C) for 30 minutes before performing the PI activity assay.

To check the effect of the metal ions on PI activity, 10 metal salts (barium chloride, manganous chloride, lead chloride, nickel chloride, mercuric sulfate, chromium chloride, cadmium chloride, cupric sulfate, and ferric chloride) were taken in 5 mM and 10 mM concentrations and added to 1 mL of *T. cordifolia* stem PBS extract and incubated at 37°C for 30 min before performing the PI activity assay.

Purification of PI by Ammonium sulfate precipitation

Tinospora cordifolia was sourced from Lalbagh Botanical Garden nursery. The stem was weighed and 80 g was ground with 400 mL of 0.1 M PBS of 7.2 pH. The resulting mixture was filtered using a muslin cloth, and then the filtered sample was centrifuged at 10000× g at 4°C for 10 min, to remove any insoluble plant material. The supernatant was collected and measured. The amount of extract was 380 mL which was divided equally into 2 conical flasks. For 40% and 80% saturation, 46.67 g and 107.73 g of ammonium sulfate was added respectively, at 4°C and kept under constant stirring for about 12 hours. The solution was centrifuged at 6000× g at 4°C for 30 min to separate the precipitate and the supernatant. The precipitate was dissolved in 0.1 M PBS of 7.2 pH and further processed.

Dialysis

The PI precipitated by ammonium sulfate was dialyzed in PBS (0.01M) with a pH of 7.2 to remove ammonium sulfate from it. [11] The dialysis membrane used was from HIMEDIA (LA 401-1 mt). The samples after dialysis were pipetted out and stored in vials at 4°C for further analysis. The PI activity against trypsin was analysed as mentioned earlier.

MTT ([3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide])-assay

To assess the cytotoxicity of the PI on MCF-7 and HepG2 cancer cell lines, MTT assay was carried out according to the standard methodology.[12] The cancer cell lines ($1x10^4$ cells/mL) were seeded onto the wells of 96-well microtiter plates, after trypsinization. The crude extract and ammonium sulfate precipitated (40% & 80%) PIs were added (10 µl) after 24 h at varying concentrations (1:1, 1:10, 1:25 and 1:50 dilutions) for 24, 48 and 72 hours. After the incubation, MTT assay was performed and the optical density was recorded at 540 nm. The viability percentage was calculated as given below:

$$\text{Percentage viability} = \frac{\text{OD of sample}_{(540)} \times 100}{\text{OD of control}_{(540)}}$$

Assessment of cytotoxicity by lactate dehydrogenase (LDH) activity

The degree of toxicity of the PI in the treated cancer cells was analyzed by estimating LDH enzyme activity in the cytosol.[13] The increase in the LDH enzyme in the treated cells reflects the number of lysed cells. The assay was carried out based on the instructions provided in the kit manual.

Relative protein quantification using ESI-nanoLC-MS/MS (Proteomics)

Protein extraction

Confluent cancer cell lines (70%) were cultured in serum-free medium for 12 h, and later lysed using 2% SDS in 50 mM triethyl ammonium bicarbonate [TEABC] as the lysis buffer, followed by sonication. The concentration of protein in this sample was determined as per standard methods.

Trypsin digestion

The samples were reduced using 100 mM 1, 4-Dithiothreitol (Sigma Aldrich), alkylated using 200 mM iodoacetamide (Sigma Aldrich), and digested over night with MS-grade trypsin (Sigma Aldrich) in the ratio 1:25 (1 µg of trypsin to 25 µg of protein). All the samples were injected in duplicate (2 injections per sample). The injection volume was 1.0 µl. The detailed in-solution protocol we employed was given in the web link.[14]

Liquid chromatography conditions

Liquid chromatography system (Instrument): nanoACQUITY UPLC® chromatographic system (Waters, Manchester, UK) was used for proteomics study with MassLynx4.1 SCN781 software for acquisition, Trap column: Symmetry® 180 µm x 20 mm C18 5 µm, waters, Analytical column: 75 µm X 200 mm HSS T3 C18 1.8 µm, (waters), Solvent flow rate: 300 nL/min, Column temperature being 35ºC, Reverse phase chromatography mode: Auto sampler temperature was 4ºC.

Mass spectrometry conditions

MS runs were performed using ion mobility-enabled separation. MS system: Synapt G2 High Definition MS™ System (HDMS^E System) Waters, with Sodium iodide calibration.

RESULTS

Residual Protease activity and PI activity of selected plant and microbial extracts against trypsin.

T. cordifolia leaves (Sample 1), *T. cordifolia* stem (Sample 2), *C. thevetia* leaves (Sample 3), *C. thevetia* stem (Sample 4), *A. vinetianus* (Sample 5), and *Streptomyces* sp. (sample 6). The positive control was the uninhibited trypsin, i.e., trypsin untreated with any plant extract. According to the results of this investigation, *T. cordifolia* stem extract at 1:5 dilution had the highest inhibition of trypsin activity (62.87%), followed by the same extract at a 1:10 dilution (**Fig 1a**). At any of the tested concentrations, the leaves of *T. cordifolia*, the leaves and stem of *C. thevetia* did not exhibit any inhibitory effects on the protease enzyme trypsin, which resulted in enhanced enzyme activities (with negative values for protease inhibition $-43.64\%, -52\%\ and\ -106\%$ respectively). Additionally, at all the investigated concentrations, microbial isolates of *A. vinetianus* and *Streptomyces* sp. did not show any inhibitory effects on trypsin with negative values as shown in **Fig 1a**.

