

RESEARCH ARTICLE

Phytochemical analysis, and antioxidant and antibacterial activities of *Alstonia scholaris* from Mizoram, India

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Abstract

The Devil's tree (*Alstonia scholaris* (L.) R.Br.), a member of the Apocynaceae family, is recognised in various traditional systems for its efficacy in treating several diseases. In the Mizo traditional medicines of India, the bark extract is utilised as a remedy for bacterial and parasitic infections, among other ailments. To validate the therapeutic claim of the Mizo people, a methanolic extract of the bark was prepared and its chemical composition was analysed. The extract was found to contain alkaloids, carbohydrates, flavonoids, glycosides, phytosterols, saponins, tannins, and reducing sugars. The antioxidant components of the extract were quantified, revealing a phenolic content of 13.563±0.09 mg/g quercetin equivalent, a flavonoid content of 31.64±2.50 mg/g gallic acid equivalent, and a total antioxidant of 10.48±0.84 mg/g ascorbic equivalent. These findings underscore the plant's cellular protective capacity. Furthermore, the antioxidant activities were assessed using 2,2-diphenyl-1-1-picryldrazyl (DPPH) and ferric reducing antioxidant power (FRAP) assays. The plant extract exhibited significant antioxidant properties, with a half-maximal inhibitory concentration (IC₅₀) value of 11.01 against free radicals generated from the DPPH reaction. Notably, the extract demonstrated broad-spectrum antibacterial activity against Gram-negative bacteria, including *Escherichia coli* and *Salmonella typhi*, as well as Gram-positive species such as *Bacillus cereus*, *Enterococcus faecalis* and *Staphylococcus aureus*. This study establishes *A. scholaris* as a medicinal plant with promising antimicrobial and pharmacological properties, containing chemical components that can be harnessed for therapeutic purposes.

Keywords

Alstonia scholaris; antibacterial activity; antioxidant activity; medicinal plant

Introduction

A major challenge in current medical management of infections and the accompanying pharmaceutical progress lies in the discovery and development of new medications. This dilemma is exacerbated by the widespread evolution of antibiotic resistance in nearly all pathogenic microbes. Various bacteria have developed drug resistance to such extreme levels, known as multi-drug resistance, that the most commonly available and used antibiotics have become ineffective and obsolete (1). This situation is considered the most serious threat to the effective management of infectious diseases, leading to increased mortality rates (2). Among the promising sources of novel drugs, plants are recognised as key molecules of

pharmacological interest, and their applications established in traditional medicines have become a central focus of research attention (3-5). Plants are known to offer practical advantages due to their minimal or lack of adverse effects, and those used in Indian traditional systems are appreciated as the best sources in terms of both quantity and diversity (6).

Alstonia scholaris (L.) R.Br. is an evergreen tree belonging to the family Apocynaceae, native to Africa, Australia, Bangladesh, Bhutan, Cambodia, China, India, Malaysia, Myanmar, Nepal, Pakistan, Papua New Guinea, Queensland, Sri Lanka, Thailand, and Vietnam (7). The species is characterised by oblong leaves arranged in three to ten whorls (8). In India, this tree typically has whorls of seven leaves, earning it the common name *Saptaparna*, meaning seven leaves (9). It is also known as the Devil's tree due to severe allergic reactions caused by its pollens during the flowering season. In various Indian traditional systems, the leaves, latex, and bark of this tree are used to treat chronic diseases such as asthma, cancer, jaundice, leprosy, and malaria (10, 11). The latex is specifically employed for wounds healing, ulcers, and as an analgesic in rheumatoid arthritis (9). The plant is documented to possess analgesic, anticancer, anthelmintic, anti-inflammatory, anti-tumour and laxative properties (12). Extracts from the leaves have shown antifungal, antibacterial properties (13, 14), herbicidal activities (15), as well as antitussive, expectorant and anti-asthmatic activities (16). Due to its multi-faceted applications, it is well-documented in the Ayurvedic Pharmacopoeia of India, British Pharmacopoeia, British Pharmaceutical Codex, French Pharmacopoeia, and Indian Pharmacopoeia (12).

The Mizo people inhabit one of the most remote regions of mainland India, situated within the Indo-Burma biodiversity hotspot. In contrast to the general occurrence of seven-whorled leaves in other parts of India, the *A. scholaris* variety found in Mizoram stands out with precisely eight leaves on each whorl (Fig. 1). Due to this unique feature, the Mizo people refer to it *thumriat*, which means *eight-whorled*. In Mizo traditional medicine, various

parts of this tree are employed for treating wounds (17), and as remedies for conditions such as diarrhoea, dysentery, ear infection, heart diseases, hypertension, typhoid fever (18), and malaria (19). The extensive list of medicinal applications strongly suggests that the plant possesses important chemical properties and antibacterial functions. However, precise knowledge about its medicinal properties remains limited. Therefore, this study was designed to investigate the fundamental phytochemical components, antioxidant properties, and antibacterial activities of the *A. scholaris* variety found in Mizoram.

