

## Review

# The Emergence of Microbial Protease as a Potential Anti-Cancer Agent

 Rupak Mahapatra,  Tanmoy Paul

Ramakrishna Mission Vivekananda Centenary College, Kolkata, West Bengal, India

## Abstract

Cancer is one of the most dominant causes of human death globally. The most common mechanism responsible for the growth and survival of cancer cells involves inhibition of apoptosis. Numerous cytotoxic agents are used to induce apoptosis in a targeted manner. An array of conventional therapies including surgery, radiotherapy, chemotherapy and hormonal treatments targeting apoptosis exists in contemporary time which also culminates in relapse of cancer, multi-drug resistance and hazardous toxic effects on normal healthy tissues, arising from non-targeted collateral damage to normal healthy tissues.

In recent time, microbial protease seems to be a promising candidate for anti-cancer therapeutic management because of their specificity and efficiency in the targeted way of action. Microbial proteases are enzymes secreted from the various microbes hydrolysing the peptide bonds of proteins resulting in an inhibitory effect on growth, proliferation, invasion, metastasis, angiogenesis and motility of cancer cells. The present review will assess the anti-cancer potential of different microbial proteases reported in contemporary time. Whether microbial protease could lead to targeted onco-therapy in different human cancer will also be assessed.

**Keywords:** Apoptosis, cytotoxicity, microbial protease, onco-therapy, targeted therapy

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Cancer is considered as one of the major causes of human death throughout the world in recent time. Contemporary treatment for cancer involves surgery, chemotherapy and radiotherapy coupled with systemic administration of chemotherapeutic drug. Therapy with systemic cytotoxic drug involves low molecular weight compound to induce selective cytotoxicity to the tumour cells resulting in inhibition of their growth and proliferation. Presently available chemotherapeutic agents used for cancer treatment are categorized depending on the target of action which include agents that interacts with DNA (e.g., cisplatin, doxorubicin), agents interacting with nucleic acid synthesis called anti-metabolites (e.g., methotrexate), hormones,

agents with anti-tubulin activity (e.g., taxanes), and agents which target several molecules and pathways.<sup>[1]</sup> Despite of the various applications for cancer treatment, there are several major disadvantages of treatment with chemotherapeutic drugs, which include relapse of cancer, resistance to chemotherapeutic drugs and hazardous toxic effects on normal healthy tissues including bone marrow suppression, gastro-intestinal tract lesions, loss of hair, nausea etc. resulting in the life impairment of patients.<sup>[2]</sup> These side effects occur because of the non-targeted cytotoxic effects on both tumour and normal cells.<sup>[3]</sup> To overcome these problems it is very essential to discover new effective anticancer agents with greater efficacy and high specificity for tumour cells.

**Address for correspondence:** Tanmoy Paul, Ph.D. Ramakrishna Mission Vivekananda Centenary College, Kolkata, West Bengal, India  
**Phone:** +91 9477455669 **E-mail:** dr.paultanmoy@gmail.com

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Various studies have been reported which demonstrated that microbes secrete different proteases with properties for induction of cellular apoptosis which could also restrict tumour growth.<sup>[4, 5]</sup> Microbial proteases are degradative enzymes, present in different microbes, which can hydrolyse the peptide bond of proteins.<sup>[6]</sup> These proteases are beneficial because they don't result in issues related to chemotherapy, like toxicity, lack of selectivity and multi-drug resistance.<sup>[7-9]</sup> Thus microbes are more advantageous because of their abundance in the earth with a variety of naturally occurring compounds having different structural characteristics. In addition to these properties, purification of these compounds are also very cost effective than chemically synthesised drugs.<sup>[10, 11]</sup> The present review focuses on the anti-cancer activity of various different microbial proteases with very little or no toxic side effects on normal cells. The different cellular processes targeted by microbial proteases will also be explored.

