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# Assessment of Cytotoxic Effects of Plant Protease Inhibitors on Cancerous Cell Lines

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***Authors' contributions***

*This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.*

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## ABSTRACT

Protease inhibitors, whether natural or synthetic, show promise in cancer treatment by targeting dysregulated proteolytic activity linked to tumor progression and metastasis. This study evaluated trypsin inhibitory activity and cytotoxic effects of selected plant extracts. Screening twenty-six plant extracts revealed twelve extracts contain trypsin inhibitors, and eighteen plant extracts showed cytotoxicity to the HT29 cell line, with the highest cytotoxicity shown by *Senna alatta* extract (98.45 ± 0.44 %). When plant extracts containing protease inhibitors were subjected to proteinase K digestion, the protease inhibitor activity as well as cytotoxicity was reduced. For example, extracts

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from *Chassalia curviflora, Senna alata*, and *Grewia nervosa* showed high trypsin inhibition as well as cytotoxicity, both of which were reduced by proteinase K treatment. This indicates that there is some correlation between cytotoxicity and trypsin inhibition (r = 0.52) and proteinaceous trypsin inhibitors or other proteins are involved in the cytotoxicity. These findings highlight the potential role of trypsin inhibitors in cytotoxicity against cancer cell lines.

*Keywords: Plant protease inhibitor; trypsin inhibition; cytotoxicity; plant extract.*

## 1. INTRODUCTION

Proteases, the proteolytic enzymes, are pivotal in orchestrating various proteolytic cascades, ensuring precise control to prevent excessive degradation of proteins. In animals, proteases play key roles in diverse cellular activities, including inflammatory responses, cellular apoptosis, hemostasis, and hormone processing. Nonetheless, the proteolytic potential of these enzymes necessitates stringent regulation to avert undesirable outcomes [1].

The protease inhibitors (PIs) are indispensable for modulating proteolytic activity, thereby exerting significant influence on metabolic and cellular physiological processes. In animals, certain PIs are identified as growth modulators and integral components of cell signaling cascades, with some of them demonstrating anticancer activity. Conversely, malfunctioning PIs underlie various inherited disorders such as emphysema and certain forms of epilepsy, underscoring their undeniable role in maintaining cellular homeostasis [2].

In plants, PIs feature prominently in diverse physiological processes, including the mobilization of reserve proteins, regulation of endogenous enzymatic activities, apoptosis regulation, and strengthening of defense mechanisms against predators and pathogens [3]. Notably, plants serve as prolific sources of PIs, particularly those belonging to the serine protease inhibitor category, which exhibits remarkable efficacy against proteases like trypsin and chymotrypsin. They play crucial roles in both endogenous and exogenous defense mechanisms [4]. The major pests on plants are the larvae of lepidopteran insects and their gut is rich in trypsin–like proteases. Thus, naturally occurring plant protease inhibitors (PPIs) serve as defense molecules in plants [5]. Also, their ability to target specific proteases, PIs holds significant promise in biotechnology and medicine, particularly in combating diseases such as cancer, AIDS, and cardiovascular conditions [6].

Cancer remains a global health challenge, claiming millions of lives every year. In spite of advances in therapy, issues such as drug side effects and resistance continue to hinder effective cancer management. Therefore, there is a growing interest in developing novel anticancer agents that are both effective against cancer cells and less prone to resistance development [7].

Plant protease inhibitors like soybean Bowman–Birk protease inhibitor [8] and plant lectins [9] have shown anticancer properties and are extensively studied. Additionally, many plant species remain untapped sources of PIs with potential anticancer effects. The inhibition of proteases has been linked to anticancer properties, particularly through the use of plant derived inhibitors. These inhibitors have demonstrated the ability to inhibit the proliferation of cancer cells, positioning them as potential candidates for cancer therapy [10]. Studies by Magee et al. isolated protease inhibitor concentrates from various leguminous sources and evaluated their effects on breast and prostate cancer cell proliferation *in vitro*. Specifically, the protease inhibitor from *Cicer arietinum* L. exhibited anticancer activity against various cell lines, including MDAMB-231 breast cancer and PC-3 and LNCaP prostate cancer cell lines [11]. Additionally, *Lavatera cashmeriana* protease inhibitors showed *in vitro* anticancer activity on THP- 1 (leukemia), NCIH322 (lung), and Colo205, HCT-116(Colon) cancer cell lines [12]. The mature Bowman-Birk inhibitor (BBI) from *Lavatera cashmeriana* was found to inhibit the growth of human colon adenocarcinoma HT29 cells and colonic fibroblast CCD-18Co cells at concentrations as low as 19µmol/L in a concentration dependent manner, without affecting the CCD-18Co cells [6]. Overall, the literature on plant derived protease inhibitors underscores their multifaceted roles in biological processes, from regulating programmed cell death in plants to inhibiting cancer cell proliferation.

