Akta Agrosia. 2025. 28(1):8-14
Doi: 10.31186/aa.28.1.8-14

Akta Agrosia
https://ejournal.unib.ac.id/Agrosia

The Inhibitory Properties of the Single-Chain 2S Albumin Seed Storage Protein from *Theobroma cacao*

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Cited this as:
Karim,N.A., C. Budiman, A. Awang, and K.F. Rodrigues . 2025. The inhibitory properties of the Single-Chain 2S Albumin seed storage protein from *Theobroma cacao*. Akta Agrosia 28(1):8-14. Doi: 10.31186/aa.28.1.8-14

ARTICLE INFO

Keywords:
2S albumin protein,
antibacterial,
antifungal
heavy-chain peptide,
Theobroma cacao

Article history:
Received: Oct 06, 2024
Accepted: Feb 16, 2025

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ABSTRACT

2S albumin seed storage proteins from *Theobroma cacao* (Tc-2S) are known for their roles in plant defense mechanisms due to their antimicrobial properties. However, it remains unclear whether both the light and heavy chains of Tc-2S are required for this activity. This study develops an expression system for the single-chain precursor of Tc-2S and evaluate its antimicrobial activity. Specifically, the heavy-chain subunit (Tc-9M), corresponding to residues 78 to 150 of the Tc-2S precursor, was cloned and expressed in a heterologous system. The resulting Tc-9M protein, expressed as a fully soluble protein, was purified via column chromatography, yielding 24 mg of pure protein from 300 mL of the expression culture. Antibacterial and antifungal activity was assessed using the Kirby-Bauer disc diffusion method, revealing that Tc-9M remarkably inhibited the growth of several bacterial strains, including Salmonella sp., Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, and Bacillus cereus. Additionally, Tc-9M inhibited the growth of the yeasts Saccharomyces cerevisiae and Pichia pastoris but showed no activity against the fungus Trichoderma asperellum. These findings ndicate that the single-chain Tc-2S may be sufficient for antimicrobial defense in plants. Because the characterization of the Tc-9M protein has not been previously reported, this work provides a basis for further exploration of the biological significance of the 2S albumin subunit in plant defense.

INTRODUCTION

During seed development, plants accumulate large amounts of storage proteins that are rapidly hydrolyzed to provide reduced nitrogen and other essential elements for early seedling growth (Krishnan and Coe, 2001; Autran et al., 2001). These major storage proteins are classified into four solubility-based categories: albumins (water-soluble), globulins (salt-soluble), prolamins (alcohol-soluble), and glutelins (soluble in dilute acids or alkali). Different plant taxa contain varying proportions of albumins, globulins, prolamins, and glutelins (Osborne, 1916; Voigt et al., 1993).

In *Theobroma cacao* (cocoa), albumin is the predominant seed protein fraction, accounting for 52% of the total seed proteins (Zak and Keeney, 1976). Research has largely focused on the well-characterized 21-kDa albumin known as the cocoa albumin trypsin inhibitor (CoATi). In contrast, the 19-kDa albumin, the second most abundant seed storage protein in cocoa, has been less extensively studied.

ISSN: 1410-3354 / e-ISSN:2615-7136

Kochhar et al. (2001) identified this protein as a 2S albumin seed storage protein, referred to hereafter as the Tc-2S precursor.

Like other 2S albumins, Tc-2S was initially synthesized as a 19-kDa precursor consisting of a signal peptide, an N-terminal extension peptide, a 4-kDa light-chain subunit (Tc-4M), a linker peptide, a 9-kDa heavy-chain subunit (Tc-9M), and a C-terminal extension peptide. The Tc-2S precursor undergoes post-translational modifications to produce a mature 2S albumin, comprising the Tc-4M and Tc-9M proteins. This mature protein has a typical heterodimeric structure stabilized by intra- and inter-disulfide bridges formed through a conserved cysteine motif (Kochhar et al., 2001; Kochhar et al., 2005).

