RESEARCH ARTICLE

# Variation in Antimicrobial Activity and Seed Storage Proteins in Three Species of the Medicinal Plant *Alstonia*

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## Abstract

The family *Apocynaceae* comprises three species of the substantially important *Alstonia* plant, *viz., A. scholaris, A. venenata, and A. macrophylla*. The investigation of proteins contained within seeds, that has the potential to provide both precise details and a structural basis for characterizing diversity. The utilization of an electrophoretic technique for protein analysis has been observed in recent scholarly investigations. This implies that certain protein bands exhibit variability, with their presence or absence being detected across different seed arrangement levels in the gel. Furthermore, this implies that the protein bands have been segregated into distinct categories. A study was conducted to analyze the seed storage protein profiles of three distinct *Alstonia* species through the application of sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The resolution of the seed storage protein of the *Alstonia* species using 15% SDS was found to alter the banding pattern of the polyacrylamide gel. The SDS-PAGE analysis revealed differential up-regulation of proteins across distinct bands. Throughout the examination, it was determined that the three aforementioned species exhibited a common band, in addition to a protein with a molecular weight of 34 kDa. The number of protein bands attached to *A. venenata* was the highest (ten bands), while the number of protein bands that adhered to *A. scholaris* was the lowest (five bands). Further, powdered leaves of *A. scholaris*, *A. venenata*, and *A. macrophylla* were investigated for antibacterial and antifungal activities. In accordance with the results, *A. macrophylla* leaf powder is most effective against *Staphylococcus aureus*, followed by *A. venenata* and *A. scholaris* at 1 µg/mL. In addition, the findings support the inference that the *A. scholaris* leaf powder effectively inhibited the growth of *Aspergillus niger*. *A. venenata* and *A. macrophylla*, both at 1µg/mL, are, nonetheless, also effective against *A. niger*. *A. macrophylla* has the largest zone of inhibition (11.5 mm) against *A. niger.*


**Keywords:** SDS-PAGE, relative mobility, gel image analysis, *Alstonia*, antimicrobial,antifungal activities.

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## Introduction

The taxonomic classification of *Alstonia* is within the family *Apocynaceae* and comprises a collection of perennial trees and shrubs that retain their foliage throughout the year. The *Alstonia* genus comprises approximately 40–60 species that are indigenous to the tropical and subtropical regions of Southeast Asia, Africa, Polynesia, America, and Australia, with a majority of the species found in the Malaysian region. *Alstonia longifolia* is a solitary species found in Central America, primarily growing in shrub form, as documented by Markgraf (1974) and Forster (1992).

According to Khare *et al.* (2007), the *Alstonia* plant species have a long history of being exploited as therapeutic agents in a variety of traditional medical systems including Ayurveda, Unani, Siddha, and Tamil. *Alstonia* is a plant that has a long history of usage in traditional medicine as a therapy for a variety of conditions, including fever (Rajakumar *et al*., 2010), malaria, jaundice, hepatitis, tumours, cancer, and skin problems (Moilik *et al*., 2010). In addition to detecting and characterising diversity in seed storage crop cultivars, the study of macromolecules may also provide insights into the molecular processes behind the acquisition (Nisar *et al*., 2007; Tanksley *et al*., 1981; Thanh V.O.C. and Hirata, 2002). These benefits can be attributed to the work of Nisar, Tanksley, and Hirata.

Furthermore, scholarly research has demonstrated that the analysis of macromolecule bands can yield valuable insights into the correlation between seeds procured and utilised from diverse geographical locations (Ghafoor *et al*., 2003; Satija *et al*., 2002; Asghar *et al*., 2003). The storage proteins present in the seeds of various plant species exhibit significant variation in their quantities. The use of electrophoresis in protein analysis is a highly effective method for detecting genetic variability. Among the various electrophoretic techniques, SDS-PAGE is considered to be particularly reliable due to the fact that seed storage proteins exhibit a high degree of independence from environmental fluctuations. This assertion is supported by studies conducted by Javid *et al.* (2004) and Iqbal *et al.* (2005).