In light of the urgent need for more efficient cancer therapies, the current study focuses on

identifying PIs from various plants and evaluating their anticancer activity against the human colorectal carcinoma cell line, HT29.

## 2. MATERIALS AND METHODS

### 2.1 Materials Used

HT29 (human colorectal carcinoma cell line) was purchased from the National Centre for Cell Science (NCCS), Pune, foetal bovine serum (FBS) from Gibco-ThermoFisher Scientific, USA , McCoy's 5A medium from Sigma Aldrich, USA; MTT ([3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]) from Himedia, India; Proteinase K from Himedia, India and trypsin (origin-bovine pancreas) from Sigma Aldrich, USA. All other reagents and chemicals used were of analytical grade.

### 2.2 Collection of Plants

A variety of plants and plant parts were gathered from various locations in Malappuram and Palakkad, Kerala, India. The specimens were identified by Dr. A. K. Pradeep, Assistant Professor, Department of Botany, University of Calicut.

### 2.3 Plant Extract Preparation

The leaves or other plant parts (Table 1) were rinsed with clean water, dried to remove excess moisture, and then immersed in 10 mM phosphate buffered saline with a pH of 7.4 (1 mL/g of tissue). After overnight soaking, the materials were homogenized. The homogenates were then centrifuged at 10,000xg for 10 minutes at 4ºC. The supernatant with soluble proteins was carefully recovered and stored at -20 ºC until use.

### 2.4 Protein Estimation

The protein estimation of plant extracts was determined by Bradford's dye binding method using bovine serum albumin as a standard [13].

### 2.5 Preparation of Trypsin

A solution of trypsin was made by dissolving 1 mg of bovine trypsin in 1 mL of 1 mM HCl and kept frozen until use.

### 2.6 Protease Assay

To conduct the protease assay, 5 µL of trypsin (1µg/µL) with 78 µg azocasein was incubated in a total volume of 20.2µL at 37ºC for 30 minutes. Following incubation, the reaction was halted by adding 80µL of 5% trichloroacetic acid and centrifuged at 10,000xg at 4ºC for 10 minutes. Fifty microliters of the supernatant were subsequently mixed with 150µL of 0.5 M NaOH, and the absorbance was measured at 440nm [14].

$$\text{Protease activity (in \%)} = (100 \times (A_{\text{Inhibited test}} - A_{\text{Inhibitor alone}})/A_{\text{Trypsin}})$$

### 2.7 Protease Inhibition Assay

For protease inhibition assay, the plant extract was pre-incubated with trypsin for 10 minutes before the addition of the substrate. The subsequent steps were conducted in the same manner as in protease assay described elsewhere.

$$\text{Protease inhibition (in\%)} = 100 - \text{Protease activity}$$

### 2.8 Proteinase K Treatment

To confirm the proteinaceous nature of the PI in the extract, proteinase K digestion of the extract followed by protease inhibition assay was performed. Initially, 90µL of plant extract was incubated with 10µL of proteinase K (2.3 µg) at 56ºC over night. The proteinase K was inactivated by heating the mixture at 96ºC for 10 minutes. Finally, the resulting mixture was used for protease inhibition assay to assess the inhibitory activity of the PI.

### 2.9 Cell Culture

HT29 (human colorectal carcinoma cell line) was cultured in McCoy's 5A medium supplemented with 10% FBS and 1% antibiotic-antimycotic solution under 5% carbon dioxide at 37 ºC temperature.

### 2.10 MTT Assay for Cytotoxicity

MTT assay was performed to evaluate the cytotoxicity of the extracted PIs on the HT29 cancer cell line. Initially, the cells were seeded in 96-well microplates ($1x10^4$ cells/mL) in a total volume of 100 µL McCoy's 5A medium. After 24 hours of incubation, 20µL of the inhibitor was added at a protein concentration of 1mg/mL to each well and the cells were further incubated for 48 hours. Subsequently, 20µL of MTT solution (5 mg/mL) was added to each well and the plates

were incubated in a dark chamber for 3 hours to allow formazan crystal formation. To dissolve the formazan crystals, 100µL of lysis buffer was added and in an ELISA plate reader, the absorbance was recorded at 570nm. The percentage viability was calculated using the formulae [15]:

Cell viability (in %) = ($A_{Test}$/ $A_{Control}$) x 100

Percentage mortality = 100 – Cell viability

## 2.11 Statistical Analysis

All experiments were replicated thrice and the results are expressed as mean±SEM. The results were analysed statistically using Graphpad Prism 10.

