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ORIGINAL ARTICLE

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

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ABSTRACT

Objectives: Dysregulation of proteolysis underlies diseases like cancer. Protease inhibitors (PIs) regulate many biological functions and hence have potential anticancer properties. With this background, the current study aimed to identify the PI from natural sources such as plants and microbes against trypsin (a protease), which was assayed against casein, using an ultraviolet spectrophotometer-based methodology.

Materials and Methods: PI extracted from a few plants and microbial samples were screened for their PI activity against trypsin. The PI from the most promising source in our study, Tinospora cordifolia (Willd.) Hook. f. and Thoms. stem, was partially purified using ammonium sulfate precipitation followed by dialysis. The PI activity of the 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. Liquid Chromatograography Mass Spectrometry -based proteomic studies were performed on HepG2 cells to understand the signaling pathways affected by the PIs in the liver cancer cell line.

Results: Among the samples tested the PIs from *T. cordifolia* stem extract had the highest inhibitory activity (72.0%) against trypsin along with cytotoxicity to HepG2 cells. After partial purification by 80.0% ammonium sulfate precipitation, PI had increased inhibitory activity (83.0%) against trypsin and enhanced cytotoxicity (47.0%) to HepG2 cells. Proteomic analysis of the PI-treated HepG2 cells revealed that BAG2 and FAT10 signaling pathways were affected, which may have caused the inhibition of cancer cell proliferation.

Conclusion: PI from *T. cordifolia* stem has promising anticancer potential and hence can be used for further purification and characterization studies toward cancer drug development.

Keywords: *Tinospora 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 of the mechanism of proteolysis could lead to many underlying diseases such as cancer, cardiovascular disease, and neurological disorders. Regulation of proteolysis is therefore crucial for the regular developmental activities of an organism.[1,2]

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The functioning of proteases has been 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 use PIs in oncology.[3]

Surgery, chemotherapy, and radiotherapy are the three main cancer treatment techniques. Although these treatment modalities play an important role in controlling cancer to a certain extent, the associated adverse 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 the family Menispermaceae, can be found all over tropical regions like India and China, growing at altitudes up to 300 m. 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 PI (2081) from *Streptomyces* sp. 2081 exhibited potent inhibition against papain and significant inhibition of tumor cell migration.[7] Thus, studies on the protease landscape associated with cancer have resulted in the emergence of better clinical technologies for diagnosis and therapy that use pathologic patterns of proteolytic activity.[8] Although much work has been conducted in the area of PIs for human immunodeficiency virus (HIV) and other viral diseases, their utility toward cancer therapy has not been extensively explored.[9] Hence, analyzing the ability of PIs for cancer treatment appears to be a very promising approach. The current study is aimed to isolate compounds from medicinal plants and screen them for their PI activity against trypsin and chymotrypsin, along with checking their cytotoxicity on the HepG2 (liver cancer) cell line.

MATERIALS AND METHODS

T. cordifolia cordifolia (Willd.) Hook. f. and Thoms. stem and leaves, *Cascabela thevetia* (L.) Lippold stem and leaves, and meth seeds (*Trigonella foenum-graecum* L.) were collected from Lalbagh in Bangalore in 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; *T. 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, trichloroacetic acid (TCA), sodium bicarbonate, ethylenediaminetetraacetic acid (EDTA) (1 mM), diphenyltetrazolium bromide and dimethyl sulfoxide. All analytical grade chemicals used in this study were procured from Himedia, India.

Extraction of the PI

All plant materials were separately dried at room temperature, and the stem, leaves and seeds were separately ground using a blender. Stem/leaf/seed powder (10 g) was mixed in 100 mL of PBS (0.1 M) having a pH of 7.2, and incubated at room temperature for 30 minutes. The mixture was then centrifuged to remove all the insoluble material at 6000 x g at 4 °C for 10 minutes. The clear supernatant was stored at 4 °C for further analysis. Microorganisms were cultured in nutrient broth for 48 hours, centrifuged at 6000 x g at 4 °C for 10 minutes. 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[10] with minor modifications, the PI activity against the proteases trypsin, chymotrypsin, and collagenase was assessed. 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.[11] One unit of enzyme activity is the amount of enzyme that releases 1 µmole/min/mL of tyrosine under the assay conditions. One unit of PI activity unit 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.[12]

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 of these extracts were analyzed on trypsin for PI activity. The samples extracted with distilled water (DW) (TD) 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 °C, 50 °C, 75 °C, 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 (TP) and incubated at 37 °C for 30 minutes before performing the PI activity assay.