While 2S albumins are primarily utilized as nutrient sources during seed germination and growth (Moreno and Clemente, 2008; Higgins, 1984), recent studies have demonstrated additional biological activities, such as roles in plant defense mechanisms (Nawrot et al., 2014; de Souza Cândido et al., 2011). The involvement of the Tc-2S precursor and its subunits in these defensive roles, however, remains underexplored. In particular, it is unclear whether the entire structure of the Tc-2S precursor is required for these properties. This study aimed to investigate whether the single chain of the mature Tc-2S region, specifically the Tc-9M protein, is independently capable of eliciting plant defense responses.

MATERIALS AND METHOD

Gene Identification, Optimization, and Synthesis

The heavy-chain peptide gene sequence of the 2S albumin (Tc-9M) was identified based on Kochhar et al. (2001). The sequence was optimized using OptimumGene™ and chemically synthesized by GeneScript (NJ, USA). The synthetic Tc-9M gene was delivered in the pUC18 plasmid.

Cloning, Expression, and Purification of Recombinant Tc-9M

The Tc-9M gene was cloned into the pET-32a(+) expression vector at the BamHI and XhoI sites using conventional cloning methods. The recombinant Tc-9M/pET-32a(+) was overexpressed in *E. coli* BL21 (DE3) cells induced with 1 mM IPTG at an OD 600 of 0.4 - 0.5, followed by incubation for 5 h at 37°C with constant shaking. The recombinant Tc-9M protein was purified using an ÄKTA™ pure system (GE Healthcare, Sweden) through a series of chromatographic steps, affinity chromatography (5 mL HisTrap HP column), ion-exchange chromatography (HiTrap Q FF column), and gel-filtration chromatography (HiLoad 16/600 Superdex 75 pg column).

Antibacterial Activity Assay

The antibacterial activity of the Tc-9M protein was tested against Gram-negative bacteria (*Salmonella* sp. ATCC 14028, *E. coli* ATCC 259922, *P. aeruginosa* ATCC 27853) and Gram-positive bacteria (*S. aureus* ATCC 25923, *B. cereus* ATCC 10987). The bacterial suspensions were mixed with molten sterile LB agar, poured into sterile Petri dishes, and allowed to solidify.

The Tc-9M protein was prepared in varying concentrations (10, 5, 1, 0.5, and 0.1 $mg.mL^{-1}$), with ampicillin serving as a positive control. Sterile paper discs (0.5 cm diameter) were placed on the solidified agar, and different concentrations of Tc-9M or ampicillin were pipetted onto each disc. Plates were incubated for 5-10 minutes at room temperature (~25°C) and then inverted for incubation at 37°C overnight for less than 12 h. The diameter of the inhibition zones was measured and recorded.

Antifungal Activity Assay

The antifungal activity of Tc-9M was assessed against *S. cerevisiae*, *P. pastoris*, and *T. asperellum*. For *S. cerevisiae* and *P. pastoris*, a single colony was cultured in 10 mL YPD medium and incubated at 30°C for 12 -16 hours with constant shaking. The resulting culture was evenly spread on the YPD agar plates. For *T. asperellum*, pure cultures were obtained from a collaborator, and the fungal mycelium was inoculated onto PDA medium and incubated at 28°C for 48 h before the application of the Tc-9M protein.

The antifungal activity was evaluated based on the inhibition zones produced by the Tc-9M protein at concentrations of 10, 5, and 0.1 mg/mL. Sterile paper discs were placed on solidified agar containing fungal cultures, and different concentrations of Tc-9M protein or antifungal agents (positive control) were applied. Plates were incubated for 5-10 minutes at room temperature (~25°C) and then inverted for incubation at 37°C overnight for less than 12 hours). The inhibition zones were measured and recorded.