T. cordifolia stem buffer (PBS) extract was serially diluted before the protease inhibition assay was conducted (the OD was measured at 280 nm), and as per the formula protease inhibitory activity was calculated and presented graphically (**Fig 1b)**. The *T. cordifolia* stem extract has good PI activity with 70.97% inhibition at 1:2 dilution (highest PI activity), followed by the undiluted stock sample with 60.2%.

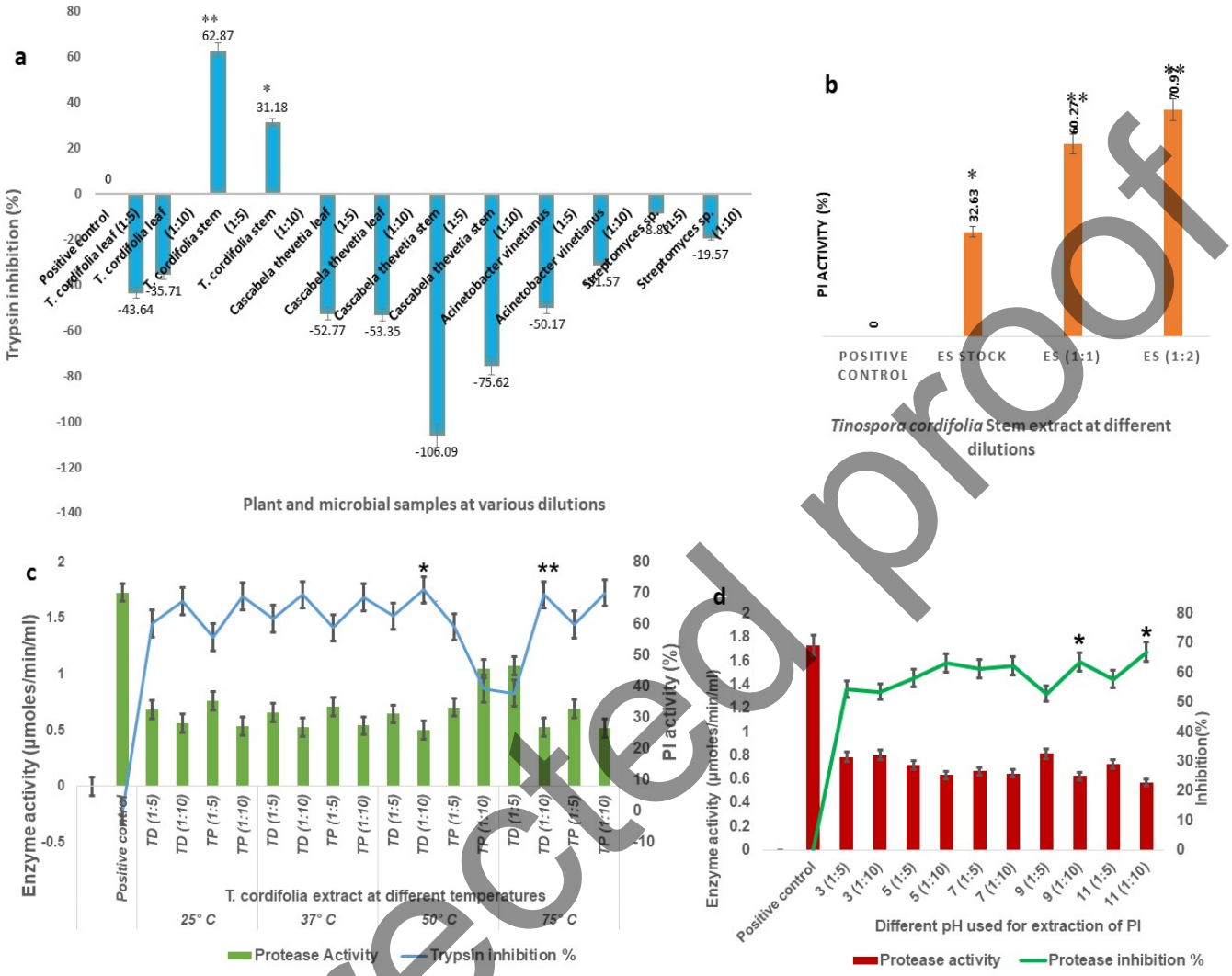

Fig 1: a) Screening the PI activity of plant and microbial samples, **b)** PI activity of *T. cordifolia* stem extract **c)** Effect of temperature on PI activity of distilled water (TD) and buffer extracts (TP) of *T. cordifolia* **d)** Effect of pH on the PI of *T. cordifolia* stem extract

Effect of temperature on PI activity of *T. cordifolia*

Phosphate buffer (PBS) and distilled water (TD) extracts of the stem of *T. cordifolia* were checked at varying temperatures of 25°C, 37°C, 50°C, and 75°C for their effect on PI activity (**Fig 1c**). According to the findings, at 50°C, the distilled water (TD) extract at a dilution of 1:10, showed the highest protease inhibition with 71.08%, followed by the PBS extract at 75°C, at a dilution of 1:10 with 70.10% inhibition.