## 3. RESULTS AND DISCUSSION

Protease plays crucial roles in cancer progression, from benign states to malignancy. Proteases also involved in various processes such as cancer cell escape through tissue barriers facilitating metastasis [16]. The intricate process of proteolysis influences inflammatory responses, immune cell recruitment, proliferation, and apoptosis in cancer [17]. Regulation of protease activity involves interactions with endogenous inhibitors like tissue inhibitors of metalloproteases, serine proteases, and cysteine proteases [18]. Understanding the role of proteases and their inhibitors in cancer progression expands the therapeutic opportunities for cancer treatment [19]. Comprehensive insights into protease functions in tumour progression, metastasis, and cancer development will help in the development of innovative therapeutic strategies against cancer.

Cancer metastasis entails a complex series of events, including adherence, migration, extracellular matrix degradation, and establishment of cancerous cells at new sites. Proteases, notably serine proteases and matrix metalloproteinases (MMPs), are pivotal in this process, forming a complex cascade system. MMPs, in conjugation with activated trypsin,

**Table 1. List of plants used for the screening of protease inhibition and cytotoxicity against the HT29 cancer cell line**

| | Plant Name | Family | Plant Part | Collection locality |
|---|---|---|---|---|
| 1 | *Aerva lanata* (L.) Juss. | Amaranthaceae | Leaves | Palakkad |
| 2 | *Anamirta cocculus* (L.) Wight & Arn. | Menispermaceae | Leaves | Palakkad |
| 3 | *Chassalia curviflora* (Wall.) Thwaites | Rubiaceae | Seeds | Palakkad |
| 4 | *Clitoria ternatea* L. | Leguminosae | Leaves | Palakkad |
| 5 | *Cyperus rotundus* L. | Cyperaceae | Leaves | Palakkad |
| 6 | *Desmodium triflorum* (L.) DC. | Leguminosae | Whole plant | Palakkad |
| 7 | *Euphorbia heterophylla* | Euphorbiaceae | Inflorescence | Malappuram |
| 8 | *Ficus racemosa* L. | Moraceae | Inflorescence | Palakkad |
| 9 | *Grewia nervosa* (Lour.) Panigrahi | Malvaceae | Seeds | Palakkad |
| 10 | *Hamelia patens* Jacq. | Rubiaceae | Leaves | Palakkad |
| 11 | *Jasminum coarctatum* Roxb. | Oleaceae | Leaves | Malappuram |
| 12 | *Jasminum grandiflorum* (L.) | Oleaceae | Leaves | Palakkad |
| 13 | *Lantana camara* (L.) | Verbenaceae | Leaves | Malappuram |
| 14 | *Leucas aspera* (Wild.) Link | Lamiaceae | Leaves | Malappuram |
| 15 | *Macaranga peltata* (Roxb.) Müll. Arg. | Euphorbiaceae | Leaves | Malappuram |
| 16 | *Micrococca mercurialis* (L.) Benth. | Euphorbiaceae | Whole plant | Malappuram |
| 17 | *Pedilanthus tithymaloides* (L.) Poit. | Euphorbiaceae | Leaves | Palakkad |
| 18 | *Phyllanthus amarus* (Schumach. & Thonn. | Phyllanthaceae | Whole plant | Palakkad |
| 19 | *Rhaphidophora pertusa* (Roxb.) Schott | Araceae | Leaves | Palakkad |
| 20 | *Senna alata* (L.) Roxb. | Leguminosae | Seeds | Palakkad |
| 21 | *Stachytarpheta indica* (L.) Vahl | Verbenaceae | Leaves | Malappuram |
| 22 | *Talinum portulacifolium* (Forssk.) Asch. Ex Schweinf | Talinaceae | Leaves | Palakkad |
| 23 | *Terminalia catappa* L. | Combretaceae | Seeds | Malappuram |
| 24 | *Thunbergia erecta* (Benth.) T. Anderson | Acanthaceae | Leaves | Palakkad |
| 25 | *Wattakaka volubilis* (L.f.) Stapf | Asclepiadaceae | Leaves | Malappuram |
| 26 | *Ziziphus oenoplia* (L.) Miller | Rhamnaceae | Leaves | Malappuram |

promote cell invasion and metastasis. These proteases are essential drivers of cancer cell dissemination and are integral to the metastatic process [20,21].