The utilization of macromolecule patterns has been identified as a potentially effective method for the targeted cultivation of particular strains of crops, as noted by Jha and Ohri (1996) and Seferoglua *et al.* (2006). According to Magni *et al.* (2007), there is a higher protein concentration in mature seeds compared to other plant tissues. As a result, the SDS-PAGE patterns of seed storage macromolecules in specific species exhibit polymorphisms supported by protein intensity variations among different genotypes. This work aims to investigate the interrelationships among three species belonging to the class magnoliopsida, which are of medicinal importance.

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Additionally, the study aims to identify and describe any variations or inconsistencies observed. The three species under consideration are *A. scholaris, A. venenata, and A. macrophylla*. The SDS-PAGE technique was utilised to analyse variations in seed macromolecules individually.

## Material And Methods

### Protein Extraction

The protein was isolated by using Lowry's technique, which was described in 1951. Using a cold mortar and pestle, a seed sample that weighed 100 mg was homogenized with 1-mL of extraction buffer solution. After the sample was homogenized, it was placed in a microcentrifuge tube with a capacity of 1.5 mm and centrifuged. After that, the tube was centrifuged at a rate of 8000 rpm for a period of 5 minutes to separate the contents within. The liquid part of the sample is collected and utilized in the calculation of the total protein content as well as in the SDS-PAGE analysis.

### Protein Estimation

After adding 0.1–0.5 mL, of working standard to a series of labeled test tubes. After that, the distilled water needed to bring the volume of each test tube up to 1 mL. After transferring a volume of 0.1 mL of the protein sample into a separate test tube, the volume of the sample was diluted with water to reach a final volume of 1-mL. After that, 5 mL of an alkaline copper solution was added to each of the test tubes, including the control. The tube contents were subjected to vortex mixing and subsequently incubated for 10 minutes at ambient temperature under low light conditions. Subsequently, a volume of 0.5 mL of Folin's reagent (also known as Folin-Ciocalteu) that had been diluted was introduced and allowed to incubate for the duration of 30 minutes under conditions of darkness and at ambient temperature. Following incubation, the optical density (OD) was measured at 630 nm for both the standard and sample in comparison to the blank. The linearity regression equation helped determine the protein concentration of each sample in mg/mL, considering the dilution factor.

### Electrophoretic Analysis (SDS-PAGE)

The Lowry test (Lowry 1951) was used to analyze the seed proteins derived from a variety of different seed sources. Bovine serum albumin (BSA) standard served as the evaluation's point of reference. The Laemmli (1970) methodology was followed as directed when doing the SDS-PAGE analysis. An extraction buffer containing 0.5 M Tris HCl, 5M urea, 0.2% SDS, and 1% mercaptoethanol was used to homogenize the seeds of the three different species. In order to carry out the PAGE procedure, a 15% separating gel was used. This gel was made up of a 30% acrylamide solution, a 1.5M tris-HCl buffer with a pH of 8.8, 10% SDS, and 10% ammonium persulfate (APS). During the course of the experiment, a stacking gel was produced by combining 30% acrylamide/bis, a 0.5M tris-HCl buffer with a pH of 6.8, and 10% SDS in a solution.

For the purpose of conducting an SDS analysis, a vertical slab electrophoresis system manufactured by Bio-Rad and known as the SDS-PAGE unit was used. In the course of the experiment, the lower reservoir of the apparatus had the protein electrode buffer solution added to it. In order to avoid the creation of bubbles at the bottom of the gel plates, the gel plates had to be precisely positioned inside the apparatus. With the use of a micropipette, we filled each well of the gel with an equal quantity of protein that had been extracted from each of the samples, as well as a protein molecular weight marker. The apparatus was connected to a reliable supply of electrical current during its operation. Until the tracking dye had migrated into the separating gel, electrophoresis that employed a constant current of 15 milliamperes was carried out. Following that, the electrical current was raised to a level of 20 milliamperes and maintained at that level for three to four hours. Following electrophoresis, bands of protein were observed by staining them with 0.1% Coomassie Brilliant Blue CBB R250. Following this, the protein bands were destained using a solution that included methanol: acetic acid:water in the proportions 4:1:5, respectively.