Materials and Methods

Specimen collection and identification

Alstonia scholaris was collected from Lungdai village, Kolasib district of Mizoram, India, located at 23°52' N 92° 44' E. The fresh leaves and flowers were used to prepare a herbarium, which was authenticated at the Botanical Survey of India (BSI), Shillong, India (no. BSI/ERC/Tech/2023-24/102-17-05-23). A reference specimen with accession number PUC-A-23-01 is preserved in the herbarium section of the Department of Botany, Pachhunga University College, Aizawl, India.

Chemicals and reagents

All chemicals and reagents used were of analytical grades procured either from HiMedia Laboratories Private Limited or SD Fine Chemicals Limited, both of Mumbai, Maharashtra, India.

Preparation of plant extract

The barks of *A. scholaris* were collected, washed in distilled water, cut into fine pieces and dried under shade for four weeks. 350 g of the dried samples were packed into the Soxhlet apparatus for extraction using methanol as a solvent. Continuous extraction was done for 72 hr. The crude extracts were concentrated in a vacuum rotatory evaporator, Buchi Rotavapor® R-100, which removed and recycled the solvent. The plant extracts were maintained in a refrigerator at 4°C and later used for phytochemical tests, antioxidant assays, and antibacterial tests.

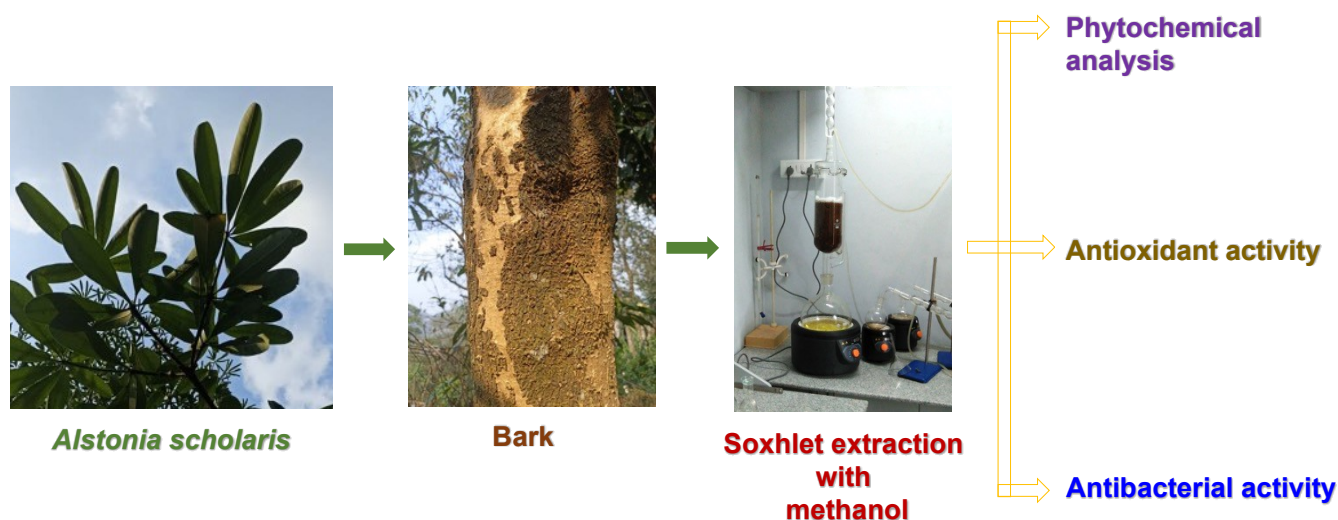


Fig. 1. *Alstonia scholaris* from Mizoram and workflow of the study. Note the leaves are arranged in whorls of eight (left inset).

Qualitative phytochemical tests

Phytochemical identification of *A. scholaris* bark extract was performed according to the standard protocols based on 11 groups tests (20). In outline: alkaloids were tested by Hager's test, Wagner's test, Mayer's test, and Dragendorff's test; flavonoids by alkaline reagent test, lead acetate test, and Shinoda's test; reducing sugar by Fehling's test and Benedict's test; carbohydrates by Molisch's test, Benedict's test, iodine test, Fehling's test; glycosides by Liebermann's test, Keller-Kiliani test; saponins by foam test; protein and amino acid by Biuret test and ninhydrin test; phytosterols by Salkowski test and Liebermann-Burchard's test; gums by alcohol test; anthraquinones by Borntrager's test and ammonium hydroxide test.