### 3.1 Trypsin Inhibition by Plant Extracts

Twenty six different plant extracts were assessed for their ability to inhibit trypsin activity using azocasein as a substrate. Of these, 12 plant extracts demonstrated trypsin inhibition (Fig. 1). The highest percentage of trypsin inhibition was given by *Terminalia catappa* seed extract (85.08 ± 0.79 %), followed by the *Senna alata* (81.97 ± 1.06 %) leaf extract and *Micrococca mercurialis* (77.83 ± 3.30 %) whole plant extract. Of the 26 plant extracts tested, 12 extracts gave more than 50% inhibition of trypsin activity (Fig. 1). A heat-stable trypsin inhibitor (86% inhibition) was previously identified from the bark of *Terminalia catappa* [22]. As the seeds of this plant are edible, the inhibitor is a dietary source for humans [23]. *Senna alata* is a medicinal plant, and from its seeds two potential inhibitors are isolated [24]. Previous research has identified trypsin inhibitors in many food plants like potatoes (*Solanum tuberosum*), sweet corn (*Zea mays*), sweet potato (*Ipomea batatus*), spinach (*Spinacia oleracea*), broccoli (*Brassica oleracea* var.botrytis), Brussels sprouts (*Brassica oleracea* var. gemmifera) [25], cucumber (*Cucumis sativus*) [25,26], red bryony (*Bryonia diotica*), figleaf gourd (*Cucurbita ficifolia*), spaghetti squash (*Cucurbita pepo*), water melon (*Citrullus vulgaris*) [26] and bitter gourd (*Momordica charantia* L.) [27], green gram (*Vigna radita*), cashew nuts (*Anacardium occidentale*), jack fruit seeds (*Artocarpus heterophyllus*) [28] and other plants like red lucky seed (*Adenanthera pavonina* L.), babul (*Acacia nilotica L.*) [27], *Dalbergia latifolia* (85.09±0.40%) [29], etc. Trypsin inhibitors have been also isolated from sweet corn [25] from the seeds of cucumber (*Cucumis sativus*), red bryony (*Bryonia diotica*), figleaf gourd (*Cucurbita ficifolia*), spaghetti squash (*Cucurbita pepo*) and water melon (*Citrullus vulgaris*) [26], *Spatholobus parviflorus* [30] *Moringa oleifera* [31,32] etc. No protease inhibitors were reported from the remaining plants in the present study.

### 3.2 Trypsin Inhibition of Plant Extracts After Proteinase K Treatment

Treatment of extracts from *Talinum portulacifolium, Terminalia catappa, Micrococca mercurialis, Senna alata, Grewia nervosa, Chassalia curviflora, Stachytarpheta indica, Macaranga peltata, Aerva lanata* and *Ficus racemosa* with proteinase K resulted in a significant reduction in trypsin inhibition (Fig. 2). This finding indicates the proteinaceious nature of the trypsin inhibitors in these plant extracts. Conversely, the trypsin inhibition profiles of extracts from other plant extracts, *Rhaphidophora pertusa* and *Pedilathus tithymaloides* remained unaltered, implying that the trypsin inhibitor in those extract may be non-protein in nature. Proteinaceous trypsin inhibitor was reported from *Lawsonia inermis* leaf extract [33] and inhibitor against Spodoptera gut proteases from *Ardisia solanaceae*, *Acasia concina* [34] and *Areca triandra* [35]. Non–proteinaceous trypsin inhibitors like Myricitrin, quercetin, Isoquercitrin, rutin, Kaempferol, Betulinic acid, Caffeic acid, Andrographolide, Faradiol 3-O-palmitate, etc were reported from different plants [36].

### 3.3 Cytotoxicity of Plant Extracts to the Cancer Cell Line, HT29

Twenty-six plant extracts were screened for their cytotoxicity against HT29 cancer cell line. Cytotoxicity was assessed after 24, 48 and 72 hour intervals. Among the plants screened, 18 exhibited significant cytotoxicity against the HT29 cancer cell line. *Senna alata* displayed the highest cytotoxicity with 98.45±0.44% mortality after 24 hours incubation at a protein concentration of 1mg/mL (Fig. 3). Ethyl acetate extract from the seeds of *S. alata* has been reported to inhibit cancer cell proliferation in in many cell lines including HT29 [37]. Also anticancer properties have been observed in the hexane extract of *S. alata* against breast cancer cell line MCF7, SK – BR3, bladder carcinoma T24, colorectal carcinoma Col 2, and non-small cell lung adenocarcinoma A549 [38]. However, the proteinaceous anticancer properties of *S. alata* have not been thoroughly explored. The other plant extracts which gave more than 85% mortality in HT29 cancer cell line includes *Cyperus rotundus* (98.37±0.26%), *Clitoria ternatea* (98.03±0.27%), *Leucas aspera* (97.76±1.90%), *Stachytarpheta indica* (96.54±1.50%), *Chassalia curviflora* (96.13±1.16%), *Phyllanthus amarus* (94.83±0.13%), and *Micrococca mercurialis* (88.03±4.57%) (Fig. 3). *In vitro* studies using extracts from the rhizome of *Cyperus rotundus* demonstrated cytotoxic effects against various cell lines, including HT29 [39]. Additionally more than 50% mortality in HT29 cell line were

