Chelonian Conservation And Biology

Vol. 19 No. 1 (2024) | https://www.acgpublishing.com/ | ISSN - 1071-8443

DOI: doi.org/10.18011/2024.01(1).1513-1529

EVALUATION OF ANTIMICROBIAL AND ANTICANCER EFFICACY OF PURIFIED SERRAPEPTASE ENZYME

Dalia Elebeedy[1], Aml Ghanem[2], Marwa El-Sayed[3], Ingy Badawy[1], Alya Redhwan[4,*], Mashail A. Alghamdi[5], Tahani M. Alqahtani[5], Enas A. Almanzalawi[5], Majid Al-Zahrani[6], Asmaa S. Abdelgeliel[7]

[1] College of Biotechnology, Misr University for Science and Technology (MUST), 6th of October City, Egypt. dalia.elebeedy@must.edu.eg, ingy.badawy@must.edu.eg.

[2] School of Biotechnology, Badr University in Cairo, Badr City, Cairo 11829, Egypt. amal.ghanim@buc.edu.eg

[3] Department of Microbiology and Immunology, Faculty of Medicine, South Valley University, Qena 83523, Egypt. nooreleiman1.me@gmail.com

[4] Department of Health, College of Health and Rehabilitation Sciences, Princess Nourah bint Abdulrahman University, P.O. Box 84428, Riyadh 11671, Saudi Arabia. AMRedhwan@pnu.edu.sa

[5] Department of Biological Sciences, Faculty of Science, King Abdulaziz University, Jeddah 21589, Saudi Arabia. mamalgamdi2@kau.edu.sa, talkahtani@kau.edu.sa, ealmanazalawy@kau.edu.sa

[6] Biological Science Department, College of Science and Art at Rabigh, King Abdulaziz University, Rabigh, Saudi Arabia. maalzahrani4@kau.edu.sa

[7] Department of Botany & Microbiology, Faculty of Science, South Valley, University, Qena 83523, Egypt. asmaa.elgafari@sci.svu.edu.eg

Correspondence should be addressed to Alya Redhwan; amredhwan@pnu.edu.sa

Abstract

Background: Proteolytic enzymes have been used for different therapeutic purposes. Serrapeptase has versatile authenticated applications due to its immense properties. Our study aimed to investigate the antibacterial and anticancer effect of serrapeptase enzyme.

Methods: Serrapeptase was purified from the supernatant of bacterial culture and precipitated using ammonium sulfate. Antibacterial activity was assessed against multiple bacterial strains by microdilution method. Anticancer activity of serrapeptase against three cancer cell lines (A549, Caco, MCF7) was evaluated by measuring cytotoxicity through MTT assay. Serrapeptase induced apoptosis was assessed by measuring activity of caspase-3 and flow cytometry measurement using annexin kit.

Results: The lowest MIC (minimum inhibitory concentration) of serrapeptase was for *K. pneumonia* (2.94 µg/mL) while the highest values were for *P. aeruginosa*, and MRSA (MIC= 47 µg/mL). *S. aureus, E. coli, S. enterica, P. mirabilis*, and *C. albicans* had the same MIC (5.88 µg/mL). MTT assay showed that the lowest IC_{50} (half-maximal inhibitory concentration) values were for A549 and MCF7 cell lines (IC_{50} =1.59 µg/mL and 1.15 µg/mL respectively). Assessment of apoptosis revealed increase caspase-3 activity and percentage of early apoptotic cell in treated A549 and MCF7 cell lines.Conclusion: This study showed promising effects of serrapeptase as an antibacterial and anticancer agent that need to be validated by more in-vivo and mechanistic studies

.**Keywords:** [serrapeptase; antibacterial; anticancer; MCF7; A549; Caco.]