Total phenolic content

The total phenolic content of *A. scholaris* bark extract was estimated after the method of Singleton *et al.* with minor modifications (21). Gallic acid was used as a benchmark compound to estimate the amount of phenols. In brief, 1 mL of the plant extract (at a concentration of 100 µg/mL) and standard gallic acid of different concentrations (*viz.* 10, 20, 40, 60, 80, and 100 µg/mL) were made. Then, 5 mL of Folin-Ciocalteu reagent (FCR) was added to all samples. Reaction was allowed to run at ambient temperature for 3 min; after which 4 mL of a 0.7 M sodium carbonate solution was added and then mixed in rotary mixer. The samples were left undisturbed at room temperature for 1 hr. A blank concentration was made from 1 mL of methanol, 5 mL of FCR, and 4 mL of sodium carbonate. The absorbances of all samples were recorded at the wavelength of 765 nm in a UV-Vis spectrophotometer. A calibration curve for gallic acid was generated. The total phenolic content was calculated as gallic acid equivalent in milligrams per gram (GAE mg/g) of dry weight of the plant sample. The experiment was carried out in triplicate.

Total flavonoid content

The total flavonoid content of *A. scholaris* bark extract was determined by aluminium chloride assay based on the method of Zhishen *et al.* (22). Quercetin was used as a reference flavonoid compound. 1 mL of the plant extract (100 µg/mL) and quercetin of different concentrations (*viz.* 10, 20, 40, 60, 80, and 100 µg/mL) were prepared, and 2 mL of distilled water was added to each sample. After letting remain for 5 min, 3 mL of 5% sodium nitrite and 0.3 mL of 10% aluminium chloride were added. Reaction was allowed for 6 min, after which 2 mL of 1 M sodium hydroxide was added. The final volume of each sample was made up to 10 mL by adding distilled water. After 1 hr, the absorbances were measured at 510 nm. From the quercetin standard curve, the total flavonoid concentration was calculated and presented as quercetin equivalent in milligrams per gram (QE mg/g) of the dry weight. The samples were analysed in triplicates.

Total antioxidant content

By slightly modifying a standard procedure of Prieto *et al.* (23), the total antioxidant activity of *A. scholaris* bark extract was assessed by phosphomolybdate reaction. Ascorbic acid was used as a reference antioxidant. 1 mg/

mL of the plant extract was dissolved in distilled water to create a stock solution. 1 mL (100 µg/mL) of the extract from the stock solution and different concentrations of ascorbic acid (at 10, 20, 40, 60, 80, and 100 µg/mL) were prepared. A reagent solution was made by mixing 0.6 M sulphuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate. 3 mL of the reagent solution was added to 1 mL of the plant extract and ascorbic acid of different concentrations. They were incubated for 90 min at 95°C. After allowing to cool down to room temperature, the absorbances were taken at 695 nm. From the calibration curve of ascorbic acid, the total flavonoid content was estimated and given as ascorbic acid equivalent in milligrams per gram (AAE mg/g) of the dry weight. The analysis was carried out three times.

DPPH free radical-scavenging activity assay

The antioxidant activity of *A. scholaris* bark extract was studied using the method developed by Blois after certain modifications (24). Butylated hydroxytoluene (BHT) served as the reference antioxidant. The free radical-producing substrate, DPPH (2,2-diphenyl-1-picrylhydrazyl), was employed as the target of antioxidation reaction. Both the plant extract and standard BHT were prepared at different concentrations, specially 10, 20, 40, 60, 80, and 100 µg/mL. In each sample, 3 mL of the extract was mixed with 0.5 mL of 1 mM DPPH. The control sample consisted of a mixture of 3 mL methanol and 1 mL DPPH. All samples were incubated and allowed to react for 30 min at 37°C. The absorbances were measured at 517 nm. The following formula was then used to translate the values in absorbance into a percentage of antioxidant activity:

Scavenging activity(%)=

$\frac{\text{Absorbance control} - \text{Absorbance of extract}}{\text{Absorbance of control}} \times 100$

Absorbance of control

IC₅₀ values were calculated from Prism GraphPad version 8.0.2. The log dose was determined from the concentrations, 1, 0.5, and 0.25 mg/mL of the extract.