### Data Analysis

The inclusion and exclusion of each band was considered in all three cultivars under investigation. Jaccard's similarity index was computed utilizing the binary data matrix. The dendrograms of the genetic association among all analyzed cultivars were generated using the UPGMA and Neighbour Joining methods of PyElph software, as described by Pavel and Vasile (2012). The process of determining the molecular weight for each band involved the utilization of GelAnalyzer 19.1 software in constructing a standard curve. This was achieved by referencing the Molecular weight of protein with a ladder using a quadratic type curve.

### Evaluation of Antibacterial and Antifungal Activity

The well-diffusion method was used to determine the inhibitory zone in mm for the in-vitro antimicrobial evaluation of the *Alstonia scholaris, A. venenata,* and *A. macrophylla* leaf powder. The antibacterial activity of the sample was evaluated against *Staphylococcus aureus*.For the isolation of bacteria, initially, Muller Hinton Agar was made according to the standard composition provided by HI-media, which was 38 gm of the media suspended in 1L DW and sterlized at 121°C and 15 psi for 15 minutes in an autoclave (Gentex India Pvt. Ltd.). After the sterilization, media was poured into sterile glass petriplates inside the laminar airflow (Toshiba, India) using the aseptic techniques, each plate was poured with 20mL of the culture media. After allowing the plates to solidify appropriately, the media was administered with the appropriate bacterial isolate by spread plate technique, for which 100 µL of the culture broth of isolate was added over the media and uniformly spread using a sterile glass rod. After allowing the biofilm to expand for ten minutes, it was transferred to the media plates using sterile forceps. Subsequently, each disc was filled with the appropriate sample and put on separate plates. During this time, control was established with one of the biofilms. After allowing the samples to diffuse through the disc into the medium, the plates were covered with paraffin and placed in an incubator at 34  for 24 hours. The plates each had two discs, with one disc as a positive control containing 20 L of ciprofloxacin at a concentration of 0.8 ppm, and the other a negative control containing distilled water. The next day, after the plates had been incubated, they

were inspected to locate the zone of inhibition, which is a clear region encircling the disc. The diameter of these areas was measured in mm and the results were recorded. These tests were carried out thrice.

## Results And Discussion

The seed proteins originating from various seed sources were evaluated using the Lowry assay (Fig. 1) with a bovine serum albumin (BSA) standard as the basis for the determination.

The analysis of storage protein in seed was conducted via denaturing method of electrophoresis using a 15% polyacrylamide gel. Table 1 displays variations in the total protein content among three Alstonia species. The protein's SDS banding pattern exhibited a distribution of 24 bands across all species, identified using a marker with a molecular weight ranging from 16 kDa to 250 kDa, as depicted in Fig 2.A. scholaris (AS), A. venenata (AV) and A. macrophylla (AM) showed 10, 10 and 4 protein bands, respectively. A maximum of 10 protein bands were observed in both A. venenata and A. scholaris. A minimum of bands were observed in A. macrophylla that exhibited 4 bands. In addition to the typical bands observed within the analyzed taxa, it was observed that 78, 33 and 23 kDa bands of proteins were universally present across all three species.

Two ubiquitous bands were observed across all inspected species, with molecular weights of 34 kDa for band No. 4 and band No. 5, respectively. The second, fifth and sixth bands, with a molecular weight of 78, 33 and 23 kDa each, having Rf of 0.224, 0.482 and 0.666 were observed in all three species under investigation. Three bands of 21, 16 and 13 having Rf of 0.698, 0.794 and 0.866 were shared among A. scholaris (AS), A. venenata (AV) but absent in A. macrophylla. AM showed presence of a unique band at Rf of 0.599 with a molecular weight 28 KDa.