Introduction

Enzymes are essential biomolecules with a particular catalytic function. They have an important impact on the human body because of their safety, selectivity, efficiency, and their broad therapeutic application. Microorganisms are considered as rich purses of enzymes in comparison to animal origin or plant due to remarkable growth rate, reproduction, easy of modification and its viability [1]. New medical biotechnology like genetic engineering and gene manipulations of microorganisms using recombinant DNA technology play a fantastic role to increase the rate of enzyme production [2]. Many applications in paper, textile, leather, food, medicine and pharmaceutical industries are found related to the microbial enzymes due to their greater efficiency, so its demands are increased [3]. Serratiopeptidase is a natural bacterial enzyme that's usually used in Europe and Asia as alternative to salicylates and ibuprofen, for its antiinflammatory, fibrinolytic, mucolytic and anti-bacterial biofilm properties. It has also been used in the post-operative period to improve healing of surgical wound and to reduce inflammation [2]. Its widely used in treating fibrocystic breast disease, arthritis, carpal tunnel syndrome, sinusitis and bronchitis due to its ability to digest blood clost, cysts, arterial plaque, non-living tissues and all other kinds of inflammations [4]. Serrapeptase is a proteolytic, metalloprotease enzyme belongs to serralysin group that originally produced from Gram negative *Serratia marcescens* and isolated from the silkworm Bombyx mori L intestine. According to growth curve analysis, serrapeptase enzyme production by the *Serratia enterobacterium* species E15 in trypticase soy broth is observed after 12 hrs from the inoculation and the maximum production happens after 2 days of growth time [5]. The term of the production of serrapeptase enzyme is unit/ml by using a special standard curve [6]. The recovery stage and enzyme purification are performed by many systems like as: high-performance liquid chromatography (HPLC), ammonium sulfate precipitation, ultra-

filtration, dialysis and aqueous two-phase systems. It can also be concentrated by using three phase partitioning in assistance with ultrasound. The enzyme has a molecular size of 52kDa and can bind with alpha-2-macroglobulin in blood at a ratio of 1:1.4. Other common names for serrapeptase are serratio peptidase, serralysin, serratiopeptidase, *Serratia* peptidase, serratiapeptase and serrapeptidase. Serratiopeptidase has a long history of use as a therapeutic enzyme, and its demand in industries has been satisfied by wild, recombinant, and mutated strains. The first report and introduction of serratiopeptidase to the world as anti-inflammatory drug happened by Japanese researchers. After its creation and its common formulations, it has become one of the most widely used medication in the world by surgeons and physicians [3].

It has vast therapeutic applications that authenticated in vitro and in vivo studies due to its magical properties including anti-inflammatory, antibacterial, antibiofilm and analgesic effects [7]. Without harming the healthy tissue, its fibrinolytic effect breaks the fibrin and other dead or damaged tissues and facilitate the penetration of antibiotics to make it effective against bacterial biofilm infection [8,9]. Thanks to serrapeptase chemical structure that prevent the attachment to healthy tissues proteins, it does not damage healthy tissues [10]. Bacteria were recognized as promising anticancer agents capable of providing direct and indirect tumoricidal effects [11]. Many approaches had been proposed including usage of bacterial products like endotoxins as cancer vaccines and even genetically modified bacteria [12,13]. Bacterial enzymes have widely been utilized for different therapeutic purposes such as in leukemia and skin ulcers [14]. Serrapeptidase has been sold in drugs markets under many trade names and has been successfully used as mucoactive and anti-inflammatory treatment [15,16]. The controversial results obtained from preclinical and clinical studies indicate that more research is needed to confirm its therapeutic applications, explore the mechanism of action, and elucidate safety doses and formulations. The aim of our study is to explore potential antibacterial and anticancer effect of serrapeptase enzyme.

Materials and Methods

Isolation of *Serratia marcescens* bacteria from Silkworm

The silkworms were collected and sterilized with (70%) ethanol until they drained out their gut contents and were put in falcon tube with 1 mL saline (0.90% w/v of NaCl), then the microorganism was isolated by striking on TSA (Trypticase soy agar) media plate by bacterial loop and incubation in 30 ℃ for 24 h. After incubation subculture each bacterial colony on plate for isolation & their purification.

Identification of *Serratia marcescens* bacteria

To identify of *Serratia marcescens* bacteria API 20E (analytical profile index) test was used (BioMeriux, France). To prepare the inoculum, in API NaCl 0.85 % Medium (5 mL) a well isolated single colony inoculated and mixed well to be sure for a homogeneous bacterial suspension then used directly. To inoculate the API strip, all tubes were filled with the bacterial suspension and

created an-aerobiosis in the tests: URE (Urease enzyme test test), H2S (production of hydrogen sulfide), ADH (Amino acid arginine decarboxylation by arginine dihydrolase), ODC (Amino acid ornithine decarboxylation by ornithine decarboxylase) and LDC (Amino acid lysine decarboxylation by lysine decarboxylase), incubate at 36°C for 24 hrs. after overlaying with mineral oil. Then after the incubation period is completed, the results can be obtained by reading the strip referring to reading table.