observed with extracts from *Macaranga peltata, Grewia nervosa, Hamelia patens, Aerva lanata, Thunbergia erecta, Wattakaka volubilis, Desmodium triflorum, Jasminum coarctatum* and *Talinum portulacifolium.* With increase in duration of incubation, the extracts gave higher mortality rates (Fig. 3).

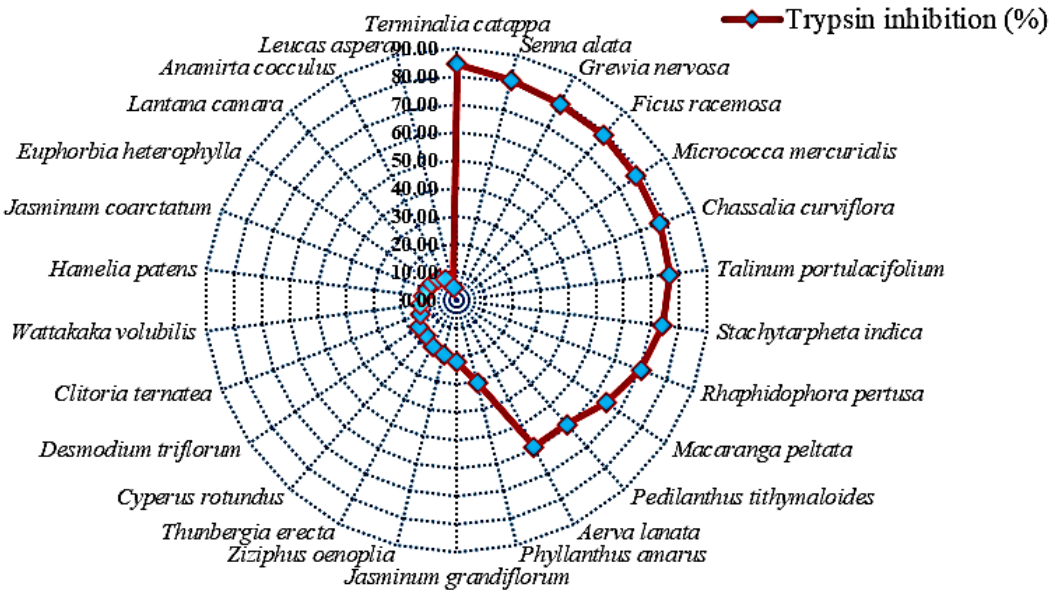


**Fig. 1. Diagram showing percentage trypsin inhibition exhibited by plant extracts**

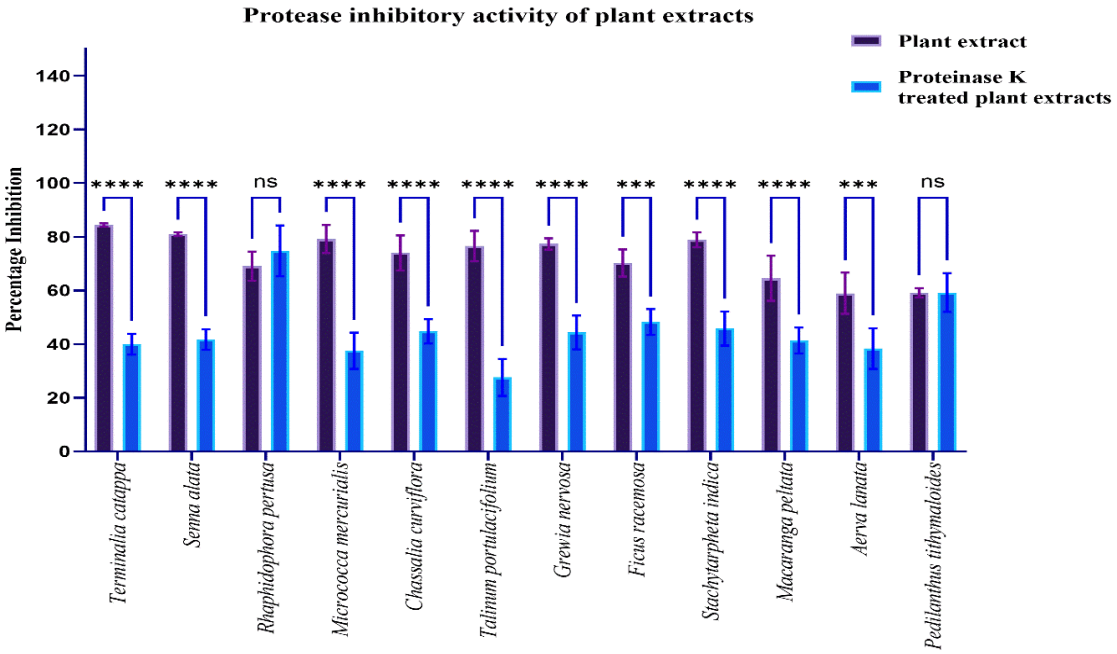