Statistical Analysis

Data were analyzed using analysis of variance (ANOVA) in SAS version 9.4. Significant means were separated using the Least Significant Difference (LSD) at $p > 0.05$.

RESULTS AND DISCUSSION

Direct isolation of the Tc-9M protein from *Theobroma cacao* seeds is a complex and labor-intensive process. Consequently, a heterologous expression system was employed as a more efficient alternative. The Tc-9M gene was successfully cloned into the pET-32a (+) expression vector and expressed as a fully soluble recombinant protein in the *E. coli* BL21 (DE3) system. Using column chromatography, 24 mg of highly pure Tc-9M protein (evidenced by a single band in 15% SDS-PAGE analysis) was obtained from a 300 mL expression culture (data not shown). This study provides a preliminary characterization of the Tc-9M protein's role in plant defense by evaluating its antibacterial and antifungal activities.

Evaluation of the Antibacterial Activities

The initial findings indicate that the recombinant heavy-chain peptide of cocoa 2S albumin, Tc-9M, can independently inhibit the growth of all the tested pathogenic bacteria. The antibacterial activity of Tc-9M, measured by the diameter of the inhibition zone, was significant ($P \leq 0.01$) (Table 1). There was also interaction between the type of bacteria and the concentration of Tc-9M protein (B × C). The diameters of the inhibition zones for each tested bacterium are presented in Figure 1.

Overall, the Tc-9M protein demonstrated the ability to inhibit the growth of all tested pathogenic bacteria in a concentration-dependent manner, as shown in Figure 1. Notably, the Tc-9M protein exhibited significantly higher antibacterial activity against *Salmonella* compared with the other tested bacteria. These findings suggest that the single-chain subunit of 2S albumin, Tc-9M, can effectively inhibit the growth of pathogenic bacteria. This study is the first to report the antibacterial activity of a single-chain subunit of 2S albumin against *Salmonella*, *E. coli*, *P. aeruginosa*, *S. aureus*, and *B. cereus*.

The 2S albumin seed storage proteins isolated from various sources, such as *Ricinus communis*, *Raphanus sativus*, *Brassica napus*, *Triticum aestivum*, *Hordeum vulgare*, *Cucurbita maxima*, and *Putranjiva roxburghii*, have also demonstrated strong antibacterial activity against a wide range of bacterial species, including human pathogens (Sharma et al., 2017; Souza et al., 2016; Tomar et al., 2014). However, as mentioned earlier, studies on the antibacterial properties of the 2S albumin family have largely been limited to the precursor proteins and/or their mature regions. This study is the first to identify and report the antibacterial activity of a single-chain subunit of 2S albumin against *Salmonella*, *E. coli*, *P. aeruginosa*, *S. aureus*, and *B. cereus*.

In general, plant antimicrobial peptides are categorized into several families but share common properties, such as a high number of positively charged residues, the presence of disulfide bridges that stabilize their structure, and a mechanism of action that targets the outer membrane structures (Nawrot et al.,

Table 1. Mean square error from the analysis of variance (ANOVA) of Tc-9M protein against tested bacteria

Source of variation	df	$F_{Calculated}$
Bacteria (B)	4	1.49**
Concentration (C)	6	5.79**
B × C	24	0.30**
Error	70	