Effect of pH on the PI activity of *T. cordifolia* stem extract

T. cordifolia stem buffer extract was analysed for protease activity (trypsin) and PI activity in relation to pH. The stem of *T. cordifolia* was extracted in buffers of different pH. The pH 11 extract (1:10 dilution) exhibited the maximum protease inhibition with 67.1%, followed by pH 9 (1:10 dilution) with 63.79%, and again the pH 9 extract at 1:5 dilution with 52.85% (**Fig 1d**).

Metal ions effect on the PI activity of *T. cordifolia*

T. cordifolia stem extract was incubated with 10 different metal ions at 5- and 10-mM concentrations for analyzing their effect on PI activity. As per the results, it was seen that all metal ions had significantly inhibited the PI activity of *T. cordifolia* stem extract (**Fig 2a**).

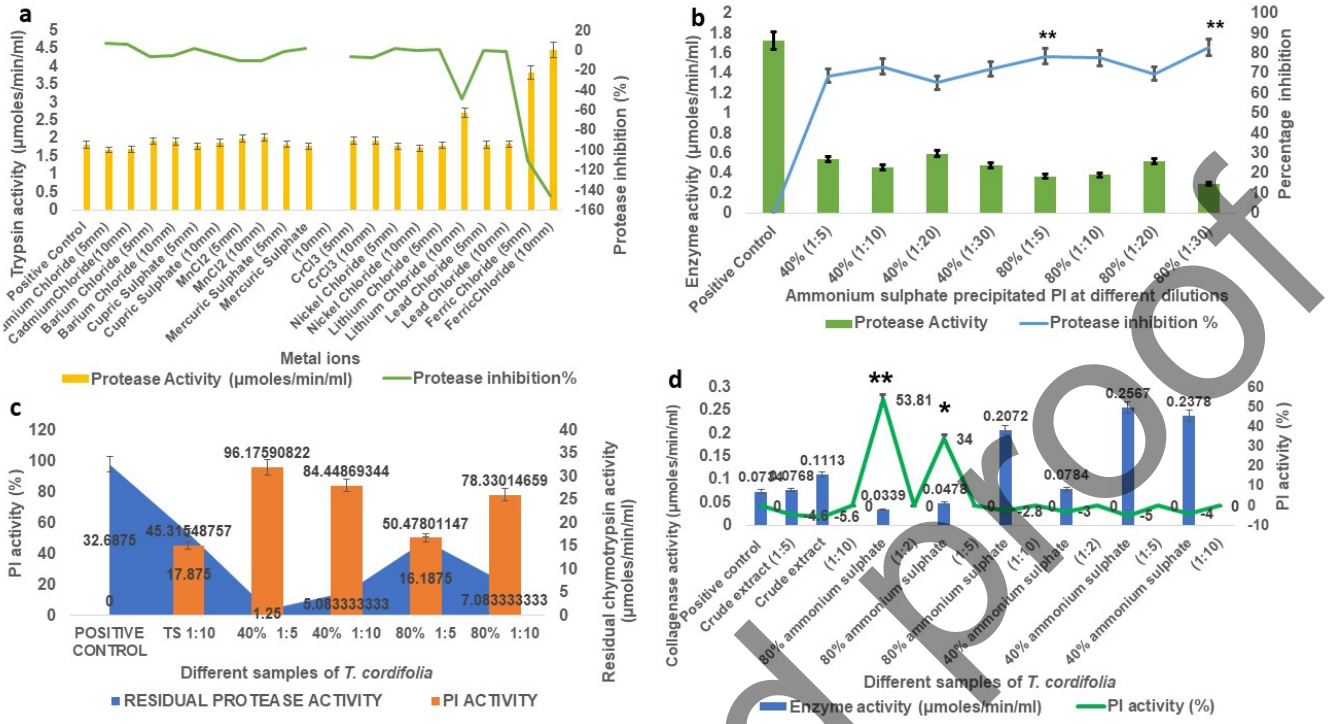

Fig 2: a) Effect of metal ions on PI activity of *T. cordifolia* **b)** PI activity of ammonium sulfate precipitate of *T. cordifolia* stem extract **c)** Chymotrypsin inhibitory activity of the PI from *T. cordifolia* **d)** Collagenase inhibitory activity of the crude and partially purified PI from *T. cordifolia* stem.

Partial purification of the PI by salting out

For partial purification ammonium sulphate precipitation method was used. The *T. cordifolia* stem extract was added with ammonium sulfate to 40% and 80% saturations and the precipitated inhibitor was used to perform the PI activity assay. **Fig 2b** displays the study's findings, with the 80% sample at 1:30 dilution had the highest PI activity with 83.07% inhibition, followed by the 80% sample at 1:5 dilution with 78.54% inhibition. PI activity was maximum in the 80% ammonium sulphate precipitate, and was also noticeable in the 40% precipitate.