**Fig. 2. Graph showing the change in trypsin inhibition of the plant extracts after proteinase K treatment. Data is represented as mean±SEM and Statistical significance: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 and ns – not significant**

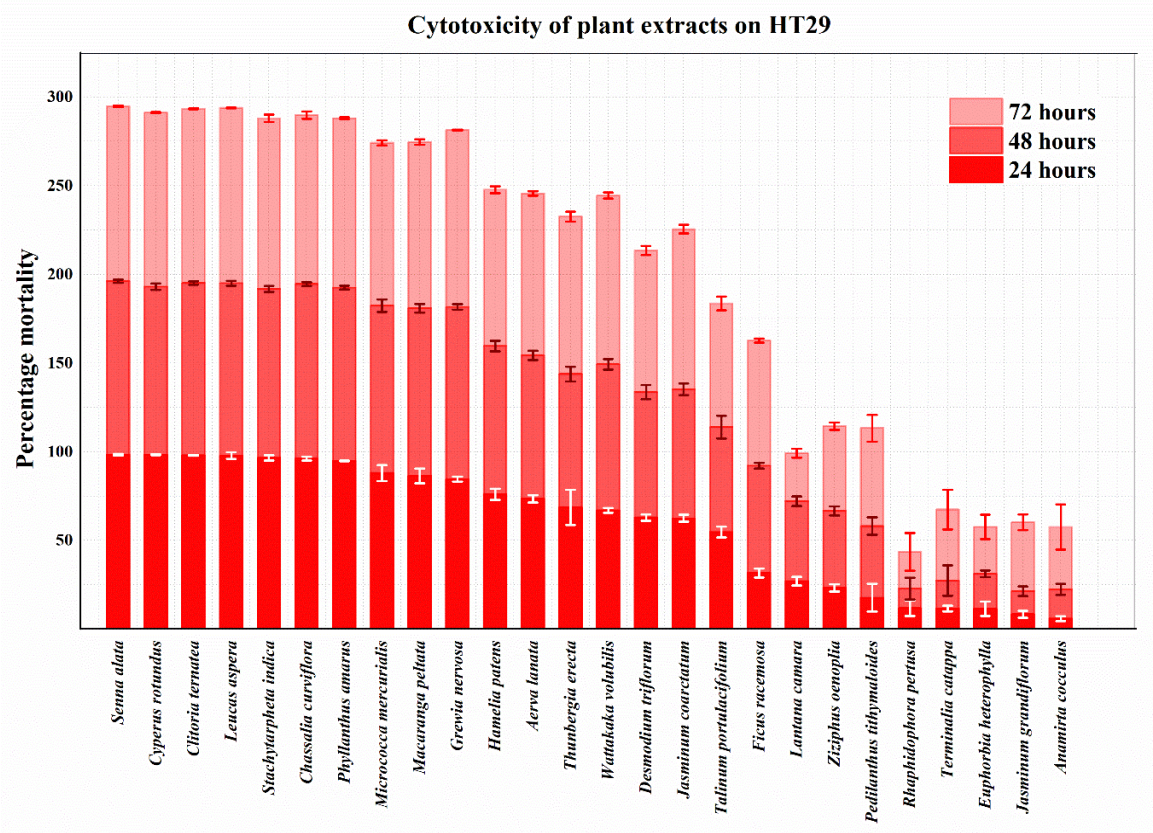


**Fig. 3. Diagram showing the percentage mortality of HT29 cancer cell line after treating with the plant extracts for different durations. Data is represented as mean±SEM**