** indicates significance at $P \leq 0.01$.

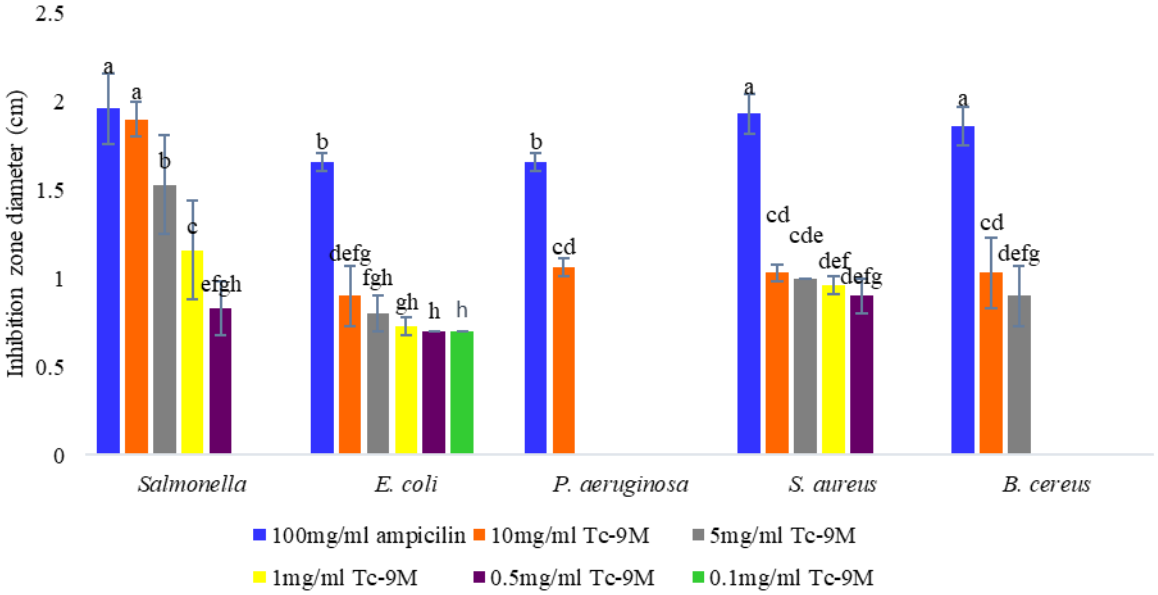

Figure 1. Mean inhibition zone diameter for different concentrations of Tc-9M protein against the tasted bacteria. Different superscripts indicate differences among the Tc-9M protein antibacterial activity. Error bars represent the standard deviation.

2014; Hammami et al., 2008; Barbosa Pelegrini et al., 2011). Consistent with these common properties of antimicrobial peptides, the 2S albumin from *T. cacao* appears to share similar characteristics, such as molecular weight, cationic residues, and disulfide bonds (Kochhar et al., 2001; Kochhar et al., 2005). These findings support the conclusion that the Tc-9M protein possesses antibacterial activity and may play a crucial role in the defense mechanisms of *T. cacao*. However, the precise mechanism by which the Tc-9M protein exerts its antibacterial activity remains unknown, and further studies are needed to elucidate this mechanism.

Evaluation of the antifungal activity

The antifungal activity of the Tc-9M protein was assessed based on its inhibition zone against the tested fungi, including *S. cerevisiae*, *P. pastoris*, and *T. asperellum*. As shown in Table 2, the antifungal activity of the Tc-9M protein, measured by the diameter of the inhibition zone, was significant ($P \leq 0.01$).

A significant interaction was also observed between the type of fungi and the concentration of Tc-9M protein (F×C). The diameters of the inhibition zones against the tested fungi are shown in Figure 2.

Figure 2 shows that the Tc-9M protein is capable of inhibiting the growth of *S. cerevisiae* and *P. pastoris*. At a concentration of 10 mg/mL, the Tc-9M protein exhibited antifungal activity comparable to the positive control, with no significant difference in inhibition observed between them. However, the Tc-9M protein was unable to inhibit the mycelial growth of *T. asperellum*, even at the highest tested concentration of 10 $mg.mL^{-1}$. The inhibitory activity of 2S albumin proteins from various species against a range of yeast strains has been reported in several studies (Ribeiro et al., 2012; Agizzio et al., 2006; Nawrot et al., 2014).

For instance, a detailed study by Ribeiro et al. (2007) on 2S albumin isolated from *Capsicum annuum* L. (chili pepper), named CaNap, demonstrated significant inhibition properties against *C. guilliermondii*, *S.*

Table 2. Mean square error from the analysis of variance (ANOVA) of Tc-9M protein against tested fungi.