PI's ability to inhibit Chymotrypsin and Collagenase enzymes

Due to the high PI activity of the partially purified inhibitor from *T. cordifolia* stem, it was tested on other proteases like chymotrypsin and collagenase. As per the results, chymotrypsin inhibitory activity was highest for the 40% ammonium sulfate precipitate of the PI with 96.17% inhibition, followed by the 80% sample with 78.3% inhibition, and slightly lower activity was observed with the crude PI with 45.3% inhibition (**Fig 2c**). Whereas the collagenase inhibitory potential was exhibited only by the 80% sample (53.8% inhibition), and both the crude extract and the 40% sample were not able to inhibit the activity of collagenase (**Fig 2d**).

Cytotoxicity studies on HepG2 liver cancer cell line

As *T. cordifolia* stem extract and the ammonium sulphate precipitated samples have shown good PI activity, these samples were treated on to the liver cancer HepG2 cell line to assess their potential anticancer activity. According to the findings, after 48 hours of incubation, ethanol extract exhibited 17.31% cytotoxicity, whereas the buffer extract displayed 13.0% cytotoxicity to HepG2 cells (**Fig 3a**).

Cytotoxicity of partially purified samples (PI) from *T. cordifolia* stem extract

When the partially purified PI (40% and 80%) of *T. cordifolia* was analyzed for cytotoxicity to HepG2 (liver cancer) cells for varied lengths of time (24h, 48h, and 72h), the partially purified PI (40% sample) demonstrated considerable cytotoxicity at all the time periods with 81%, 80% and 65% viabilities respectively at 1:10 dilution. The maximum cytotoxicity was 35% (**Fig 3b**). The highest cytotoxicity was displayed by the PI (80% sample) at 72 hours of incubation and at 1:20 dilution. The treated liver cancer cells had a viability of 53.2% with a cytotoxicity of 46.8% (**Fig 3c**), and other dilutions also demonstrated considerable cytotoxicity after a 72-hour incubation period. The viability was 93.5% at 24 hours, 79.6% at 48 hours, and 69.82% at 72 hours of incubation by the 1:5 diluted PI.