Propagation & Production of Protease

Colonies of Serratia marcescens bacteria were taken by swab form agar plate & inoculated it in 500mL TSB broth media flask cascade then kept it in 30°C for 24 h. To determine proteolytic activity 5% gelatin is used as a substrate for protease enzyme. 100 µl of culture supernatant was added in diffusion wells in agar containing gelatin plate media. Then incubate the reaction mixture for 24 hrs at 30 °C. After incubation mixture of (15g of HgCl and 20 mL of 6N HCl mixed well then completed with water to 100 ml)

was added to detect protease activity by the presence of clear zone then clear zone was measured by mm [17].

Protease Purification

To obtain supernatant, the culture of bacterial was centrifuged at 4 °C at 2500 xg for 20 min, then let the supernatant precipitated by ammonium sulfate. At 67% saturation level (The high appropriate level of saturation) of the protease precipitation, slowly was added to the resulted bacterial culture supernatant with keeping striking for 1 h at 4 °C. Then centrifuged for 20 min at 8000 xg to collect the precipitate. At distilled water with 1:3 ratio the resulted pellet from centrifugation was dialyzed then the separated protein dialyzed again against phosphate buffer saline at pH=7 [18,19].

SDS-PAGE electrophoresis

Prepare polyacrylamide gel was prepared according to standard protocol. Samples were loaded and run at 25 mA in 1x SDS Running Buffer. At this point, the gel was stained with Coomassie then placed in a plastic container and covered with isopropanol fixing solution with shaking at room temperature. After pouring off fixing solution, gel was covered with Coomassie blue staining solution and shaken at room temperature for 2 hr. After pouring off staining solution, gel was washed with 10% acetic acid to destain and shaken at RT. Gel documentation system was used for analysis of the gel. The Prism Protein Ladder 10–175 kDa (Abcam, UK) was used as a molecular weight marker [20].

Determination of total Protein Content by Biuret kit

One tube labeled as the blank tube and for tube labeled for each tested sample. 0.2 mL from Sodium Chloride Solution 0.85% was added in blank tube. In the appropriately labeled test tube, 0.2 mL of a test sample solution prepared was added. 2.2 mL of the Biuret Reagent was added to each tested tube. After mixing well, the tubes allowed to stand for at least 10 min. Then added to each tube 0.1 ml of Folin and Ciocalteau's Phenol Reagent, immediately mixed well then allowed again to stand for 30 min at room temperature. The absorbance at 650 nm measured for all tested samples and the blank content as a reference after transferring to microplate. The protein concentration (mg/mL) of each tested sample was determined from the standard curve.

Culture of bacterial strains and fungus

From VACSERA, all the following strains were obtained; *Staphylococcus aureus* strain ATCC 25923, MRSA USA300, *Bacillus subtilis* ATCC 6051, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 25923, *Salmonella enterica* ATCC BAA-710, *Candida albicans* ATCC 18804, *Klebsiella pneumonia* ATCC 13883, *Proteus mirabilis* ATCC 29906. Then by streaking methods cultured on TSA or NA plates, at 37° C incubated for 24 hrs., then the single isolated colonies cultured at 37° C incubated for 24 hrs. brain heart infusion broth. Strains had been chosen to cover Gram positive and negative bacteria and fungi.

Antimicrobial activity of serrapeptase

96 well microtiter plates were used for determining the minimum inhibitory concentration (MIC) of the of serrapeptase. By dissolving 188 mg/mL of sterilized distilled water, active metabolite stock solution was prepared then make several dilutions (188,94 ,47,23.5, 11.7 5, 5.88,2.94, 1.47). 50 µL of each dilution of the solution was dispensed in to each well of the microtiter plates. To each well, 50 µL of the tested microorganism contain 1.5×10^5 CFU/mL was added. 100 µL of the Mueller Hinton broth media was added in case of bacteria, sabroud broth medium in case of yeast to each well of the microtiter plates that were incubated at 37 ºC for 24 hours in case of bacteria ,30 ºC for 24 hrs in case of yeast. Negative wells prepared from several dilutions without serrapeptase was included in the plate. The microtiter plates were read after the end of the incubation period by using microtiter plates reader (ELISA reader).