Purification of PI by ammonium sulfate precipitation

T. 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 at pH 7.2. The resulting mixture was filtered using a muslin cloth, and then the filtered sample was centrifuged at 10000 x g at 4 °C for 10 minutes to remove any insoluble plant material. The supernatant was collected and measured. The amount of extract was 380 mL, which was divided equally into two conical flasks. For 40.0% and 80.0% saturation, 46.67 g and 107.73 g of ammonium sulfate were added, respectively, at 4 °C and kept under constant stirring for approximately 12 hours. The solution was centrifuged at 6000 x g at 4 °C for 30 minutes 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 (0.01 M) with a pH of 7.2 to remove ammonium sulfate from it.[13] 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 analyzed as mentioned earlier.

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

To assess the cytotoxicity of PI on MCF-7 and HepG2 cancer cell lines, MTT assay was performed according to the standard methodology.[14] The cancer cell lines (1 x 10^4 cells/mL) were seeded onto the wells of 96-well microtiter plates after trypsinization. The crude extract and ammonium sulfate precipitated (40% and 80%) PIs were added (10 µL) after 24 hours at varying concentrations (1:1, 1:10, 1:25 and 1:50 dilutions) for 24, 48, and 72 hours. After incubation, the MTT assay was performed, and the optical density was recorded at 540 nm. The viability percentage was calculated as follows:

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

where, OD represents the optical density of the sample and control at 540 nm

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.[15] The increase in the LDH enzyme levels in the treated cells reflects the number of lysed cells. The assay was performed based on the instructions provided in the kit manual.

Relative protein quantification using electrospray Ionization-nano Liquid Chromatograography Mass Spectrometry (LC-MS/MS) (Proteomics)

Protein extraction

Confluent cancer cell lines (70%) were cultured in a serum-free medium for 12 hours and later lysed using 2% sodium dodecyl sulfate in 50 mM triethyl ammonium bicarbonate 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 overnight with MS-grade trypsin (Sigma Aldrich) in a 1:25 ratio (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 is given in the web link.[16]

Liquid chromatography conditions

LC-MS/MS (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 20 mm C18 5 µm, water, Analytical column: 75 µm 200 mm HSS T3 C18 1.8 µm, (waters), Solvent flow rate: 300 nL/min, Column temperature being 35 °C, reverse phase chromatography mode: Autosampler temperature was 4 °C.

Mass spectrometry condition

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.

Analysis of identified proteins by Bioinformatics

Making use of the STRING database (https://string-db.org), the differentially expressed proteins were subjected to network analysis. Ingenuity Pathway Analysis (IPA) (https:// ingenuity.com/products/ipa) was made use of for analysing molecular pathways and canonical pathways. The proteomics data were deposited at the ProteomeXchange Consortium.[17]

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 (Figure 1a). At any of the tested concentrations, the leaves of *T. cordifolia*, and 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

-44.0%, -52.0% and -106.0% respectively). In addition, at all the investigated concentrations, microbial isolates of *A. venetianus* and *Streptomyces* sp. did not show any inhibitory effects on trypsin with negative values, as shown in Figure 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, PI activity was calculated and presented graphically (Figure 1b). The *T. cordifolia* stem extract has good PI activity with 71.0% inhibition at 1:2 dilution (highest PI activity), followed by the 1:1 diluted sample with 60.0%.

Effect of temperature on the PI activity of T. cordifolia

Phosphate buffer (PBS) and DW (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 (Figure 1c). According to the findings, at 50 °C, the DW (TD) extract at a dilution of 1:10 showed the highest protease inhibition with 71.0%, followed by the PBS extract at 75 °C, at a dilution of 1:10 with 70.0% inhibition.

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

T. cordifolia stem buffer extract was analyzed 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 67.0%, followed by pH 9 (1:10 dilution) with 63.0%, and again the pH 9 extract at 1:5 dilution with 53.0% (Figure 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 to analyze their effect on PI activity. As per the results, all metal ions significantly inhibited the PI activity of *T. cordifolia* stem extract (Figure 2a).

Partial purification of PI by salting out

For partial purification ammonium sulfate precipitation method was used. The *T. cordifolia* stem extract was added with ammonium sulfate to 40.0% and 80.0% saturation, and the precipitated inhibitor was used to perform the PI activity assay. Figure 2b displays the study findings, with the 80.0% sample at 1:30 dilution having the highest PI activity with 83.0% inhibition, followed by the 80.0% sample at 1:5 dilution with 79.0% inhibition. PI activity was maximum in the 80.0% ammonium sulfate precipitate and was also noticeable in the 40.0% precipitate.