**Table 1:** Table showing the concentration of protein in mg/g for the different *Alstonia* seed samples

| *S. No.* | *Sample code* | *Concentration (mg/g)* |
|---|---|---|
| 1 | A. scholaris | 389.09 |
| **2** | A. macrophylla | 509.17 |
| 3 | A. venenata | 386.53 |

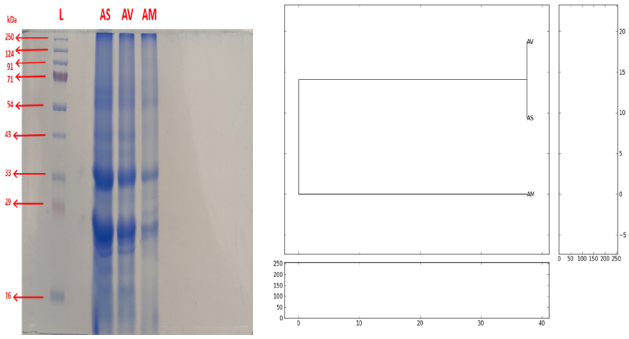


**Fig. 1:** shows SDS-PAGE gel pictures of the protein that was extracted from seed samples of Alstonia scholaris (AS), Alstonia venenata (AV), and Alstonia macrophylla (AM). The lane that is labelled with the size of the protein ladder in kilodaltons is indicated by the letter "L.". The phylogenetic tree between three cultivers AS, AV and AM generated using the Neighbour joining method using PyElph software.

According to Przybylska's research in 1995 and subsequent studies in 2000, seed proteins are typically considered the final product of genetic ordering and are comparatively less influenced by environmental factors. According to Davis and Heywood (1963) and Ladizinsky and Hymowitz (1979), the substantial stability of protein characteristics, especially in seeds, gives them a formidable weapon in illuminating the origin, evolution, and interrelationships of taxa. This is something that both of these researchers have highlighted. The presence or absence of protein bands has been recognized by Ladizinsky and Hymowitz (1979) as a diagnostic trait that may be used to classify an organism into a certain taxon or group. The findings pertaining to the protein types present in Alstonia samples collected from various locations in Bangalore indicate a discernible variance in the total number of proteins that were separated and visualized on the gel bands.

According to Wong *et al*. (2004), the process of protein synthesis during seed development occurs in a reduced state, while maturation and drying result in the oxidation of the proteins. Shutov *et al*. (2003) articulated that although storage proteins are sheltered from very early degradation, there is limiting proteolysis in dry seeds. The use of SDS-PAGE for seed storage protein profiling holds promise for facilitating differentiation among various species. The use of seed storage protein profile clustering yields insights into the phylogenetic associations among organisms. This disparity in the quantity of proteins is indicative of a significant difference in the protein content across the samples. The separation of *Alstonia* seeds into multiple bands through the use of SDS-PAGE and protein-page was noted, and there is a dearth of comprehensive research on the proteins in *Alstonia* seeds.

### Analysis of Antibacterial Activity

The antibacterial activity of *A. scholaris, A. venenata,* and *A.macrophylla* leaf powder against gram-positive bacteria namely, *S. aureus*. Fig. 2 shows the antibacterial activity of *A. scholaris, A. venenata,* and *A.macrophylla* leaf powder against *S. aureus*.

Further, Table 2 shows zone of inhibition formed by *Alstonia scholaris, Alstonia venenata,* and *Alstonia macrophylla* leaf powder against *S. aureus*.

**Table 2:** Antibacterial activity as zone of inhibition against *S.aureus* by powder-dried leaves of different species of *A.*

| *S.No* | *Sample (Powdered leaves)* | *Concentration (µg/mL)* | *Zone of Inhibition (mm)* |
|---|---|---|---|
| 1 | *A. scholaris* | | 12 |
| 2 | *A. venenata* | 1 | 11.9 |
| 3 | *A. macrophylla* | | 13..2 |

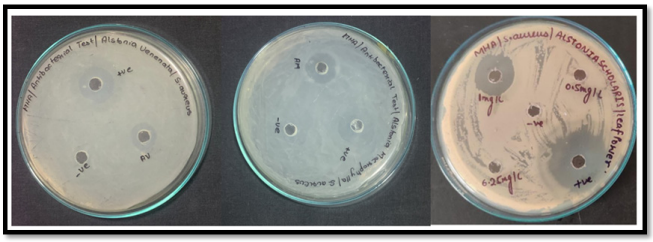

**Fig. 2:** Antibacterial activity of *A. venenata*, *A. macrophylla*, and *A. scholaris* leaf powder extracts against *S.aureus*