## Microbial Proteases

Proteases generally defined as degradative enzymes which catalyse the hydrolysis of peptide bond in proteins forming smaller peptides or liberating free amino acids.<sup>[6]</sup> Proteases belong to the generic hydrolase class of enzymes, which act in a very specific and selective way in protein modification.<sup>[12, 13]</sup> Proteases or peptidases are generally classified depending upon the site of cleavage in target peptides bond and are categorised into endopeptidases or endoproteases, which hydrolyse peptide bond in the middle of a polypeptide chain and exopeptidases or exoproteases, catalyses hydrolysis of peptide bond near the terminal end of the polypeptide chain. There are various protease families, depending upon the active site functional groups which include asparagine, cysteine, glutamine, serine, threonine and mixed proteases. Depending upon the optimum functional pH, proteases are also grouped into alkaline, neutral and acidic proteases.<sup>[14, 15]</sup> The elaborate function of all the classes still remains to be deciphered.

Microbes are generally rich in several proteases with various functions. These microbial proteases are broadly used in the field of biotechnology for different applications mainly due to their attributes like high production rate, genetic manipulation, less time consumption and cost-effectiveness.<sup>[16, 17]</sup> About 60% commercial proteases throughout the world are produced from microbial sources and these microbial proteases are chosen over the plant proteases and animal proteases due to the fact that they have all the desired properties for industrial applications.<sup>[18, 19]</sup> Microbes are able to produce both intracellular and extracellular proteases, which have a great role in differentiation, hormone regulation, turnover of proteins, and cellular protein pool.

Scientific studies have reported a variety of different applications of microbial protease in different diseases like inflammation, cardiovascular diseases, digestive disorders, respiratory tract disorders, thrombolytic disease, cystic fibrosis and other diseases.<sup>[19-21]</sup> Beside the different roles in industrial applications, microbial protease could also serve as anti-cancer agents as evident from different contemporary scientific observations.

## Role of Microbial Proteases in Anti-Cancer Therapies

Recent literatures have portrayed different microbial proteases with cytotoxic capabilities through the network of multiple cellular signalling pathways (Table 1). The different microbial proteases with potentialities for anti-cancer therapy are listed.

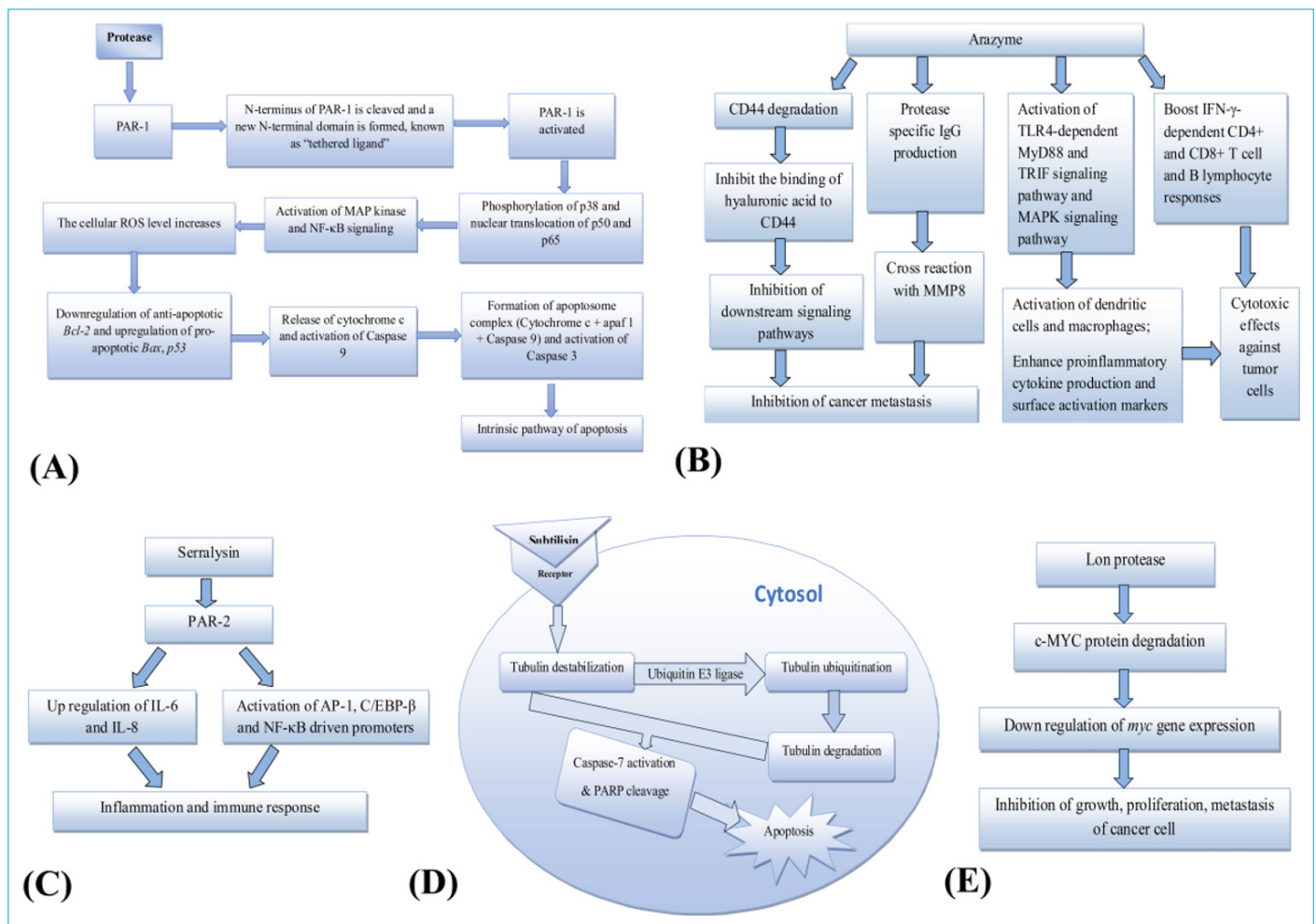
### Anti-Cancer Activity Associated with Protease Isolated from *Vibrio cholerae*