### 3.4 Toxicity of Plant Extracts to HT29 Cells After Proteinase K Treatment

To assess whether proteins in the extract contribute towards cytotoxicity, the plant extracts were digested with proteinase K followed by checking the cytotoxicity. Specifically, for *Senna alata, Stachytarpheta indica, Chassalia curviflora, Micrococca mercurialis, Macaranga peltata, Ficus recemosa, Aerva lanata, Thunbergia errecta, Grewia nervosa, Wattakaka volubilis* and *Talinum portulacifolium* extracts, the cytotoxicity decreased after digestion with proteinase K (Fig. 4). This suggests that at least to a certain extent, the cytotoxicity is due to protein component in the extract. Conversely, for all remaining extracts, cytotoxicity was unaffected by proteinase K digestion indicating that non–proteinaceous molecules in the extract is responsible for the cytotoxicity. Non–proteinaceous anticancerous compounds reported from plants include resveratrol, curcumin, quercetin, rutin, betulinic acid, artemisinin, etc [40].

### 3.5 Relation between Cytotoxicity and Trypsin Inhibition

Among the plant extracts under study revealed their trypsin inhibitory activity and cytotoxic activity against HT29 cell line. Out of 26 plant extracts tested, nine of them showed both cytotoxicity and trypsin inhibition, indicating a possible involvement of the trypsin inhibitor in the cytotoxicity (Table 2). Upon proteinase K treatment of these nine extracts, considerable decrease in both cytotoxicity and trypsin inhibition was observed. This may be due to proteinaceous protease inhibitor contributing towards cytotoxicity or other proteins in the extract impart cytotoxicity or a combined effect of both. Many PPIs have been identified as protein molecules with anticancer properties. The protease inhibitor isolated from *Enterolobium contortisiliquum* [41], Bowman Birk Inhibitor isolated from *Glycine max* [42], protease inhibitor from *Lens culinaris* [43], protease inhibitor from *Pisum sativum* [44], etc. are such well-studied PPIs having anticancer activities against HT29 cell line.

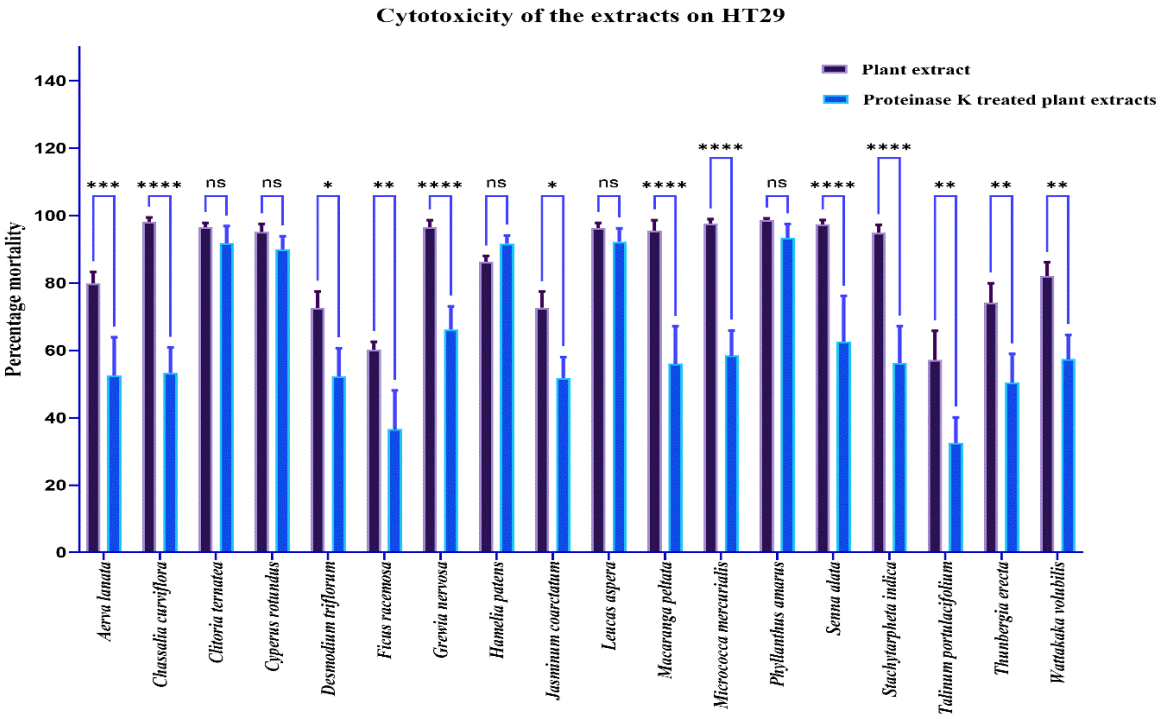


**Fig. 4. Graph showing the change in cytotoxicity of the plant extracts against HT29 cancer cell line after proteinase K digestion. Data is represented as mean±SEM and Statistical significance: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 and ns – not significant**