Cytotoxic effect of purified serrapeptase on cancer cell lines

The A549 (Lung), Caco (Colon) & MCF7 (breast) cancer cell line were Supplied by VACSERA, Egypt. In RPMI medium with 10% Fetal Calf Serum (FCS) flask, Cancer cells were seeded then incubated for 24 hrs. at 37°C in a 5% CO2. The attached cells were tyrosinated after the 24 hrs. incubation period then counted and distributed in 96 well plate, incubated at 37°C in a 5% CO2 for 24 hrs. and let the cancer cell attached very well at the bottom of the plate before the treatment with serrapeptase enzyme. In vitro cell viability assay is done by MTT assay (Sigma Aldrich, USA). Briefly, by adding MTT Reagent to each well, just incubated for 4 hrs. until obtain purple precipitate and checked clearly with microscope. Then 100 µL of DMSO (Dimethyl sulfoxide)

was added to all wells and absorbance was measured at 570 nm in a microtiter plate reader. Cancer cell lines had been chosen to cover different type of cancer cells.

Caspase-3 Activity

By Caspase-3 Colorimetric Assay (#BF3100, R&D systems), the activity of Caspase-3 was determined by Cell pellet was lysed, standing for 10 min on ice then for just 1 min, centrifuged at 10000 xg. In a 96 well flat bottom microplate, the caspase activity of the enzymatic reaction was finally carried out. Then measured with a microplate reader using a wavelength of 405 nm.

Apoptosis assays

FITC-Annexin V and propidium iodide (PI) staining were performed using the Annexin V-FITC Apoptosis Detection Kit (#88-8005, invitogen). Cells were washed once in PBS, then resuspended in binding buffer and 5 µL of fluorochrome-conjugated Annexin V was added to 100 µL of the cell suspension then incubated 10-15 minutes at room temperature. Cells were washed in and resuspended in 200 µL of binding buffer then 5 µL of Pro-pidium Iodide Staining Solution was added followed by flow cytometry analysis within 4 hours.

Statistical analysis

Data were statistically assessed utilizing IBM Statistical Package for the Social Sciences (SPSS) version 25. Laboratory characteristics were recorded as means and standard deviation for continuous data. Post hoc Bonferroni correction test was utilized to show similarities in all the examined parameters among all groups. P value was considered significant if < 0.05.

Results

Identification of bacterial sample and protease assay and purification

The biochemical identification using API 20E showed that the microorganism's name: *Serratia marcescens* (see Figure 1A). Gelatinase assay confirmed the presence of protease enzyme by the presence of clear zone around the crude enzyme with diameter equals 21 mm activity indicating digestion of gelatin substrate (see Figure 1B). After purification of serrapeptase form supernatant obtained from *Serratia marcescens* bacteria by ammonium sulphate salt using dialysis bag, we determined the total protein content by using Biuret reagent with comparison with standard curve of the total protein. Protein assay showed that the total protein content is 188 µg/mL.

A

B

Figure 1: Protease assay and purification. A: Biochemical identification using API 20E. B: Protease enzyme activity. Degradation of gelatin by serrapeptase enzyme formed clear zones of 19.5mm & 21mm in diameter.

Antimicrobial activity of serrapeptase (Microdilution methods)

MIC of serrapeptase for different microorganisms was measured by microdilution method. As shown in Figure 2 the lowest MIC for serrapeptase enzyme was for *K. pneumonia* (2.94 µg/mL) followed by MIC for *S. aureus*, *E. coli*, *S. enterica*, *P. mirabilis*, and *C. albicans* (5.88 µg/mL). The most resistant bacteria were *P. aeruginosa* and MRSA (MIC= 47 µg/mL) followed by *B. subtilis* (MIC=11.75 µg/mL).