PI's ability to inhibit Chymotrypsin and Collagenase enzymes

Because of the high PI activity of the partially purified inhibitor

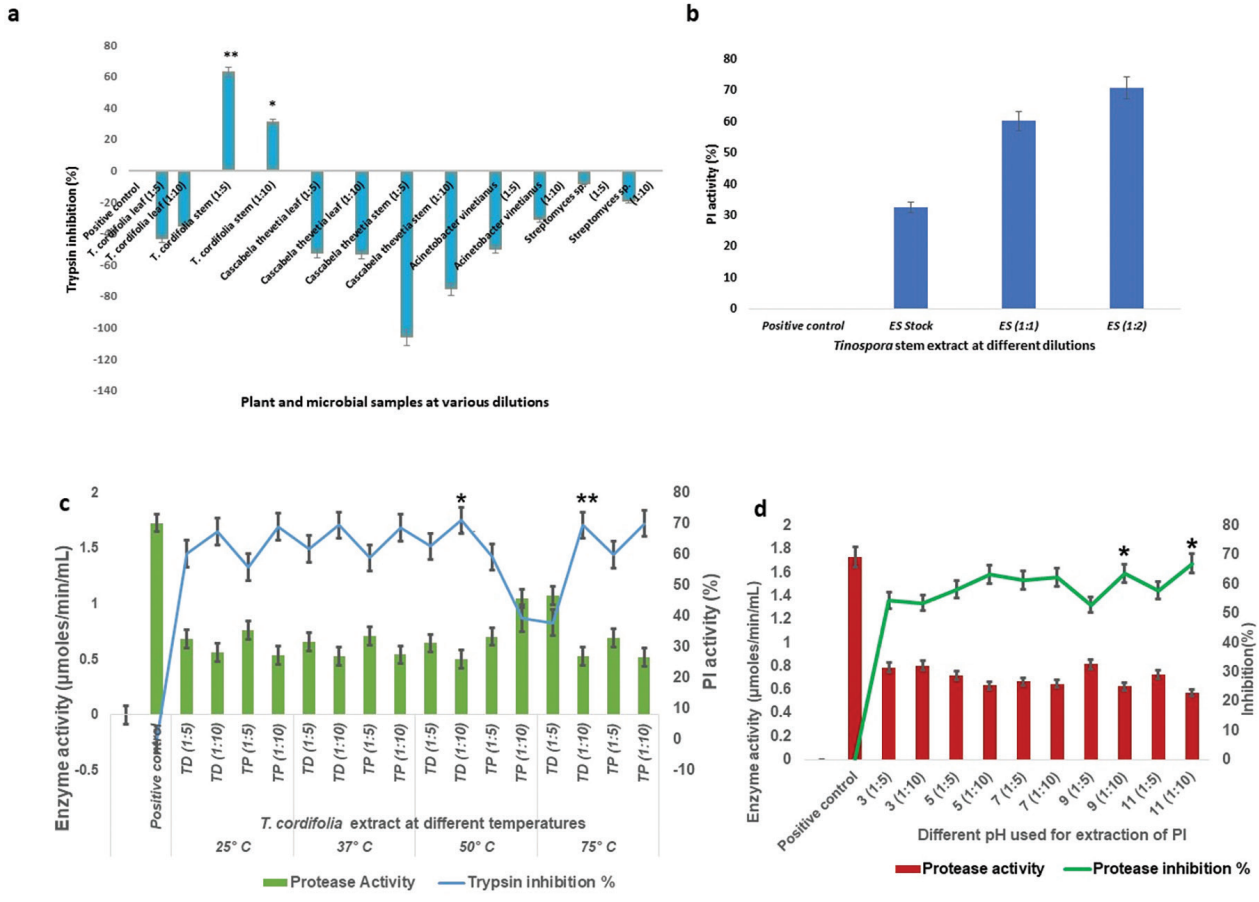

Figure 1. a) Screening the PI activity of plant and microbial samples, b) PI activity of *Tinospora cordifolia* stem extract, c) effect of temperature on PI activity of distilled water (TD) and Phosphate buffer extracts (TP) of *Tinospora cordifolia*, d) effect of pH on the PI of *T. cordifolia* stem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

PI: Protease inhibitors

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 40.0% ammonium sulfate precipitate of the PI with 96.0% inhibition, followed by the 80.0% sample with 78.0% inhibition, and slightly lower activity was observed with the crude PI with 45.0% inhibition (Figure 2c). The collagenase inhibitory potential was exhibited only by the 80.0% sample (53.0% inhibition), and both the crude extract and the 40.0% sample were not able to inhibit the activity of collagenase (Figure 2d).

Cytotoxicity studies on the HepG2 liver cancer cell line

As *T. cordifolia* stem extract and the ammonium sulfate precipitated samples showed good PI activity, these samples were treated with the liver cancer HepG2 cell line to assess their potential anticancer activity. According to the findings, after 48 hours of incubation, the ethanol extract exhibited 17.0% cytotoxicity, whereas the buffer extract displayed 13.0% cytotoxicity to HepG2 cells (Figure 3a).