The microbial protease isolated from *Vibrio cholerae* reported as hemagglutinin protease observed to induce apoptosis in breast cancer cells resulting in simultaneous reduction in tumour growth. The protease have been reported to be activated through protease activated receptors (PARs), member of the G-protein coupled receptor (GPCR) family. PARs have been categorised into four different subtypes such as PAR-1, PAR-2, PAR-3 and PAR-4. During PAR activation proteases have shown to induce proteolytic cleavage. After binding to the N-terminal domain the protease cleaves the domain and thereby activates the receptor forming an another N-terminus followed by induction through transmembrane signalling. The activated PAR then activates the mitogen activated protein (MAP) kinase and nuclear factor kappa B (NF- $\kappa$ B) signalling pathways. These signalling pathways increase the cellular reactive oxygen species (ROS) level initiating the intrinsic pathway of apoptosis (Fig. 1). In normal healthy cell, NF- $\kappa$ B and MAP kinase signalling pathways are not activated due to the much lower expression level of PAR. So, the cellular reactive oxygen species level is not elevated and the intrinsic pathway of apoptosis is inhibited.<sup>[4, 22]</sup>

### Anti-Cancer Activity Associated with Lon Protease

The Lon protease secreted from *E. coli* has also been reported to restrict tumour growth by targeting the MYC transcription factor family, which is necessary for tissue formation, metabolism and cellular proliferation. The up-regulation of *c-myc* gene causes malignancy in human with poor prognosis. In human cells and animal tissues, urinary tract pathogenic *E. coli* has the ability to break down the protein c-MYC and reduce the expression of the *myc* gene.