**Table 3:** Antifungal activity as zone of inhibition against *S.aureus* by powder dried leaves of different species of *Aspergillus niger*

| S.No | Sample (Powdered leaves) | Concentration (µg/mL) | Zone of Inhibition (mm) |
|---|---|---|---|
| 1 | *A. scholaris* | | 10 |
| 2 | *A. venenata* | 1 | 8.3 |
| 3 | *A. macrophylla* | | 11.5 |

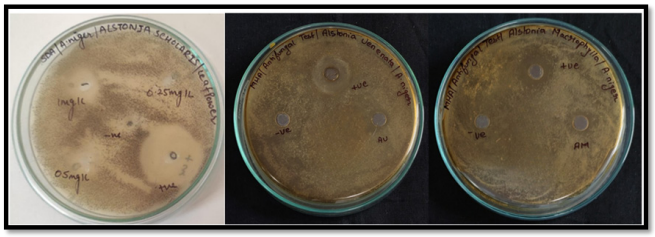

**Fig. 3:** Antifungal activity of *A. scholaris*, *A. venenata*, and *A. macrophylla* leaf powder extracts against *Aspergillus niger*

Using the data obtained, we can swiftly infer that leaf powder of *A. macrophylla* was more effective against *S. aureus* followed by *A. venenata* and *A. scholaris*. *1* µg/mL of each leaf powder was shown to be effective against *S. aureus*.

### Analysis of Antifungal Activity

The antifungal activity of *A. scholaris, A. venenata,* and *A. macrophylla* leaf powder against *Aspergillus niger.* Fig. 3 shows the antifungal activity of leaf powder of three species of *A.* against *Aspergillus niger.* Further, Table 3 shows zone of inhibition form by *A. scholaris, A. venenata,* and *A. macrophylla* leaf powder against *A.niger.*

Using the data obtained, it can swiftly infer that leaf powder of *A. scholaris* was effective against *Aspergillus niger. However, A. venenata* and *A. macrophylla* shows good potency against *Aspergillus niger* in the 1-µg/mL concentration. *A. macrophylla* has the highest potency against *Aspergillus niger* with zone of inhibition of 11.5 mm.

## Conclusion

The heritable diversity of three *Alstonia* species was investigated through light exfoliation using SDS-protein. The present results indicate that the proteins exhibit discriminatory ability among the *Alstonia* species under investigation by generating unique bands, thus facilitating the differentiation of each species. Numerous proteins are apparent in all three *Alstonia* species, indicating a degree of shared genetic material. The objective of the current investigation was to assess the protein polymorphism across diverse *Alstonia* species through an analysis of their respective species variations. The study's molecular-level findings revealed notable variations in the number of observed bands. The importance of SDS-protein in genetic analysis is apparent, as it uncovers a significant degree of genetic diversity among the diverse *Alstonia* species being studied. The protein found in the seed storehouse functions as a sturdy basis for the categorization of genotypes according to the unique pattern of polypeptide bands obtained through SDS-PAGE. The significance of SDS-Protein in genetic analysis is apparent, as it suggests a substantial level of genetic variation among the various species of *Alstonia* that were examined. We have also examined the powdered leaves of *A. scholaris*, *A. venenata*, and *A. Macrophylla* for its antibacterial and antifungal properties. Based on the results, it is clear that the *A. macrophylla* leaf powder is the most efficient against *S. aureus*, followed by the powdered leaves of the *A. venenata* and *A. scholaris*. Powdered leaves at a concentration of 1µg/mL were demonstrated to be efficient in eliminating *S. aureus*. Further, according to the results, it's easy to conclude that the A. scholaris leaf powder has no potency against the fungus *Aspergillus niger.* However, 1 µg/mL of either *A. venenata* or *A. macrophylla* is quite effective against *Aspergillus niger.* With a zone of inhibition of 11.5 mm, *A. macrophylla* is the most effective species against *Aspergillus niger.*

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## Author Contribution

Author Jyoti Kumari was involved in planning and Author Tanuja supervised the work. Author Jyoti Kumari processed the experimental data, performed the analysis, drafted the manuscript. Both the authors discussed the results and commented on the manuscript. Authors Jyoti Kumari & Tanuja reviewed the results and approved the final version of the manuscript with mutual consent.

## Conflict Of Interest

None

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