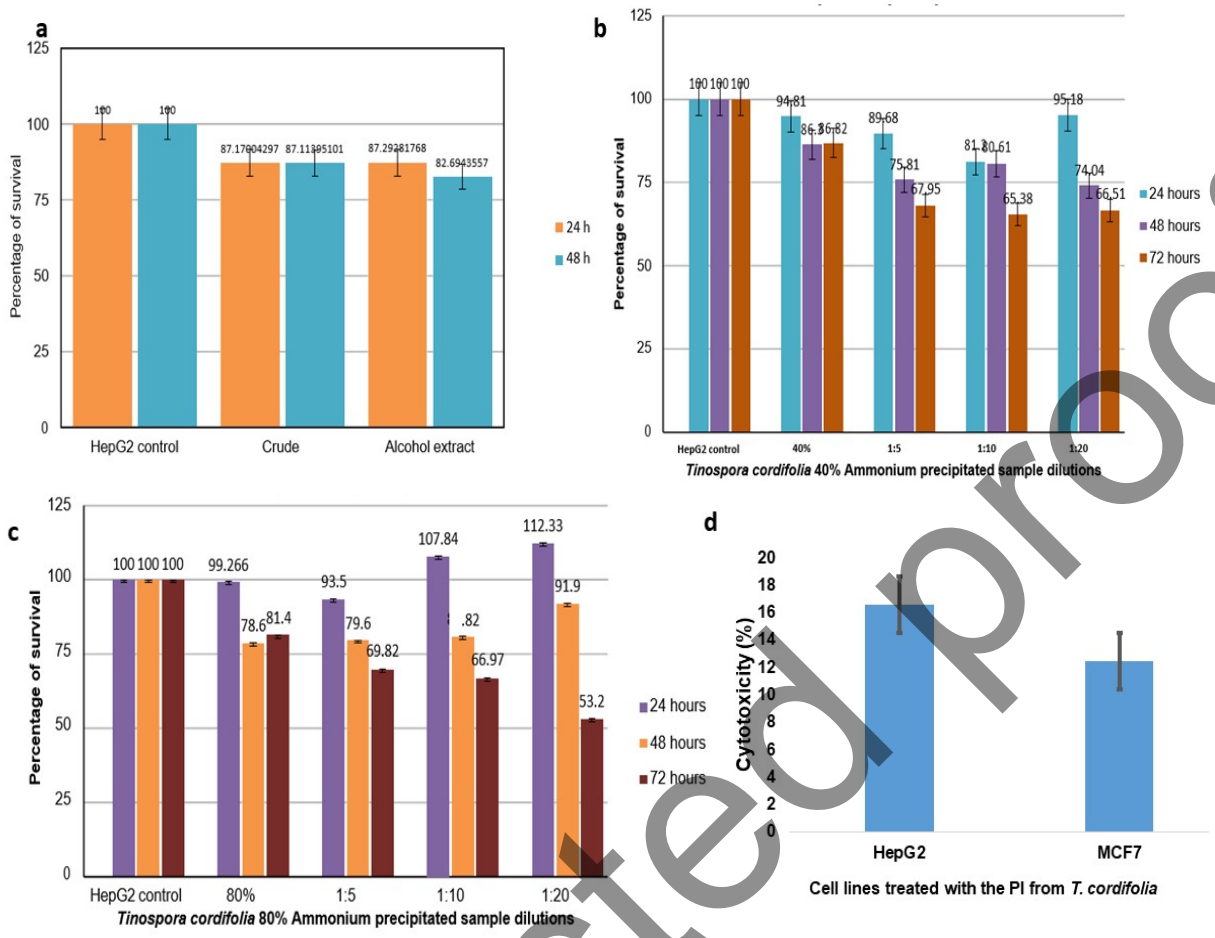

Fig 3: **a)** Effect of *T. cordifolia* stem buffer extract and ethanol extract on HepG2 cells at 24 and 48 h, **b)** Effect of *Tinospora cordifolia* 40% ammonium sulfate precipitated PI sample on Hep G2 cells, **c)** Effect of *Tinospora cordifolia* 80% ammonium sulfate precipitated PI sample on HepG2 cells, **d)** Cytotoxicity of 80% ammonium sulfate precipitate of *T. cordifolia* by LDH assay

LDH activity assay

As per LDH assay results, among the cancer cells treated with the PI (80% precipitate) from *T. cordifolia*, we observed 16% cytotoxicity on HepG2 cells and 13% cytotoxicity on MCF-7 (breast cancer) cells (**Fig 3d**).

Proteomic studies of HepG2 cells treated with PI from *T. cordifolia* stem

When the HepG2 cells treated with the PI were subjected to mass spectrometry based proteomic analysis, the data showed altered expression of several proteins. Significant overexpression of 19 proteins, downregulation of 8 proteins and remaining unaltered proteins were observed (**Fig 4a**). This proteomics data was deposited to the ProteomeXchange Consortium with the dataset identifier PXD037511.[15] A fold change cutoff of 1.5-fold and p $\leq$ 0.05 was considered to filter proteins for further analysis. As per the data, we found Proliferation-associated protein 2G4 (PA2G4) expression was 3.48 folds upregulated in treated HepG2 cells as compared to the controls. PA2G4 isoforms have opposing functions in cancer, which is well documented.[16] In our data, PEBP1 protein expression was 1.5 folds upregulated in treated HepG2 cells than the controls. EBP1 belongs to the PA2G4 family of DNA binding proteins. The growth inhibiting properties of EBP1 are multi-faceted since it binds to RNA,[17] DNA[18] and proteins.[19] Overexpression of ubiquitin-conjugating enzyme E2 L3 (UBE2L3), were reported in non-small-cell lung cancer (NSCLC) tissues. Correlation was found between high expression of UBE2L3 and advanced tumor stage and negative outcomes, while UBE2L3 knockdown was reported as to inhibit NSCLC cell growth.[20] In our current study results, UBE2L3 was downregulated 5.4 folds in the treated HepG2 cells in comparison with the control untreated cells. Mitogen-activated protein kinase kinase kinase 21 (MAP3K21) was observed to be 2.5 folds upregulated in treated HepG2 cells than controls as per our results. To understand the protein-protein interactions among the dysregulated proteins, using the STRING database [21] network analysis was carried out. As shown in the results of network analysis, mitochondrial and nuclear transport proteins, chaperon proteins, ribosomal proteins, proteasome proteins, translation initiation factors,

spliceosomal proteins, glucose metabolism proteins, and mitochondrial inner membrane proteins were majorly affected by the PI from *T. cordifolia* on HepG2 cells (**Fig 4b**).