Table 1. Brief summary of the microbial proteases with potential for anti-cancer activity.						
Microbial Protease with anti-cancer activity	EC number	Chemical nature	Co-factor	Nature	Microbial source	Signaling pathway involved
Hemagglutinin protease (HAP)	EC:3.4.24.25	Hydrolase, Protease/, Peptidase Metalloendopeptidase	Zn <sup>2+</sup>	Extra-cellular alkaline protease	<i>Vibrio cholerae</i>	HAP binds and activates PAR, causing translocation of p50 and p65 to the nucleus and phosphorylation of p38 resulting in the activation of MAPK and NFκB signaling pathway that enhances the cellular ROS level. The generation of ROS causes the intrinsic pathway of apoptosis. [4], [22], [42], [43]
Lon protease	EC:3.4.21.53	Hydrolase, Protease/ Peptidase, Serine endopeptidase	ATP	Cytosolic alkaline protease	<i>E. coli</i>	Lon protease breaks down c-MYC protein and down regulate the myc gene expression resulting in the inhibition of cancer growth and proliferation. [23], [44], [45]
Subtilisin	EC:3.4.21.62	Hydrolase, Protease/ Peptidase, Serine endopeptidase	Ca <sup>2+</sup>	Extracellular alkaline protease	<i>Bacillus amyloliquefaciens</i>	Subtilisin activates PARKIN that led to the proteasomal-mediated tubulin degradation. This led to the activation of caspase-7 through ER-stress, PARP cleavage and subsequent DNA damage which induce apoptosis [9], [46], [47]
Serralysin	EC:3.4.24.40	Hydrolase, Protease/, Peptidase Metalloendopeptidase	Ca <sup>2+</sup> , Zn <sup>2+</sup>	Extracellular alkaline protease	<i>Serratia marcescens</i>	Serralysin binds to blood α1M, breaks down the inflammatory molecules histamine, serotonin, and bradykinin, and prevents the growth of fibrin that led to the metastasis inhibition of cancer cells. Serralysin also up regulates the expression of proinflammatory cytokines (IL-6, IL-8) and activates AP-1, C/EBPβ and NF-κB via PAR-2 that led to the inflammation and immune responses against cancer. [28-30], [48], [49]
Botulinum neurotoxin type-A	EC:3.4.24.69	Hydrolase, Protease/, Peptidase Metalloendopeptidase	Zn <sup>2+</sup>	Extracellular alkaline protease	<i>Clostridium botulinum</i>	Botulinum neurotoxin type-A activates caspase-3 and -7 dependent apoptotic pathway. [32], [50], [51]
Arazyme	EC:3.4.24.40	Hydrolase, Protease/ peptidase, Metalloendopeptidase	Zn <sup>2+</sup>	Extracellular alkaline protease	<i>Serratia proteamaculans</i>	Arazyme leads to the proteolytic degradation of surface adhesion molecule CD 44 and also facilitates the protease specific IgG production which cross-reacted with the MMP-8 of tumor cells, resulting in the inhibition of tumor invasion and cancer metastasis. Additionally, Arazyme triggers the activation of dendritic cells and macrophages and increase the production of proinflammatory cytokines and expression of cell surface activation markers through the activation of TLR4 dependent MyD88 and TRIF signalling pathways and also MAPK signalling pathway. Arazyme also increases B lymphocyte, cytotoxic T cell and helper T cell mediated responses that play a crucial role in producing anti-cancer effect. [34], [52-54], [59]
UcB5	EC:3.4.21	Hydrolase, Protease/ Peptidase, serine protease	-	Extracellular alkaline protease	<i>Salmonella typhimurium</i>	Necrosis [40]



**Figure 1.** Mechanisms involved in protease mediated anti-cancer activities: **(a)** Hemagglutinin protease mediated apoptosis in human breast cancer cell. Binding of hemagglutinin protease to PAR-1 causes PAR-1 cleavage and activation of PAR-1, eventually resulting in the translocation of p50 and p65 to the nucleus and p38 phosphorylation in the breast cancer cells. This activates the MAP kinase signalling pathway and NFκB signalling pathway which increase the cellular ROS level. The generation of ROS causes the intrinsic pathway of apoptosis by decreasing the anti-apoptotic Bcl-2 expression level and by increasing the expression and activating the pro-apoptotic p53, Bax, cytochrome C, caspase 9 and caspase 3. **(b)** Arazyme inhibits cancer cells metastasis by degrading CD-44 and promoting protease-specific polyclonal antibody IgG production which cross-reacted with MMP-8 of tumor. It triggers the activation of dendritic cells and macrophages and increase the production of pro-inflammatory cytokines and expression of cell surface activation markers through the activation of TLR4 dependent MyD88 and TRIF signalling pathways and also MAPK signalling pathway. Arazyme increases B lymphocyte, IFNγ-dependent cytotoxic T cell and helper T cell mediated responses that exert cytotoxicity against cancer cells. **(c)** Serralyisin induces inflammation and host immune response by upregulation of pro-inflammatory cytokines and activation of AP-1, C/EBPβ and NF-κB via PAR-2. **(d)** Mechanism of the induction of apoptosis in human MCF-7 breast cancer cells through subtilisin protease. Subtilisin binds to its cell surface receptor, activating E3 ubiquitin ligase PARKIN which interacts with tubulin and causes ubiquitin-mediated proteasomal degradation of tubulin that led to ER-stress that activates caspase-7, PARP cleavage resulting in the apoptosis of the cancer cells. **(e)** Inhibition of growth proliferation, invasion and metastasis of cancer cells by Lon protease via c-MYC protein degradation.