Ferric reducing antioxidant power (FRAP) assay

A modified version of the FRAP method by Oyaizu was employed to assess the reducing (antioxidant) power of *A. scholaris* bark extract (25). Ascorbic acid, used as a standard antioxidant, and the plant extract were made in various concentrations, such as 10, 20, 40, 60, 80, and 100 µg/mL. In each sample, 1 mL of the extract was mixed with 2.5 mL of phosphate buffer (6.6 pH) and 2.5 mL of 10% potassium ferricyanide. The solutions were centrifuged for 10 min at 3000 rpm. For each sample, 2.5 mL of the supernatant was collected and diluted with 2.5 mL of distilled water. Subsequently, 0.5 mL of freshly prepared 0.1% ferric chloride was added and thoroughly mixed. To prepare the blank, 1 mL of distilled water was mixed with 2.5 mL of phosphate buffer and 2.5 mL of potassium ferricyanide. After allowing 10 min to pass, the absorbances were measured at 700 nm against the blank concentration.

Antibacterial activity assay

The antibacterial activity of *A. scholaris* bark extract was assessed using the well diffusion method (26). Five bacterial strains, including Gram-negative bacteria such as *Escherichia coli* (ATCC 10536) and *Salmonella typhi* (ATCC 51812), and Gram-positive species such as *Bacillus cereus* (ATCC 13061), *Enterococcus faecalis* (ATCC 29212) and *Staphylococcus aureus* (ATCC 700698) were employed. A 2% solution of ciprofloxacin, a standard antibiotic, served as a positive reference for antibacterial activity, while 1% dimethyl sulphoxide (DMSO) was used as a negative control. Three concentrations (200, 100 and 50 mg/mL) of the plant extract were prepared. After sterilizing the nutrient agar, 25 mL was poured into each petri dish. A 40 µL volume of different inoculum bacteria was evenly spread over the nutrient agar using a spreader. Wells were created by punching holes with a 6 mm sterile cork borer. In each well, 50 µL of the plant extract of varying concentrations was added, while 0.5 µL ciprofloxacin and 10 µL DMSO were placed in separate wells. The bacteria were allowed to grow for 24 hr at 37°C. The zones of inhibitions were measured, and the data were recorded as means ± standard error of the means. Tukey's multiple comparison test in Prism Graphpad version 8.2.0 was used to compare the means. Significance was considered for *p*-values less than 0.5.

Results

Qualitative phytochemical tests

The most common and important phytochemicals of the methanol extract of *Alstonia scholaris* bark were tested using 11 group tests as shown in Table 1. The chemical analysis showed the presence of alkaloids, carbohydrates, flavonoids, glycosides, phytosterols, saponins, tannins, and reducing sugars. Anthraquinones, protein and amino acids, and gums could not be detected by the specific test employed.

Table 1. Phytochemicals detected from the methanol extract of *A. scholaris* bark.

Phytochemicals	Name of test	Extract indication
Alkaloid	1. Hager's test	-
	2. Wagner's test	-
	3. Mayer's test	-
	4. Dragendorff's test	+
Flavonoid	1. Alkaline test	-
	2. Lead acetate test	+
	3. Shinoda's test	-
Reducing sugar	1. Benedict's test	-
	2. Molisch's test	+
Carbohydrate	1. Benedict's test	+
	2. Iodine test	-
	3. Fehling's test	-
Glycoside	1. Liebermann's test	-
	2. Keller-Kiliani test	+
	3. Legal's test	-
Saponin	1. Foam test	+
Tannin	1. Ferric chloride test	-
	2. Potassium dichromate test	+
	3. Lead acetate test	+
Protein and Amino acid	1. Biuret test	-
	2. Ninhydrin test	-
Phytosterol	1. Salkowski test	+
	2. Liebermann-Burchard test	+
Gums	1. Alcohol test	-
	1. Borntrager's test	-
Anthraquinone	2. Ammonium hydroxide test	-

+ indicates the presence of a compound group; - indicates the absence of a compound group

Total phenolic content

Estimation of the phenolic content of *A. scholaris* bark extract based on Folin-Ciocalteu reaction is shown in Figure 2. From the gallic acid calibration curve, the amount of phenols was found to be 13.56±0.09 GAE mg/g of the dry weight of the sample.