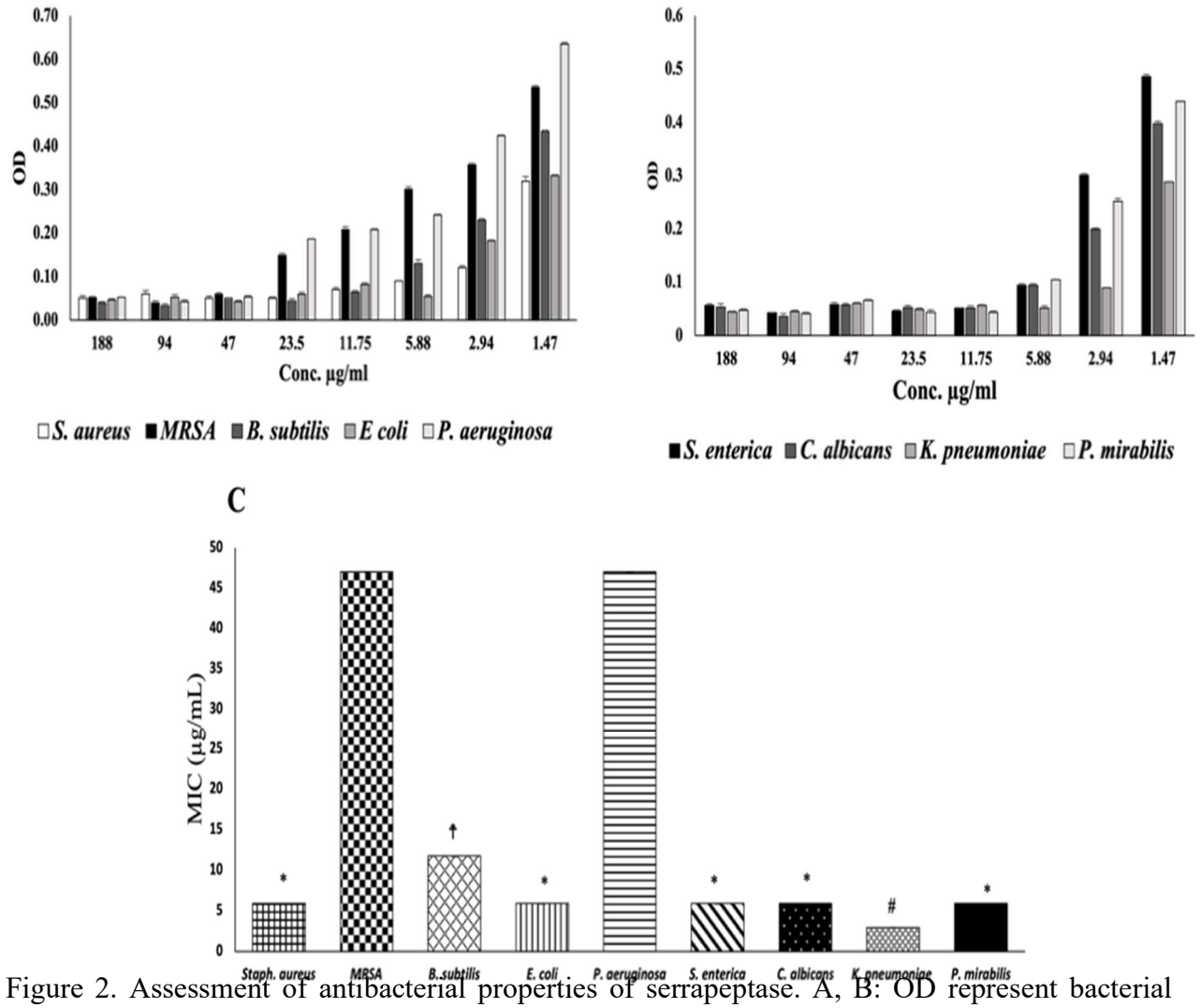

Figure 2. Assessment of antibacterial properties of serrapeptase. A, B: OD represent bacterial growth with serial dilution of serrapeptase. C&C*(Heat map analysis): MIC of serrapeptase for different micro-organisms. All data represent mean ± SEM. Results are the mean of three independent experiments: *$p < 0.05$ versus MRSA, *P. aeruginosa*, and *B. subtilis*. # $p < 0.05$ versus *P. mirabilis*. † $p < 0.05$ versus MRSA and *P. aeruginosa*

Cytotoxic effect of serrapeptase on Cancer cell lines (A549, Caco and MCF7)

The cytotoxicity of serrapeptase was measured on three cancer cell lines (A549, Caco and MCF7) by calculating viability percentage. Figure 3D represented the IC_{50} of different cell lines. The greatest cytotoxic effect was observed on A549 and MCF7 cell line (IC_{50} =1.59 µg/mL and 1.15 µg/mL respectively). The Lowest cytotoxic effect was observed on Caco cell line (IC_{50} =28.12 µg/mL).