Cytotoxicity of partially purified samples of 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 (24, 48, and 72 hours), the partially purified PI (40% sample) demonstrated considerable cytotoxicity at all the time periods with 81.0%, 80.0% and 65.0% viabilities respectively at 1:10 dilution. The maximum cytotoxicity was 35.0% (Figure 3b). The highest cytotoxicity was displayed by the PI (80.0% sample) at 72 hours of incubation and 1:20 dilution. The treated liver cancer cells had a viability of 53.0% with cytotoxicity of 47.0% (Figure 3c), and other dilutions also demonstrated considerable cytotoxicity after a 72-hours incubation period. The viability was 93.0% at 24 hours, 79.0% at 48 hours, and 69.0% at 72 hours of incubation with 1:5 diluted PI.

LDH activity assay

As per the LDH assay results, among the cancer cells treated with the PI (80.0% precipitate) from *T. cordifolia*, we observed 16.0% cytotoxicity on HepG2 cells and 13.0% cytotoxicity on MCF-7 (breast cancer) cells (Figure 3d).

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

When HepG2 cells treated with PI were subjected to LC-MS/MS-based proteomic analysis, the data showed altered expression of several proteins. Significant overexpression of 19 proteins, downregulation of 8 proteins, and the remaining unaltered proteins were observed (Figure 4a). These proteomics data were deposited to the ProteomeXchange Consortium with the dataset identifier PXD037511.[17] A fold change cutoff of 1.5-fold and $p < 0.05$ was considered to filter proteins for further analysis. As per the data, we found that proliferation-associated protein 2G4 (PA2G4) expression was 3.48-fold upregulated in treated HepG2 cells compared with

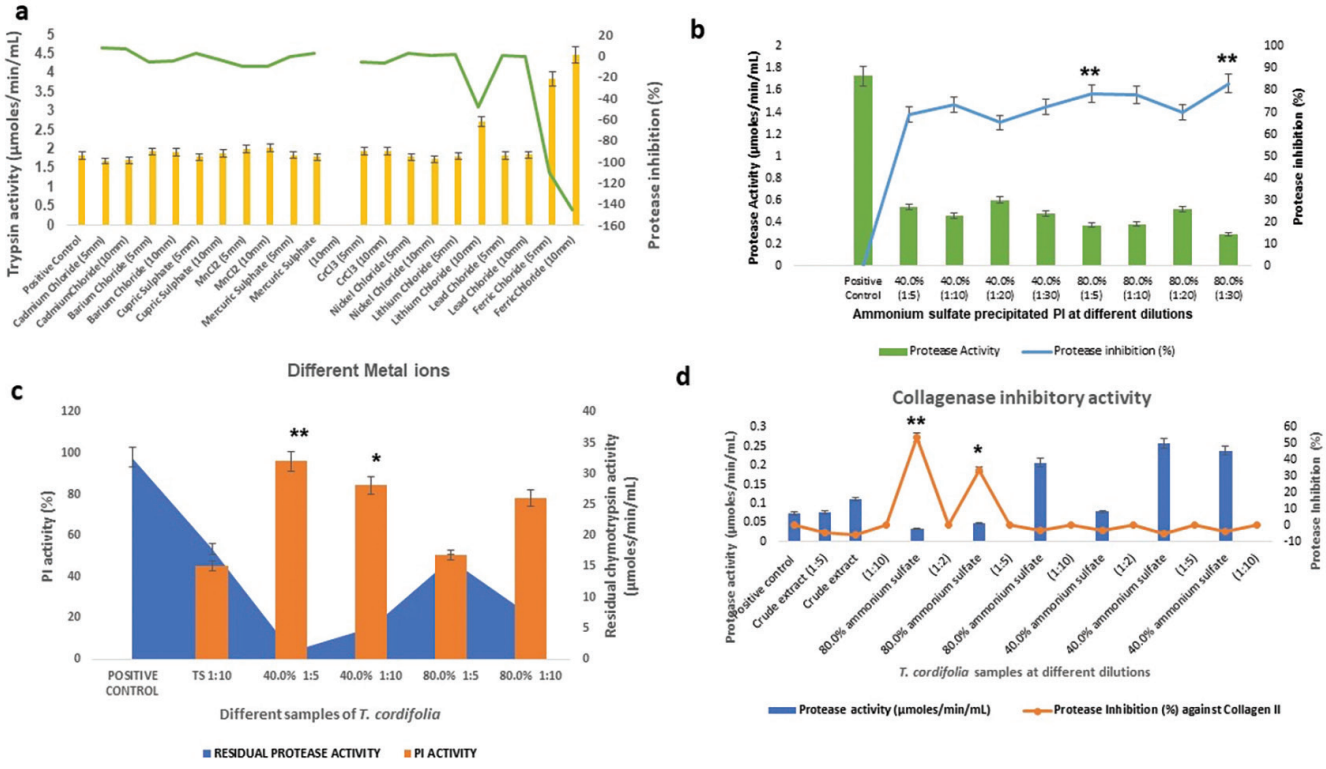

Figure 2. a) Effect of metal ions on PI activity of *Tinospora cordifolia,* b) PI activity of ammonium sulfate precipitate of *T. cordifolia* stem extract, c) chymotrypsin inhibitory activity of PI from *T. cordifolia*, and d) collagenase inhibitory activity of crude and partially purified PI from *T. cordifolia* stem