	DF	Antifungal activity of Tc-9M
Fungi (F)	2	1.64**
Concentration (C)	4	3.22**
F × C	8	0.42**
Error	30	0.02

** indicates significance at $P \leq 0.01$.

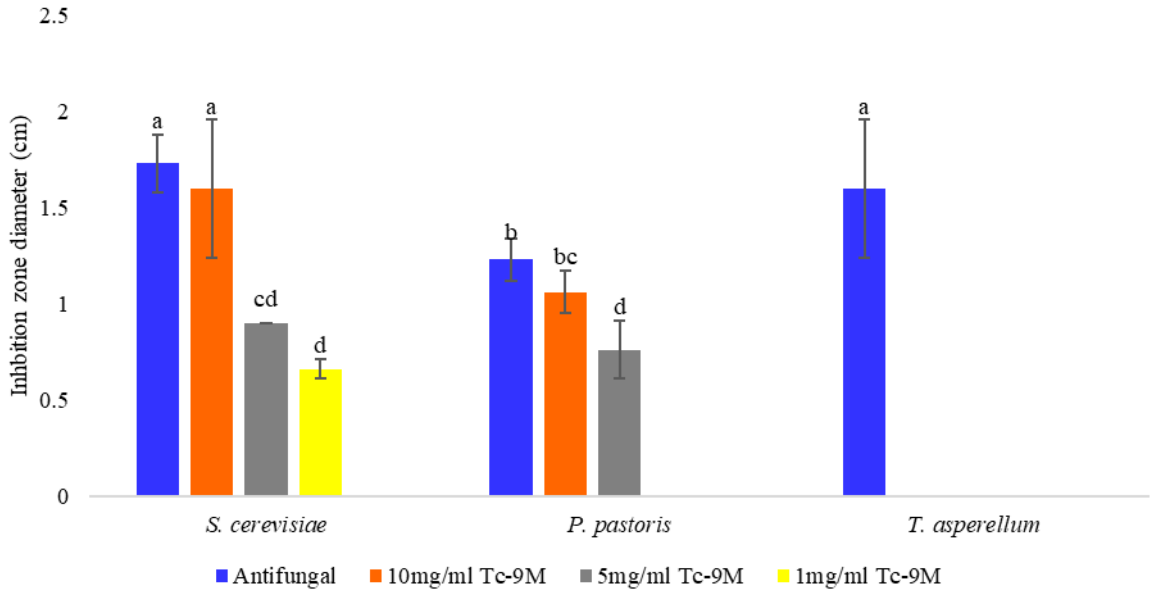

Figure 2. Mean inhibition zone diameter for different concentrations of Tc-9M protein against the tasted fungi. Different superscripts indicate differences among the Tc-9M protein antifungal activity. Error bars represent the standard deviation.

cerevisiae, *K. marxianus*, and *C. tropicalis*. The CaNap protein was shown to act on the plasma membrane of the tested yeasts, causing several morphological alterations, such as cellular agglomeration, cytoplasmic deformation, and a reduction in cell number (Ribeiro et al., 2007).

Recent studies indicate that the antifungal properties of the 2S albumin family might exhibit a broader spectrum of activity against various fungal species and other potential pathogens and may also exist in different biochemical forms. Ribeiro et al. (2012) further emphasized that different types of 2S albumins may act through distinct mechanisms, indicating a sophisticated underlying mechanism for fungal inhibition. some bacteria and fungi can develop resistance to existing inhibitory mechanisms, making the discovery of unique inhibition pathways a major research interest today. However, the specific mechanism by which the Tc-9M protein inhibits *S. cerevisiae* and *P. pastoris* is not well understood and was not investigated in this study. Therefore, further studies are required to explore the potential novel mechanisms of fungal inhibition by Tc-9M.