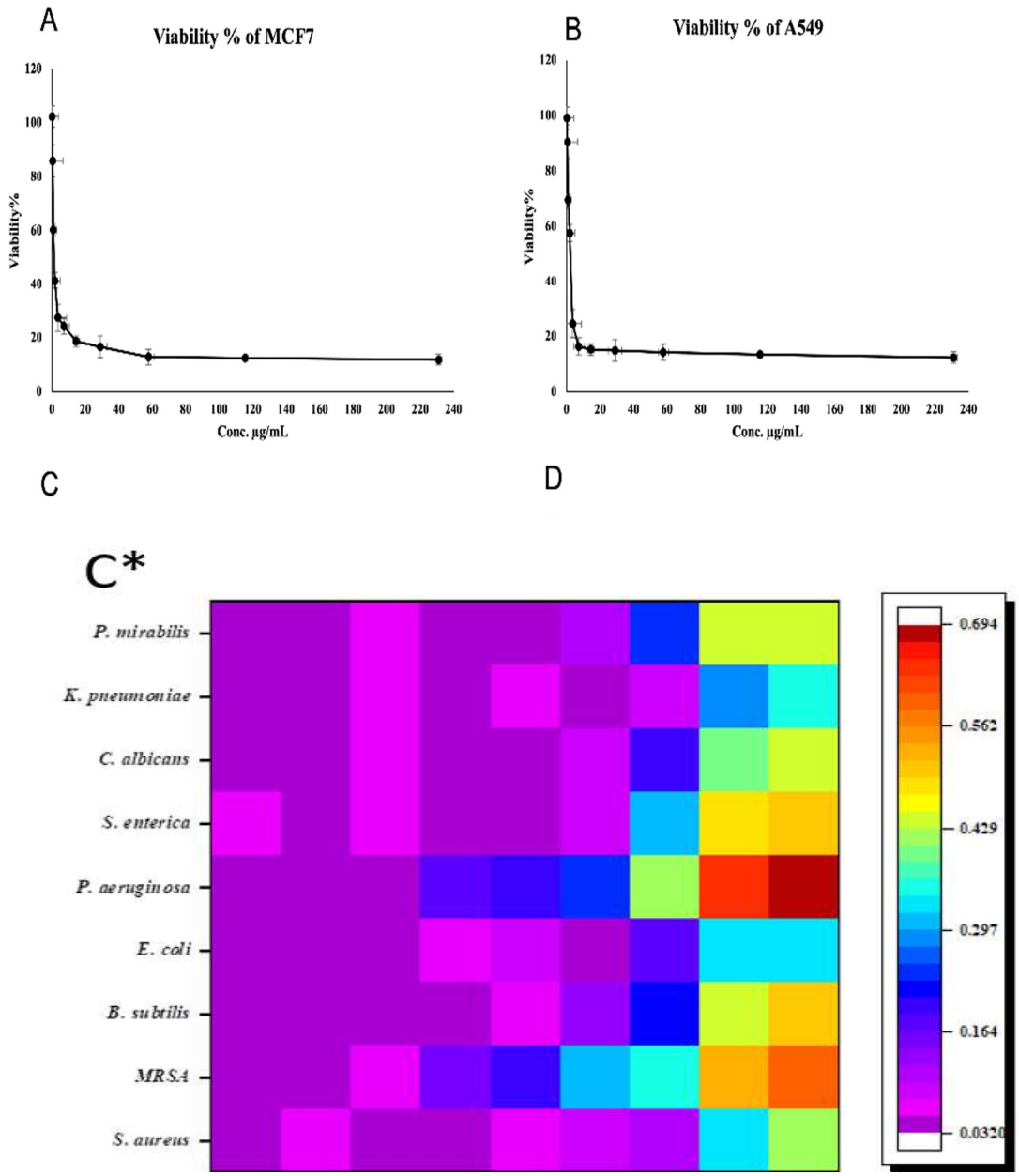

Figure 3. Cytotoxic effect of serrapeptase on Cancer cell lines (A549, Caco and MCF7). A: viability of MCF7. B: viability of A549. C: viability of Caco. D: IC_{50} of different cell lines. All data represent mean ± SEM. Results are the mean of all the three independent experiments: *$p < 0.05$ versus Caco cell line.

Serrapeptase induced apoptosis

We measured apoptosis in cancer cell line after treatment with serrapeptase by caspase-3 assay and flow cytometry using annexin kit. We found increase of caspase-3 enzyme with treatment of cell line with serrapeptase crude extract in comparison with control for each cell line. We also found that the enzyme had more effect against A549 and MCF7 than Caco (see Figure 4).

Detection of apoptosis by flow cytometry using Annexin Kit showed that serrapeptase stimulate apoptosis in A549 and MCF7 as early apoptotic cells increased from 0.57% and 1.38% in control group to 21.20% and 24.23% respectively in treated sample. The early apoptotic cells also increased in Caco cell line from 0.31% to 11.61% (see Figure 5).