PI: Protease inhibitors

the controls. PA2G4 isoforms have opposing functions in cancer, which is well documented.[18] In our data, PEBP1 protein expression was 1.5-fold upregulated in treated HepG2 cells compared with the controls. EBP1 belongs to the PA2G4 family of DNA-binding proteins. The growth-inhibiting properties of EBP1 are multifaceted because it binds to RNA,[19] DNA[20] and proteins.[21] Overexpression of the ubiquitin-conjugating enzyme E2 L3 (UBE2L3) has been reported in non-small-cell lung cancer (NSCLC) tissues. A correlation was found between high expression of UBE2L3 and advanced tumor stage and negative outcomes, whereas UBE2L3 knockdown was reported to inhibit NSCLC cell growth.[22] In our current study results, UBE2L3 was downregulated 5.4-fold in treated HepG2 cells compared with control untreated cells. Mitogen-activated protein kinase kinase kinase 21 (MAP3K21) was observed to be 2.5-fold upregulated in treated HepG2 cells compared with controls, as per our result.

To understand the protein-protein interactions among the dysregulated proteins, a STRING database network analysis was performed.[23] 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 PI from *T. cordifolia* on HepG2 cells (Fig 4b).

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

According to canonical pathway analysis, EIF2 signaling, BAG2 signaling, and FAT10 signaling pathways were found to be significantly altered in the PI-treated HepG2 cells (Figure 4c). Among these, we considered BAG2 and FAT10 signaling pathways because of the 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 upregulated, and the upregulation of cathepsins increased the formation of autophagosomes.[24,25] Further in our Ingenuity Pathway analysis, 26s Proteasome proteins were found to be downregulated (Figure 5). In nuclei, the 26s proteasome complex increases the inhibition of ubiquitinated p53 protein. As per our results, the CTSB protein was upregulated (Figure 5).

Hsp70 is an important protein and has been studied for possible effects on energy metabolism. In our data, Hsp70 is downregulated, and downregulation of Hsp70 leads to