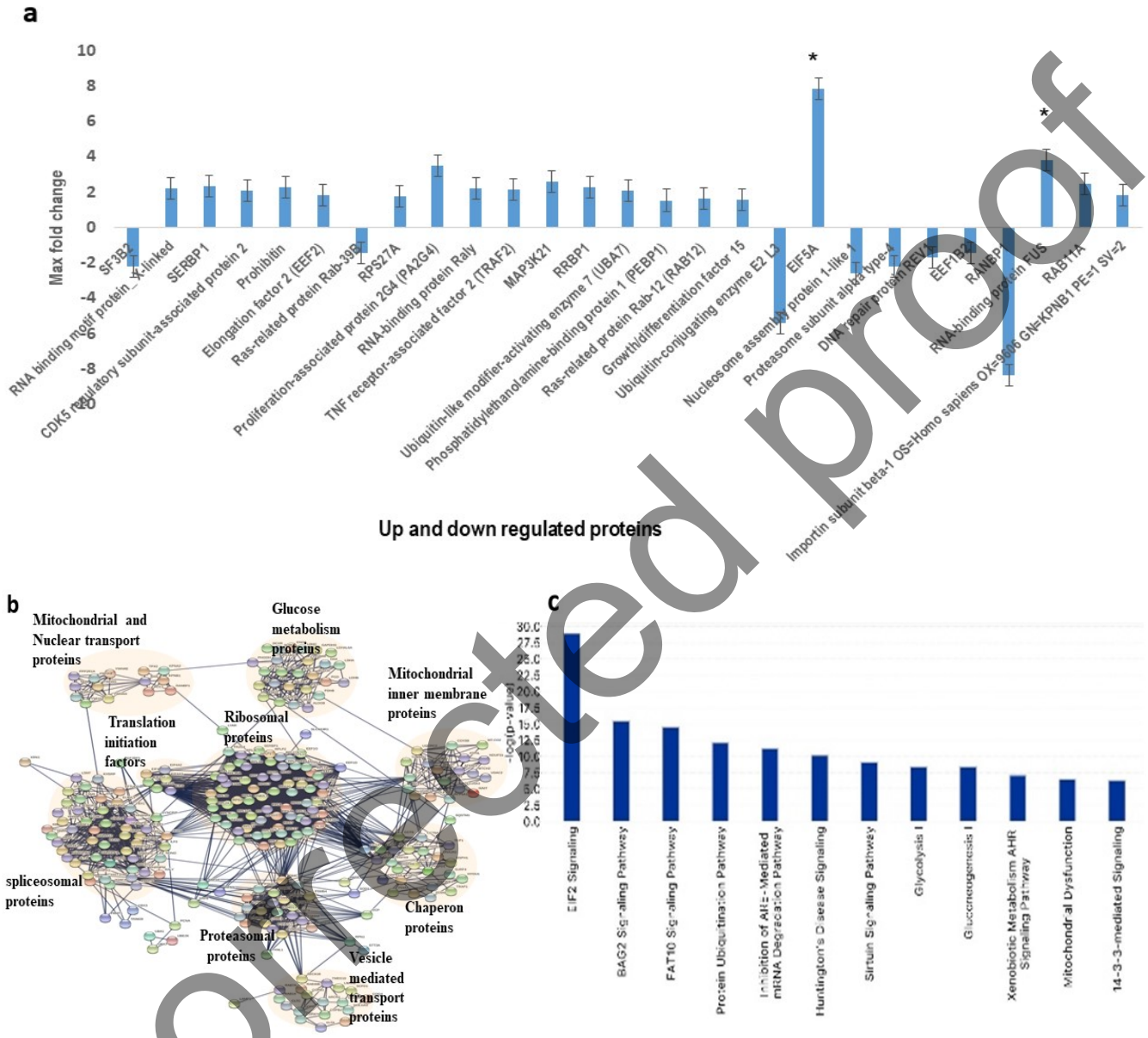

Fig 4: **a)** Up and downregulated proteins in HepG2 cells treated with *T. cordifolia* ammonium sulfate (80%) precipitated PI sample as compared to the controls, b) STRING analysis of interaction of proteins in HepG2 cells treated with *T. cordifolia* extract showing distinct molecular alterations, c) Altered canonical pathways in *T. cordifolia* PI treated HepG2 cells.

***T. cordifolia* PI treatment affects BAG2, FAT10 signaling pathways in HepG2**

According to canonical pathway analysis, EIF2 signaling, BAG2 signaling and FAT10 signaling pathways were found to be majorly altered in the PI treated HepG2 cells (**Fig 4c**). Among these, we considered BAG2 and FAT10 signaling pathways due to higher percentage of overlap (21.4 and 26.8% respectively) than EIF2 signaling, which has 17.2% overlap.

In our data, SQSTM1 and Cathepsin B (CTSB) proteins were seen as upregulated, and upregulation of Cathepsins increases formation of autophagosomes.[22,23] Further in our Ingenuity Pathway analysis, 26s Proteasome proteins were found as downregulated (**Fig 5**). In nuclei, 26s Proteasome complex increases inhibition of ubiquitinated p53 protein. As per our results, cathepsin B (CTSB) protein was found to be upregulated (**Fig 5**).

Hsp70 is one the important proteins and studied for possible effects on energy metabolism. In our data Hsp70 is downregulated and it is known that downregulation of Hsp70 leads to mitochondrial dysfunction. It can be inferred from our results that downregulation of Hsp70, induced by the PI in HepG2 cells, leads to mitochondrial dysfunction and cell death.