**Table 2. List of the plant extracts having cytotoxicity against HT29 and trypsin inhibitor**

| | Cytotoxicity on HT29 - 48 hrs (%) | | Trypsin inhibition (%) | |
|---|---|---|---|---|
| **Plant** | **Plant extract** | **Proteinase K treated** | **Plant extract** | **Proteinase K treated** |
| *Chassalia curviflora* | 98.47 ± 1.03 | 53.24 ± 4.40 | 74.36 ± 3.77 | 44.73 ± 2.65 |
| *Senna alata* | 97.68 ± 0.91 | 62.53 ± 7.82 | 81.97 ± 1.06 | 41.66 ± 2.19 |
| *Grewia nervosa* | 97.12 ± 1.60 | 66.18 ± 3.99 | 76.26 ± 1.63 | 44.34 ± 3.63 |
| *Stachytarpheta indica* | 95.21 ± 1.71 | 56.27 ± 6.27 | 75.65 ± 3.59 | 45.78 ± 3.68 |
| *Macaranga peltata* | 94.50 ± 2.44 | 56.03 ± 6.40 | 64.36 ± 4.86 | 41.34 ± 2.79 |
| *Micrococca mercurialis* | 94.29 ± 3.48 | 58.43 ± 4.31 | 77.83 ± 3.32 | 37.47 ± 3.91 |
| *Aerva lanata* | 80.81 ± 2.59 | 52.51 ± 6.55 | 59.10 ± 4.44 | 38.3 ± 4.37 |
| *Ficus racemosa* | 60.46 ± 1.68 | 36.57 ± 6.64 | 71.11 ± 3.04 | 48.24 ± 2.79 |
| *Talinum portulacifolium* | 59.14 ± 6.41 | 32.59 ± 4.36 | 75.92 ± 3.34 | 27.60 ± 3.98 |

Although, in general there is a moderate correlation (coefficient of correlation, r = 0.52) between the cytotoxicity and trypsin inhibition of the plant extracts, there are cases in which plant extracts with trypsin inhibition showing no significant cytotoxicity. For example, extracts from *Pedilanthus tithymaloides, Terminalia catappa,* and *Rhaphidophora pertusa* did not show cytotoxicity, though there was trypsin inhibition. On the contrary, plant extracts like *Phyllanthus amarus,Clitoria ternatea, Leucas aspera, Cyperus rotundus* and *Hamelia patens* are cytotoxic to HT29 cell line, but showed no significant trypsin inhibition (Figs. 1 & 3).

## 4. CONCLUSIONS

In this study, we analyzed the plant extracts for their trypsin inhibitory activity and cytotoxicity against HT29 cell line. Among the twenty-six plants investigated, eighteen plant extracts displayed cytotoxicity against the HT29 cell line, with the highest cytotoxicity shown by *Senna alata* (98.45±0.44%) followed by, *Cyperus rotundus, Clitoria ternatea,* etc. Of these 26 plant

extracts, 12 of them displayed significant trypsin inhibition, with *Terminalia catappa* (85.08 ± 0.79 %) exhibiting the highest trypsin inhibition. Of the twelve plant extracts with trypsin inhibition, nine extracts also showed higher cytotoxicity against HT29 cell line. These nine plant extracts when subjected to proteinase K digestion showed decrease in protease inhibition as well as cytotoxicity. For example, extracts from *C. curviflora, S. alata*, and *G. nervosa* showed decrease in trypsin inhibition as well as cytotoxicity when subjected to proteinase K treatment. This indicates that there is a correlation between cytotoxicity and trypsin inhibition indicating that the trypsin inhibitor or other proteinaceous components present in the trypsin inhibitor containing extracts may be contributing towards cytotoxicity. These results underscore the potential of plant extracts containing trypsin inhibitors as sources of anticancer molecules. Further purification of the trypsin inhibitor from these plant extracts is required to confirm the role played by trypsin inhibitors in these extracts in cytotoxicity on cancer cell lines. In addition, studies are warranted to identify anticancer molecules from these plant extracts to elucidate the underlying mechanisms and therapeutic potential of these plant-derived compounds.

## DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that generative AI technologies such as Large Language Models, etc have been used during writing or editing of manuscripts. This explanation will include the name, version, model, and source of the generative AI technology and as well as all input prompts provided to the generative AI technology.

**Details of the AI usage are given below:**

1. Used ChatGPT for paraphrasing.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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