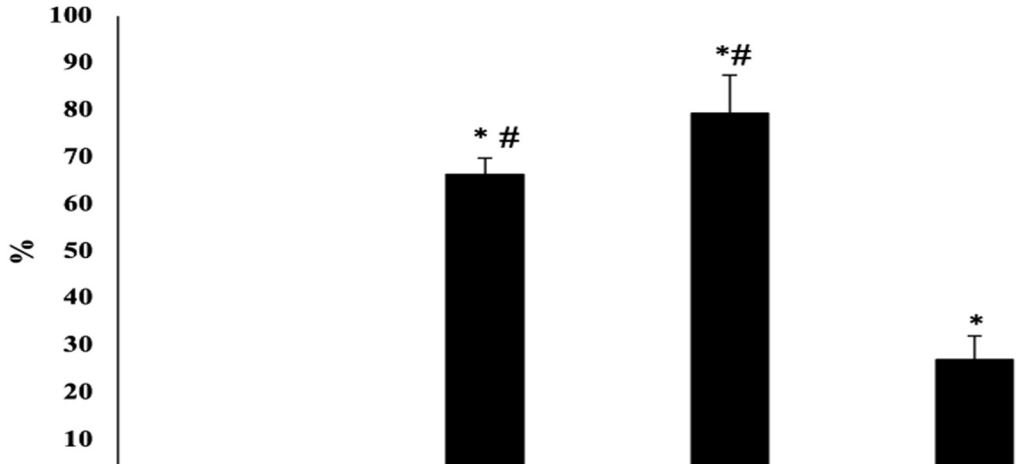

Figure 4. Caspase-3 assay. All data represent mean ± SEM. Results are the mean of the three independent experiments: *p < 0.05 versus control. #p < 0.05 versus Caco cell line.

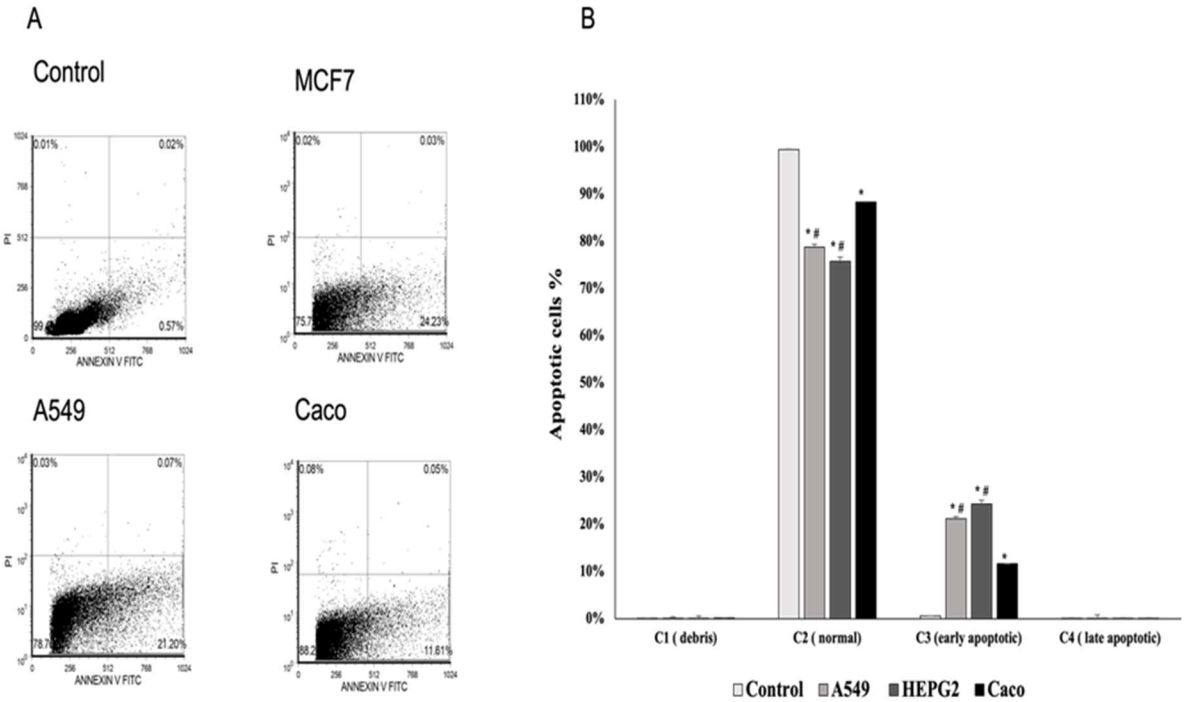

Figure 5. Serrapeptase-induced apoptosis was determined with Annexin V-FITC/PI staining by flow cytometry. A: Flow cytometry results with Annexin V-FITC/PI staining, cells were classified as normal cells (Annexin V−, PI−), early apoptotic cells (Annexin V+, PI−), late apoptotic cells (Annexin V+, PI+), and debris (Annexin V−, PI+). B: The percentage of apoptotic cells among cancer cell line. The data were presented as the mean ± SEM. Results are the mean of three independent experiments: * $p < 0.05$ versus control, # $p < 0.05$ versus Caco cell line.