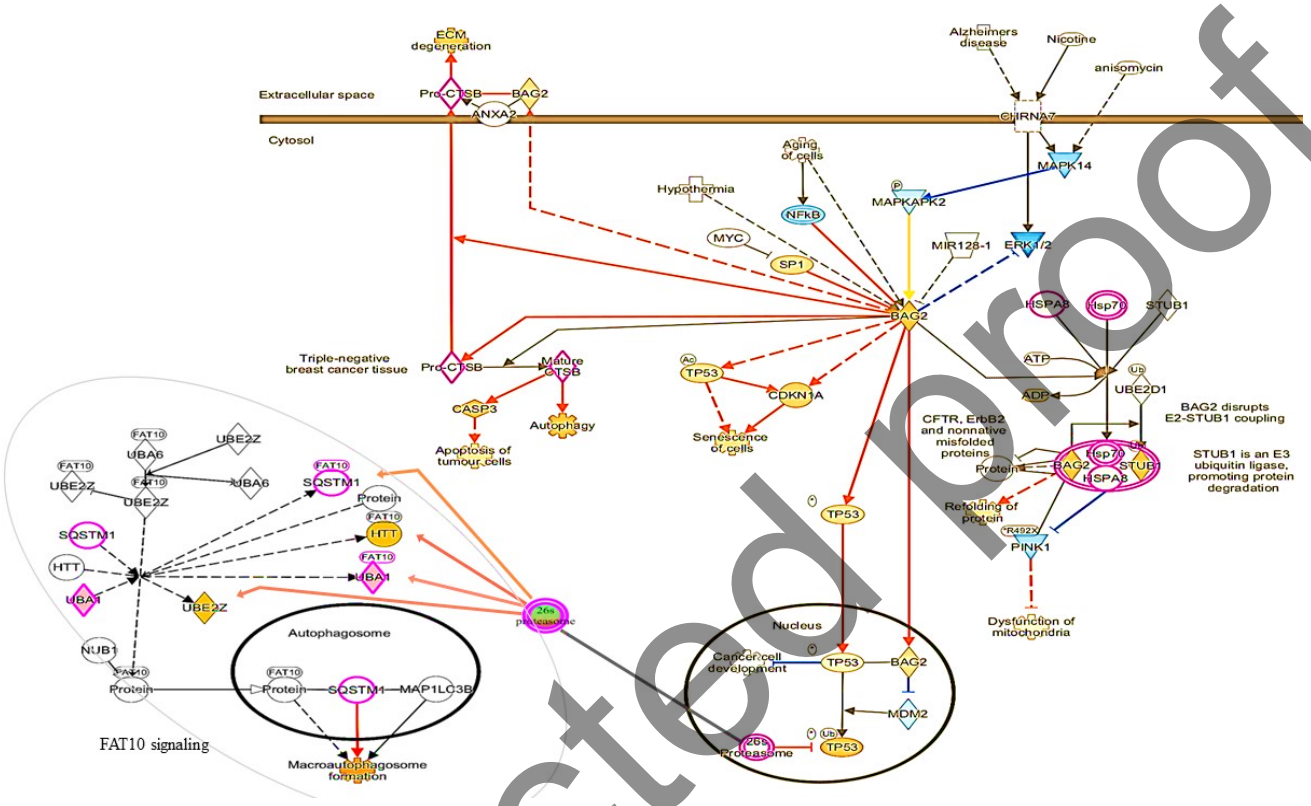

Fig 5: *T. cordifolia* partially purified PI treatment on HepG2 cells affects BAG2, FAT10 signaling pathways. Ingenuity Pathway analysis of proteins significantly dysregulated ($p \leq 0.05$) in treated HepG2/ control HepG2 cells. The signaling network of BAG2, EIF2, FAT10 pathways based on differential expression of molecules in the HepG2 cells.

DISCUSSION

Proteolysis can be considered as the most important metabolic process involved in protein turnover and processing. Proteases are involved in the degradation of stored or unwanted proteins, and these are essential for developmental processes such as differentiation and programmed cell death. Dysregulated proteolysis leads to many diseases, including cancer.[24] Hence PIs have great therapeutic potential in controlling protease-mediated diseases. Plants and microbes are major sources of PIs. Hence in our current research, many plants, plant parts, and microbial isolates were screened for their PI activity. The plants *T. cordifolia* stem and leaves, *C. thevetia* stem and leaves, and methi (*T. foenum-graecum*) seeds (sprouted), along with the microbes *A. vinetianus* and *Streptomyces* sp., were chosen for our PI activity study. All these samples were tested for protease inhibitory activity. *T. cordifolia* stem extract showed the highest PI activity against trypsin. As per our study results, *T. cordifolia* stem extract exhibited maximum inhibition of trypsin activity. *T. cordifolia* stem extract showed good PI activity, the highest inhibition value observed was 70.97%, while the methi seed extract also had good inhibitory effects with a highest of 60.8% inhibition. To analyse the effects of physico-chemical parameters on PI activity, buffers of different pH, different temperatures, and different metal ions were considered. We found that the basic pH of 11 had positive effects on the PI activity of *T. cordifolia*, with 67.1% inhibition of trypsin activity. In comparison to this observation, it was reported that plant-based PIs have a broad pH range (from 2 to 10), with the highest activity at pH 7.5, and that the plant PIs are unstable at highly acidic pH. [24] For the PI from *T. cordifolia*, in temperature studies, we observed the highest percentage of PI at 50ºC (71.08%), followed by 75ºC for the PBS extracted PI (70.10%). Most plant PIs are active at temperatures as high as 50°C. As far as the metal ions are concerned, *T. cordifolia* PI was inhibited by all tested metals at 5- and 10-mM concentrations. In a study it was reported that 1 mM Mg^{2+} addition increased the PI activity, whereas all other divalent metal ions decreased the activity. [24]