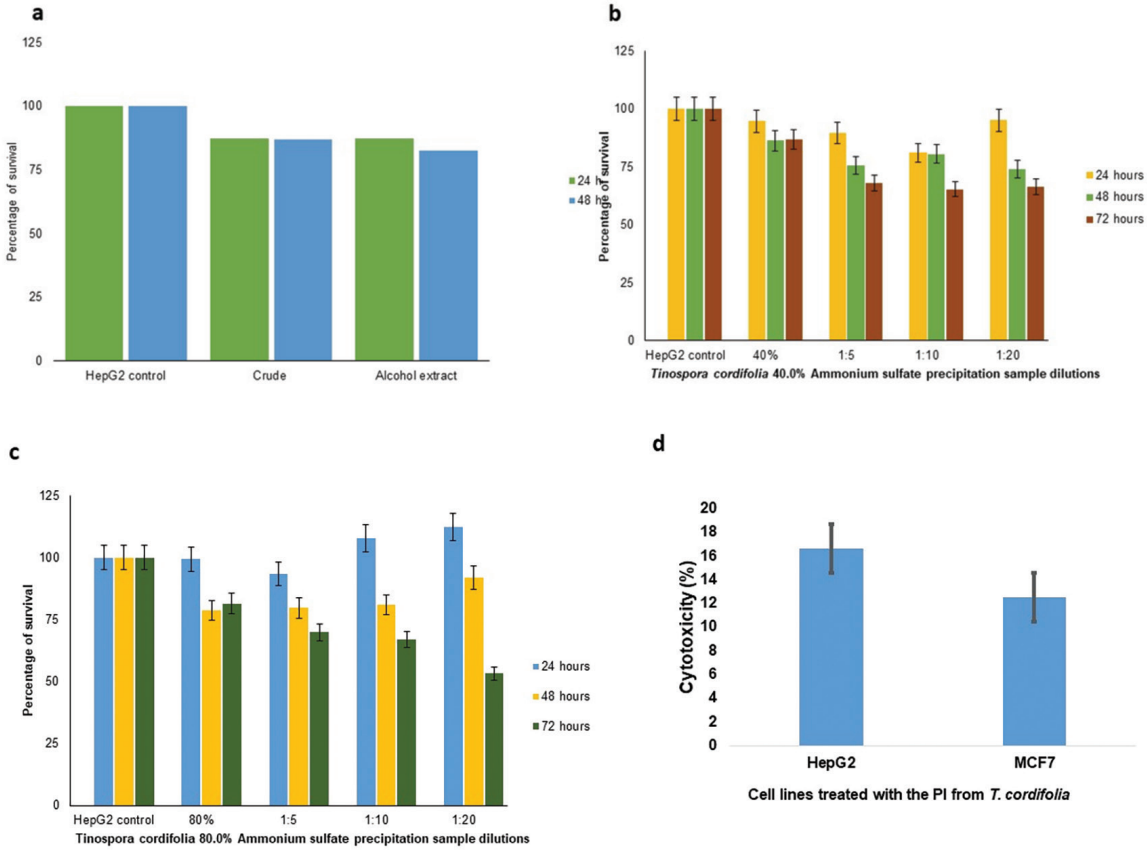

Figure 3. a) Effect of *Tinospora cordifolia* stem buffer extract and ethanol extract on HepG2 cells at 24 and 48 hours, b) effect of *T. cordifolia* 40.0% ammonium sulfate precipitated PI sample on Hep G2 cells, c) effect of *T. cordifolia* 80.0% ammonium sulfate precipitated PI sample on HepG2 cells, d) cytotoxicity of 80.0% ammonium sulfate precipitated PI of *T. cordifolia* by LDH assay

PI: Protease inhibitors, LDH: Lactate dehydrogenase

mitochondrial dysfunction. It can be inferred from our results that the downregulation of Hsp70, induced by PI in HepG2 cells, leads to mitochondrial dysfunction and cell death.

DISCUSSION

Proteolysis is considered the most important metabolic process involved in protein turnover and processing. Proteases are involved in the degradation of stored or unwanted proteins, which are essential for developmental processes such as differentiation and programmed cell death. Dysregulated proteolysis leads to many diseases, including cancer.[26] Hence, PIs have great therapeutic potential in controlling protease-mediated diseases. Plants and microbes are major sources of PIs. Therefore, 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 meth (*T. foenum-graecum*) seeds (sprouted), along with the microbes *A. venetianus* and *Streptomyces* sp., were chosen for our PI activity study. All these samples were tested for PI activity. The *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, with the highest inhibition value observed being 71.0%, while the meth seed extract also had good inhibitory effects with the highest of 61.0% inhibition. To analyze the effects of physicochemical parameters on PI activity, buffers with different pH values, different temperatures, and different metal ions were considered. We found that a basic pH of 11 had positive effects on the PI activity of *T. cordifolia*, with 67.0% 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.[26] For the PI from *T. cordifolia*, in temperature studies, we observed the highest percentage