Despite the promising antifungal properties of the Tc-9M protein, this study found that it was unable to inhibit the mycelial growth *of T. asperellum* even at the highest concentration of 10 mg/mL, as shown in Figure 2. This indicate that the inhibition of *T. asperellum* may require more complex and specific mechanisms. The inability of 2S albumin proteins to inhibit the growth of certain bacteria and/or fungi is common. Several studies have reported negative results regarding the antifungal properties of 2S albumins isolated from different plant species (Costa et al., 2015; Ngai and Ng, 2004).

Although many studies have indicated that seed storage proteins are involved in plant defense mechanisms through their antibacterial and antifungal activities, the application of these proteins remains limited due to challenges in the production of 2S albumins for industrial applications. This study may provide new insights into the future production of the Tc-9M protein using a heterologous expression system. Additionally, this study suggests that the complete structure of the Tc-2S precursor is not required to harness antibacterial or antifungal activity, and the single-chain Tc-9M protein alone may be sufficient for plant defense responses.

Because the characterization of a single-chain peptide of 2S albumin has never been reported before, this work provides a platform for further study on the biological importance

of the 2S albumin single-chain subunit. Moreover, it is interesting to speculate that 2S albumin, specifically the Tc-9M protein, could serve as an ideal candidate for natural pesticides in *T. cacao*, a valuable commercial crop.

However, despite the promising properties of the Tc-9M protein as an antibacterial and antifungal agent, this study also identified that the Tc-9M protein was unable to inhibit the mycelial growth of *T. asperellum* even at the highest concentration of 10 mg/mL. Nevertheless, this is the first study to report on the antibacterial and antifungal activity of a single-chain subunit of 2S albumin. Further studies are needed to enhance our understanding of the potential novel mechanisms in plant defense properties conferred by the Tc-9M protein.

CONCLUSION

The Tc-9M protein was found to inhibit the growth of *Salmonella*, *E. coli*, *P. aeruginosa*, *S. aureus*, and *B. cereus* in a concentration-dependent manner. The MIC analysis also supported the ability of the Tc-9M protein to inhibit the growth of all tested bacteria. In addition, the Tc-9M protein showed antifungal activity against *S. cerevisiae* and *P. pastoris*, with results comparable to the antifungal control. These findings imply that the involvement of the Tc-2S precursor in plant defensive mechanisms is regulated by the Tc-9M protein.

ACKNOWLEDGMENT

The authors are grateful to the Ministry of Education Malaysia for funding this project under the Fundamental Research Grant Scheme (Grant No. FRG0426-ST-1/2015).

REFERENCES

Agizzio, A. P., M. Da Cunha, A. O. Carvalho, M. A. Oliveira, S. F. Ribeiro, and V. M. Gomes. 2006. Retracted: The antifungal properties of a 2S albumin-homologous protein from passion fruit seeds involve plasma membrane permeabilization and ultrastructural alterations in yeast cells. Plant Science 171(4): 523-530.

Autran, J. C., N. G. Halford, and P. R. Shewry. 2001. The biochemistry and molecular biology of seed storage proteins. In: Plant nitrogen, Springer, Berlin, Heidelberg. p. 295-341.

Barbosa Pelegrini, P., R. P. del Sarto, O. N. Silva, O. L. Franco, and M. F. Grossi-de-Sa. 2011. Antibacterial peptides from plants: What they are and how they probably work. Biochemical Research International 2011.

Costa, T. G., O. L. Franco, L. Migliolo, and S. C. Dias. 2015. Identification of a novel 2S albumin with antitryptic activity from *Caryocar brasiliense* seeds. Journal of Agricultural Science 7(6): 197.

De Souza Cândido, E., M. F. S. Pinto, P. B. Pelegrini, T. B. Lima, O. N. Silva, R. Pogue, and O. L. Franco. 2011. Plant storage proteins with antimicrobial activity: Novel insights into plant defense mechanisms. The FASEB Journal 25(10): 3290-3305.