Urinary tract pathogenic *E. coli* secretes Lon protease which can accelerate the destruction of c-MYC protein and reduce the *myc* gene expression, leading to the consumption of c-MYC protein in the infected tissue, resulting in the slowing down of the progress of cancer and improving survival rate (Fig. 1). Lon protease inhibits the tumour growth in MYC-dependent bladder cancer and colon cancer.<sup>[23]</sup>

### Anti-Cancer Activity Associated with Subtilisin

Subtilisins represent one of the biggest classes of serine proteases present in living organisms including viruses.<sup>[24]</sup> The subtilisin isolated from *Bacillus amyloliquefaciens* recently showed promising anticancer property. Subtilisin causes apoptosis in colon cancer cells and breast cancer cells without affecting the normal cells. It has been shown



that subtilisin has no effect on the expression of pro-apoptotic (*Bax*) and anti-apoptotic (*Bcl-2*) molecule but it can significantly decrease the tubulin level in the cancer cell through ubiquitin-mediated proteasomal degradation. Subtilisin activates PARKIN, the E3 ubiquitin ligase which interacts with tubulin and led to the tubulin degradation.<sup>[25]</sup> The serine protease could also induce elevated expression of endoplasmic reticulum related ER-stress pro-death markers CHOP and IRE1 $\alpha$ , thereby also activating caspase-7 and subsequent DNA damage resulting in the apoptosis of the cancer cell (Fig. 1).<sup>[9, 26, 27]</sup>

### Anti-Cancer Activity Associated with Serralyisin

The Gram-negative, facultative anaerobic, rod-shaped bacteria *Serratia marcescens* secretes an extracellular zinc metalloprotease called serralyisin with anti-cancer activity. Serralyisin binds to blood alpha-1 macroglobulin ( $\alpha_1M$ ) and thereby avoids detection by the immune system by molecular camouflaging followed by movements towards the site of inflammation. The protease then breaks down the inflammatory molecules histamine, serotonin, bradykinin and inhibits the growth of fibrin thus cancer cells are unable to adhere to proteins and hence cannot enter the bloodstream.<sup>[28, 29]</sup> In cancer cells, serralyisin induces the expression of interleukin-6 (IL-6) and interleukin-8 (IL-8) mRNA and activates the activator protein 1 (AP-1), CCAAT/enhancer-binding protein  $\beta$  (C/EBP $\beta$ ), and nuclear factor- $\kappa$ B (NF- $\kappa$ B) driven promoters via protease-activated receptor 2 (PAR-2) for inflammation and host immune responses (Fig. 1).<sup>[30]</sup> That's why it is used as a potent anti-cancer drug for regional treatment of solid tumours.<sup>[31]</sup>

### Anti-Cancer Activity Associated with Protease Secreted from *Clostridium botulinum*

Botulinum neurotoxin type-A, a zinc metalloprotease induces apoptosis in human prostate cancer cells. It inhibits the growth, proliferation and progression of prostate tumour. This neurotoxin is broadly used for the treatment of benign prostate hyperplasia (BPH). Botulinum type-A toxin activates caspase-3 and caspase-7 dependent apoptotic pathway in cancer cells.<sup>[32, 33]</sup>

### Anti-Cancer Activity Associated with Arazyme

Arazyme secreted from *Serratia proteamaculans*, is a metalloprotease interferes with tumour cell metastasis by cleaving the tumour cell surface adhesion molecule CD-44 and promoting protease-specific polyclonal antibody IgG production which cross-reacted with the matrix metalloprotease-8 (MMP-8) of tumour (Fig. 1).<sup>[34]</sup> The CD-44, a glycoprotein receptor, is an important cell surface molecule which binds to hyaluronic acid required for cell adhesion.