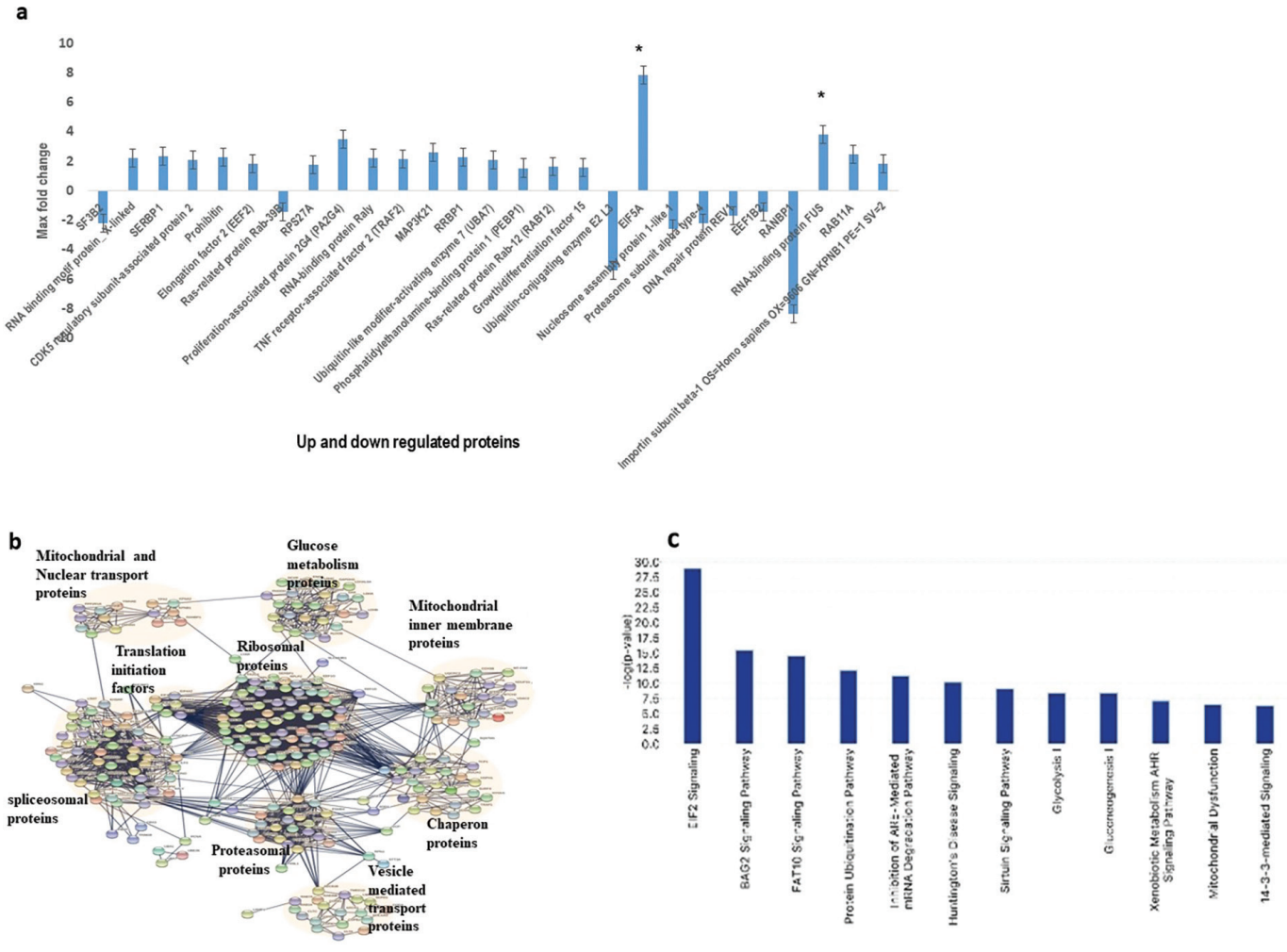

Figure 4. a) Protein expressions (having significance to cancer cell proliferation and survival) altered due to treatment with *T. cordifolia* PI sample in HepG2 cells, in comparison to the controls, b) Bioinformatic analysis of interaction of proteins using STRING database (https://string-db.org) in HepG2 cells treated with *Tinospora cordifolia* extract showing distinct molecular alterations, c) altered canonical pathways as per Ingenuity Pathway Analysis (https://ingen uity. com/ products/ipa) in *T. cordifolia* PI-treated HepG2 cells

PI: Protease inhibitors

of PI at 50 °C (71.0%), followed by 75 °C for the PBS extracted PI (70.0%). 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 previous study, it was reported that 1 mM Mg^{2+} addition increased the PI activity, whereas all other divalent metal ions decreased the activity.[26]

Because 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 40.0% and 80.0% ammonium sulfate precipitation), the highest protease inhibition was observed in the 80.0% precipitated sample. Even the 40.0% precipitated PI sample had considerable PI activity. Our results have shown that 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.[27] Compared with this report, in our current study, the 40.0% ammonium sulfate precipitated PI from *T. cordifolia* exhibited highest trypsin inhibition (78.0%) with the best cytotoxic activity (35.0%), whereas the PI sample precipitated by 80.0% ammonium sulfate exhibited 83.0% trypsin inhibition along with the highest cytotoxic activity against HepG2 liver cancer cells (47.0%). In addition, 96.0% inhibition of chymotrypsin and 54.0% inhibition of collagenase was 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, PI activity and cytotoxicity of PI from *T. cordifolia* are being reported for the first time through the current study.[28-30]

When HepG2 cells treated with 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 upregulated, and an increase in these proteins enhances the formation of autophagosome. Autophagy has both roles as a tumor suppressor and a tumor growth promoter.[24,25] In tumor suppressor mechanisms, autophagy prevents the accumulation of damaged proteins and organelles, which can promote tumor survival.[31]

In the FAT10 signaling pathway, the inhibition of fat10ylated active SQSTM1 protein occurs due to the 26s proteasome in the cytoplasm, and in our data, 26s proteasome proteins are downregulated. In contrast, in the nuclei, the 26s proteasome complex increases the inhibition of ubiquitinated p53 protein.[32] In HepG2 cells treated with PI, we could predict that the p53 protein was upregulated due to the downregulation of the 26s