Hammami, R., J. Ben Hamida, G. Vergoten, and I. Fliss. 2008. PhytAMP: A database dedicated to antimicrobial plant peptides. Nucleic Acids Research 37(suppl_1): D963-D968.

Higgins, T. J. V. 1984. Synthesis and regulation of major proteins in seeds. Annual Review of Plant Physiology 35(1): 191-221.

Kochhar, S., K. Gartenmann, M. Guilloteau, and J. McCarthy. 2001. Isolation and characterization of 2S cocoa seed albumin storage polypeptide and the corresponding cDNA. Journal of Agricultural and Food Chemistry 49(9): 4470-4477.

Kochhar, S., C. E. Hansen, M. A. Juillerat, and J. McCarthy. 2005. U.S. Patent No. 6,927,280. Washington, DC: U.S. Patent and Trademark Office.

Krishnan, H. B., and E. H. Coe. 2001. Seed storage proteins.

Moreno, F. J., and A. Clemente. 2008. 2S albumin storage proteins: What makes them food allergens?. Open Biochemistry Journal 2: 16.

Nawrot, R., J. Barylski, G. Nowicki, J. Broniarczyk, W. Buchwald, and A. Goździcka-Józefiak. 2014. Plant antimicrobial peptides. Folia

Microbiologica 59(3): 181-196.

Ngai, P. H. K., and T. B. Ng. 2004. A napin-like polypeptide from dwarf Chinese white cabbage seeds with translation-inhibitory, trypsin-inhibitory, and antibacterial activities. Peptides 25(2): 171-176.

Osborne, T. B. 1916. The vegetable proteins. Longmans, Green and Company.

Ribeiro, S. F., A. O. Carvalho, M. Da Cunha, R. Rodrigues, L. P. Cruz, V. M. Melo, and V. M. Gomes. 2007. Isolation and characterization of novel peptides from chili pepper seeds: Antimicrobial activities against pathogenic yeasts. Toxicon 50(5): 600-611.

Ribeiro, S. F., G. B. Taveira, A. O. Carvalho, G. B. Dias, M. Da Cunha, C. Santa-Catarina, and V. M. Gomes. 2012. Antifungal and other biological activities of two 2S albumin-homologous proteins against pathogenic fungi. Protein Journal 31 (1): 59-67.

Sharma, A., P. Kumar, P. Kesari, M. Katiki, M. Mishra, P. K. Singh, and P. Kumar. 2017. Purification and characterization of 2S albumin from seeds of *Wrightia tinctoria* exhibiting antibacterial and DNase activity. Protein and Peptide Letters 24(4): 368-378.

Souza, P. F., I. M. Vasconcelos, F. D. Silva, F. B. Moreno, A. C. Monteiro-Moreira, L. M. Alencar, and J. T. Oliveira. 2016. A 2S albumin from the seed cake of *Ricinus communis* inhibits trypsin and has strong antibacterial activity against human pathogenic bacteria. Journal of Natural Products 79(10): 2423-2431.

Tomar, P. P. S., N. S. Chaudhary, P. Mishra, D. Gahloth, G. K. Patel, P. Selvakumar, and A. K. Sharma. 2014. Purification, characterization and cloning of a 2S albumin with DNase, RNase and antifungal activities from *Putranjiva roxburghii*. Applied Biochemistry and Biotechnology 174(2): 471-482.

Tomar, P. P. S., K. Nikhil, A. Singh, P. Selvakumar, P. Roy, and A. K. Sharma. 2014. Characterization of anticancer, DNase and antifungal activity of pumpkin 2S albumin. Biochemical and Biophysical Research Communications 448(4): 349-354.

Voigt, J., B. Biehl, and S. K. S. Wazir. 1993. The major seed proteins of *Theobroma cacao*. Food Chemistry 47(2): 145-151.