<sup>[35, 36]</sup> Higher expression of CD-44 molecules in the tumour cell surface exerts a greater role in cancer progression as degradation of hyaluronic acid promotes tumour cell metastasis.<sup>[37]</sup> Arazyme causes the proteolytic degradation of CD-44 resulting in the reduction of tumour cells to extracellular matrix which ultimately inhibits the tumour metastasis.<sup>[34]</sup> MMP-8 is generally secreted by the neutrophils. Several studies have been reported that higher expression of MMP-8 in tumour cells downregulates the tumour metastasis and increases the tumour cell adhesion to the extracellular matrix.<sup>[38]</sup> Arazyme facilitates the IgGs production, having a greater specificity to protease. They exert cytotoxicity on tumour cells. This IgGs are cross-reacted with the MMP-8 of tumour cells, resulting in the inhibition of tumour invasion and cancer metastasis.<sup>[34]</sup> Arazyme also induces the activation of dendritic cells and macrophages and increase the production of pro-inflammatory cytokines and expression of cell surface activation markers through the activation of TLR4 dependent MyD88 and TRIF (TIR domain containing adaptor inducing IFN- $\beta$ ) signalling pathways and also mitogen-activated protein kinase (MAPK) signalling pathway. Additionally, arazyme increases B lymphocyte, cytotoxic T cell and helper T cell mediated responses and plays a crucial role in producing anti-cancer effect.<sup>[59]</sup>

### Anti-Cancer Activity Associated with Protease from *Stenotrophomonas* and *Salmonella*

Recently Thakur et al. has been reported that alkaline protease secreting gram negative bacterium; *Stenotrophomonas* sp have a greater cytotoxicity against various cancers. They suggested that the secreted protease may modify the protein structure which leads to the apoptosis, resulting in the inhibition of tumour growth and proliferation.<sup>[39]</sup>

*Salmonella typhimurium* secretes Ucb5 serine exoprotease which causes cytotoxicity against cancer cells through necrosis.<sup>[40]</sup> Simultaneously, Ucb5 hemolyse the red blood corpuscles and hemorrhage in different internal region of the body. It also affects the liver by the formation of vesicles and heterochromatin in the hepatocyte. Kotb et al reported that Ucb5 distort the nuclear membrane and fully disappear the rough endoplasmic reticulum and plasma membrane of hepatocytes.<sup>[40]</sup> It has been reported that different types of regulated necrosis could be exploited for the purpose of therapeutic intervention of cancer.<sup>[41]</sup> Thus the mentioned protease could serve as potential anti-cancer agent if it induces regulated necrosis specific signalling pathway, which necessitates further research for deeper understanding of the phenomenon.

## Discussion

Microbes are diverse and ubiquitous in their abundance in nearly every ecological niche on earth. Microbial proteases are the enzymes which have the ability to breakdown the peptide bond of amino acids. The microbial proteases are broadly used in various biotechnological purposes due to their cost effectiveness and abundance of species. Recently microbial proteases are used in several pharmacological applications.<sup>[19-21]</sup>