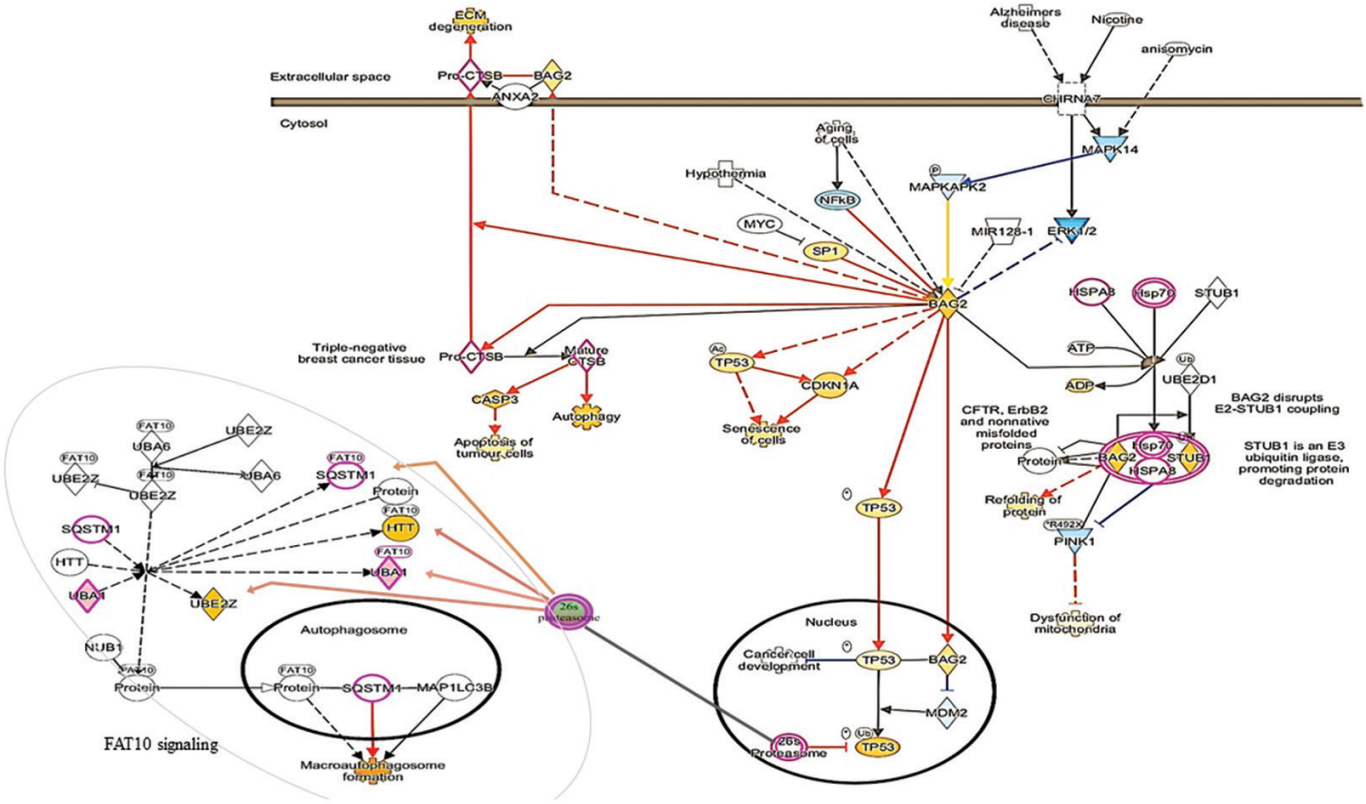

Figure 5. *Tinospora cordifolia* partially purified PI treatment on HepG2 cells affects BAG2 and FAT10 signaling pathways. Ingenuity Pathway analysis of proteins significantly dysregulated ($p \leq 0.05$) in treated HepG2/control HepG2 cells. The signaling network of BAG2, EIF2, FAT10 pathways based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

proteasome.[33] This upregulation of p53 in the HepG2 cells might be a reason 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 the 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.[34]

Hsp70 is an important protein studied for its possible effects on energy metabolism. In our data, Hsp70 is downregulated, which leads to mitochondrial dysfunction through inhibition of oxidative phosphorylation and ROS generation.[35] Because mitochondria are primarily involved in cell apoptosis, we can assume that Hsp70-mediated mitochondrial dysfunction leads to the apoptosis of HepG2 cells treated with PI in our current study. There are studies and reports about the anti-HIV and anti-severe acute respiratory syndrome Coronavirus-19 (SARS-CoV-19) activity of the *T. cordifolia* PI.[9,36] However, 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, and 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 PI from *T. cordifolia* has potent PI and anticancer effects on the liver cancer cell line HepG2. *T. cordifolia* possesses several phytochemicals with significant anticarcinogenic activities, as per many earlier *in vivo* and *in vitro* studies. Although 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 in the current study. The current study opens further scope for the complete characterization of this PI and the validation of its anticancer activity through *in vivo* and future clinical studies.

Ethics

Ethics Committee Approval: Not required.

Informed Consent: Not required.

Authorship Contributions

Concept: V.K.N., Design: V.K.N., B.S.C., K.G., M.K., N.M., N.H.K., S.L.P., Data Collection or Processing: B.S.C., K.G., M.K., N.M., N.H.K., S.L.P., S.D., Analysis or Interpretation: V.K.N., Literature Search: B.S.C., K.G., M.K., N.M., N.H.K., S.L.P., Writing: B.S.C., K.G., M.K., N.M., N.H.K., S.L.P.

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