Discussion:

Serrapeptase has been used in many therapeutic and pharmaceutical application due to its high proteolytic activity. The analgesic anti-inflammatory, antibiofilm and fibrinolytic effects of serrapeptase are widely known [21]. This study aims to further explore other therapeutic applications for serrapeptase enzyme. Consistent with other studies which investigated the effect of serrapeptase on *S. epidermidis* and *P. aeruginosa*, we showed that serrapeptase could inhibit the growth of many bacterial strains and fungi. Maximum inhibition was observed with *K. pneumonia* followed by *S. aureus, E. coli, S. enterica, P. mirabilis*, and *C. albicans*. In contrast with previous studies, *P. aeruginosa*, and MRSA had the highest MIC. This contradiction could be due to the difference in methodology where previous studies assessed the effect of serrapeptase in combination with antibiotic [22-24]. The variation in MIC with different strains could be because serrapeptase mostly has effect on phosphorus homeostasis pathways and bacterial cell walls biosynthesis and stabilization [25]. We were also interested to explore the anti-cancer properties of serrapeptase. Some studies reported anticancer potential of serrapeptase against Caco-2 cancer cell line. We demonstrated the anti-cancer potential against three cell lines: A549, Caco and MCF7. Our study showed that serrapeptase was more cytotoxic against MCF7 and A549 cell lines than Caco cell line. In agree with other studies that demonstrated that IC_{50} of Caco cell line was 24.78 µg/mL, our results showed that IC_{50} of Caco cell line was 28.12 µg/mL [26,27]. Another study reported high cytotoxicity effect for serrapeptase combined with curcumin against MCF7 (IC_{50}=0.7 µg/mL) while it had poor cytotoxic effect if used individually (IC_{50}=78.1 µg/mL). This contradiction could be attributed to the source of the enzyme; as we purify the enzyme in our lab, the previous study used a commercial powder form. The tumour suppressive activity of serrapeptase had been attributed to induction of TNFα production [27].

Consistent with cytotoxicity assay, apoptosis assay showed higher activity of casapase -3 in MCF7 and A549 cell lines than Caco cell line. Evaluation of apoptosis by flow cytometry showed a significant higher level of early apoptotic cells in MCF7 and A549 cell lines than Caco cell line indicating that indeed serrapeptase is more effective on breast and lung cancer than colon cancer.

Conclusion

We demonstrated that serrapeptase enzyme is not only anti-inflammatory and anti-fibrinolytic, but it could also be a potent antibacterial and anticancer agent, which could be reflected in clinical applications. In vivo and large-scale studies using more bacterial strains and different cancer cell

lines are still needed. Efforts to unify the source and isolation method for the enzyme as well studies on safety issues should be taken into consideration in trials to optimize serrapeptase therapy. More in vivo studies are needed to study serrapeptase in combination with other antibacterial and anticancer drugs and to explore potential mechanism through different metabolic and physiologic pathways.

Declarations

Ethics approval

This project has received ethical approval from Faculty of Science, South Valley University, Egypt, Research Ethics Committee (REC-FScSVU) with Code No. (004/05/23).

Ethics Committee, and ethical considerations and responsible practices will be observed during experimental research and field studies on bacterial strains or cell line. Our commitment to ethical principles includes respect for the intrinsic value of our ecosystems, adherence to relevant laws and ethical guidelines, and maximizing benefits while minimizing harm to pathogenic bacteria and relevant to use beneficial bacterial strains. By upholding these ethical principles, we aim to advance our understanding of bacteria biology while respecting the value of clean weather and decrease the using of chemical or antibiotics with huge problem with resistance bacteria.

Authors' contributions All authors contributed to this work

Consent for publication

Not applicable under Consent for publication.

Availability of data and materials

The data generated during this study are available from the corresponding author upon reasonable request.

Competing interests

The authors declare no competing interests.

Funding

This research was conducted without financial support.

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