Microbial components could serve various therapeutic purpose of humans including anti-cancer therapeutic modalities. In 1909, William B. Coley first demonstrated that the supernatants of heat killed *Streptococcus pyogenes* and *Serratia marcescens* could treat the 'Inoperable Sarcoma' by acting as an anticancer agent. Later the mixture is named as 'Coley's toxin'.<sup>[55]</sup> Scientific studies have also showed that microbes could have roles in both cancer progression and regression.<sup>[56]</sup> Microbes are rich in diverse metabolites with structural varieties ensuring the ability to act as pro-cancerous and anti-cancerous agent. Different microbial metabolites such as bacteriocins (Bovicin HC5, Colicins A and E1, Laterosporulin 10, Microcin E492, Nisin A, Nisin ZP, Pediocin CP2, Pediocin K2a2-3, Plantaricin A, Pyocin S2), antibiotics (Actinomycin D, Bleomycin, Doxorubicin, Mitomycin C), enzymes (L-asparaginase, Arginine Deiminase), toxins (Botulinum neurotoxin type A, Diphtheria toxin, Exotoxin A, Listeriolysin O), non-ribosomal peptides (Arenamides A, B; Ariakemicins A, B; Halolitoralins A–C, Iso-C16 fengycin B, anteiso-C17 fengycin B, mojavenisin A; Ohmyungsamycins A and B, etc) and other proteins (Azurin, p28, Entap, pep27anal2) that are cytotoxic against different types of cancer.<sup>[57]</sup>

Although conventional cancer therapies like surgery, radiotherapy and chemotherapy are the major applied treatment for cancer but these approaches often exerts greater toxic side effects on normal cells. Depending on the heterogeneous nature of cancer with high recurrence rate, the most prominent treatment of cancer is to identify a novel oncogenic target. Microbial proteases may solve this problem by targeting the cancer in a more targeted and efficient way. Microbial proteases selectively cause apoptosis, inhibits tumour growth, proliferation, invasion and metastasis. Multi-drug resistance is one of the major barriers of conventional chemotherapy in cancer treatment. Microbial proteases are more effective here, as they act in more selective and targeted way. Thus further studies will be needed to explore the phenomenon.

There are approximately 30000 cultured bacterial species and approximately 109000 identified bacteria are known.<sup>[57]</sup> But very little studies have been performed

on the anti-cancer activities of the microbial proteases. The present review has enlisted microbial candidates that could be explored for anti-cancer activities. Previous studies have shown that many of them may contain anti-cancerous natural compounds including secreted protease.

At present days, Amino Acid Deprivation Therapy (AADT) has also been developed as one of the novel approach for anticancer treatment. The principle of this therapy selectively targets metabolism of cancer cells. As the tumours develop quickly, which results in lowering the expression of particular enzymes and causes auxotrophy for certain amino acids. Amino acid depleting microbial enzymes (L-asparaginase, arginine deiminase, methionase, lysine oxidase, glutaminase and phenylalanine ammonia lyase) target these auxotrophic tumours. Such microbial enzymes causes the reduction of essential amino acids in cancer cells which affects the process of protein synthesis and cell signalling, eventually resulting in the cancer growth inhibition. For instance, the US Food and Drug Administration (FDA) has already approved microbial L-asparaginase for the treatment of acute lymphoblastic leukaemia.<sup>[58]</sup>

It has been reported that the genetically engineered bacteria exert a greater efficacy against cancer cells either alone or combine with chemotherapy.<sup>[56]</sup> Koosha et al. have been reported that recombinant arazyme (r-arazyme) have a greater cytotoxicity against cancer cells. The r-arazyme induces apoptosis and inhibits proliferation, invasion, metastasis and angiogenesis of cancer cells.<sup>[59]</sup> Further studies are also needed to ascertain the efficacy of microbial proteases either alone or when combined with chemotherapeutic agents or in recombinant form against cancer to act as a potential anticancer agent.

## Conclusion

Microbes are well abundant in nature and a variety of naturally occurring microbial compounds having different structural characteristics could be isolated from microbial source. The purification of these compounds is also very cost effective. Microbes are quite unexplored considering the possible use of microbial compounds as a potential anti-cancer agent. Thus, microbial proteases could be explored as a potential anti-cancer agent for therapeutic intervention for targeted therapy of the cancer cells. Different *in vitro* studies have already reported which needs to be further confirmed through realistic *in vivo* experiments followed by studies of toxicological parameters and clinical trials to fully explore the potentialities of microbial compounds as successful anti-cancer medicine.

## Disclosures

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**Conflict of Interest:** None declared.

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