As the PI from *T. cordifolia* had the highest activity, it was chosen for further purification studies by salting out using ammonium sulfate. Among the partially purified PI (by 40% and 80% ammonium sulphate precipitation), the highest protease inhibition was observed in the 80% precipitated sample. Even the 40% precipitated PI sample had considerable protease inhibitory activity. Our results have shown that the PI from *T. cordifolia* has potent cytotoxic and anticancer potential against the liver cancer (HepG2) cell line. In a previous study conducted on different cancer cell lines, it was reported that *T. cordifolia* showed 52-59% of protease inhibition.[25] As compared to this report, in our current study, the 40% ammonium sulfate precipitated PI from *T. cordifolia* exhibited highest trypsin inhibition (78%) with the best cytotoxic activity (34.62%), whereas the PI sample by 80% ammonium sulfate precipitation exhibited 83% trypsin inhibition along with highest cytotoxic activity against HepG2 liver cancer cells (46.8%). Besides this, 96% inhibition of chymotrypsin and 53.8% inhibition of collagenase were also demonstrated by the partially purified PI of *T. cordifolia* in our study, which substantiates its anticancer potential. As per previous reports, berberine, palmatine, tembetarine, and magnoflorine have been isolated from *T. cordifolia* stem.[4] Though anticancer properties of *T. cordifolia* were reported by earlier workers,[26,27,28,29] PI activity and cytotoxicity of the PI from *T. cordifolia* are being reported for the first time through this current study.

When HepG2 cells treated with the partially purified PI were subjected to proteomic studies, we found major signaling proteins involved in cancer to be dysregulated, such as EIF2 signaling, BAG2 signaling and FAT10 signaling pathways. In our data, SQSTM1 and CTSB were observed to be upregulated, and increase of these proteins enhances formation of autophagosomes. Autophagy has both roles as a tumor suppressor and a tumor growth promoter. [22,23] In tumor suppressor mechanisms, autophagy prevents the accumulation of damaged proteins and organelles, which can promote tumor survival.[30]

In FAT10 signaling pathway, the inhibition of fat10ylated active SQSTM1 protein[31] occurs due to 26s proteasome in the cytoplasm, and in our data 26s proteasome proteins are downregulated. Whereas, in the nuclei, 26s proteasome complex increases inhibition of ubiquitinated p53 protein. In HepG2 cells treated with the PI, we could predict p53 protein as upregulated, due to the downregulation of 26s proteasome.[32] This upregulated p53 in the HepG2 cells might be one of the reasons for the observed cytotoxicity due to the PI in the current study.

Caspases are enzymes primarily involved in mediating apoptosis, and caspase-3, the usually activated death protease, is needed for specific breakdown of several cellular proteins. In our data, CTSB protein was found to be upregulated, and according to reports, mature CTSB protein increases activation of caspase 3 protein.[33]

Hsp70 is one the important proteins studied for possible effects on energy metabolism. In our data Hsp70 is downregulated and downregulation leads to mitochondrial dysfunction through inhibition of oxidative phosphorylation and ROS generation.[34] As mitochondria are primarily involved in cell apoptosis, we can assume that the Hsp70 mediated mitochondrial dysfunction is leading to the apoptosis of HepG2 cells treated with the PI in our current study. There are studies and reports about the anti-HIV and anti- SARS-COV-19 activity of the *T. cordifolia* PI.[35] But, the current study is the first report of anticancer activity of the PI from *T. cordifolia* to the best of our knowledge. It can be concluded from the outcomes of the proteomic studies, STRING analysis, Ingenuity pathway analysis, canonical pathway analysis, that the PI from *T. cordifolia* exerts its anticancer activity by majorly altering the signaling pathways of BAG2, EIF2 and FAT10 molecules.

Conclusion

It can be concluded that the PI from *T. cordifolia* has potent protease inhibitory and anticancer effects on the liver cancer cell line HepG2. *T. cordifolia* has been reported to possess several phytochemicals with significant anticarcinogenic activities as per many earlier *in vivo* and *in vitro* studies. Though the PI activity of *T. cordifolia* was checked by earlier researchers on HIV and SARS-COV proteases, the anticancer potential of this *T. cordifolia* PI is being analyzed and reported for the first time through the current study. The current study opens further scope for complete characterization of this PI and the validation of its anticancer activity through *in vivo* and future clinical studies.

Declarations

Authors' contributions

Conceptualization: VKN; Methodology: VKN and SD; Formal analysis and investigation: BSC, KG, MK, NM, NHK, SLP, SD; Writing - original draft preparation: BSC, KG, MK, NM, NHK, SLP; Writing - review and editing: VKN; Supervision: VKN.

Availability of data and material

The data that support the findings of this study are available from the corresponding author (VKN), upon reasonable request.

Compliance with Ethical Standards

Disclosure of potential conflicts statement
The authors declare that they have no conflicts of interest.

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As this study does not involve any experiments on animals, it is not applicable for the current study.

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