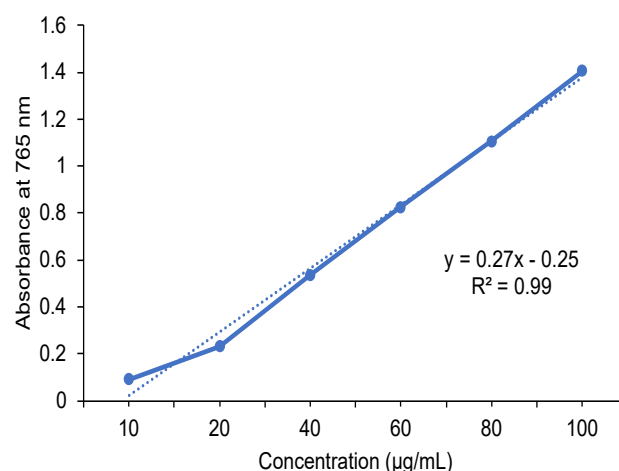


Fig. 2. Calibration curve of gallic acid for total phenol assay. The dotted line represents the linear graph. Value in means ± standard error of means (*n* = 3).

Total flavonoid content

The total flavonoid content of *A. scholaris* bark extract estimated from aluminium chloride reaction is given in Figure 3. From the linear graph of quercetin as a reference, the flavonoid concentration was determined as 31.64±2.50 QE mg/g of the dry extract.

Total antioxidant content

The absorbance at 695 nm of standard ascorbic acid at various concentrations was used to generate a standard graph (Fig. 4). The total antioxidant content calculated from the standard graph was 10.48±0.84 AAE mg/g of the dry extract.

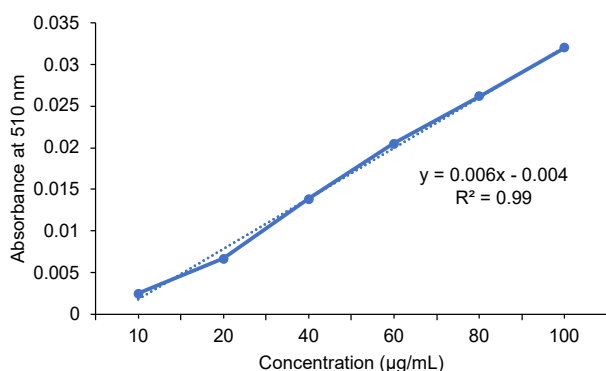


Fig. 3. Standard curve of quercetin for total flavonoid assay. The dotted line represents the linear graph. Value in means \pm standard error of means ($n = 3$).

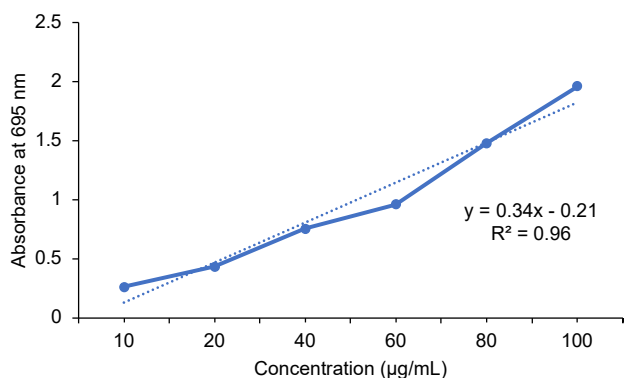


Fig. 4. Standard curve of ascorbic acid for total antioxidant assay. The dotted line represents the linear graph. Values in means \pm standard error of means ($n = 3$).

Antioxidant activity by DPPH free radical scavenging

The DPPH free radical-scavenging activity was used to evaluate the antioxidant capability of *A. scholaris* bark extract. The plant extract indicated a clear concentration-dependent antioxidation reaction, as demonstrated by the graph showing the percentage inhibition of free radicals in Figure 5. At all concentrations tested, the plant extract displayed free radical scavenging activity, albeit slightly less potent than the standard BHT. The log dose analysis revealed that the half-maximal inhibitory concentration (IC_{50}) value for BHT was 5.60, whereas for *A. scholaris*, it was 11.01, indicating good antioxidant properties, albeit with lower effectiveness than BHT (Fig. 6).

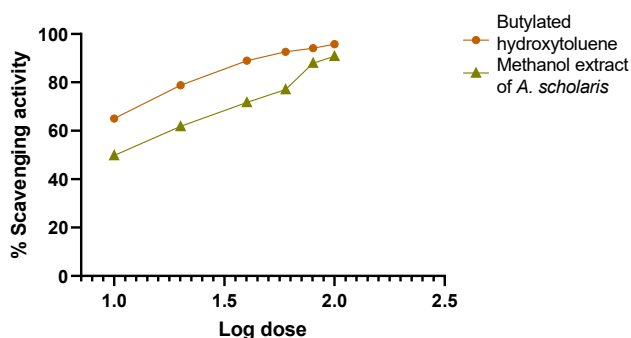


Fig. 5. The percentage inhibition of DPPH by *A. scholaris* bark extract and butylated hydroxytoluene. Data are presented as means \pm standard error of means ($n = 3$).

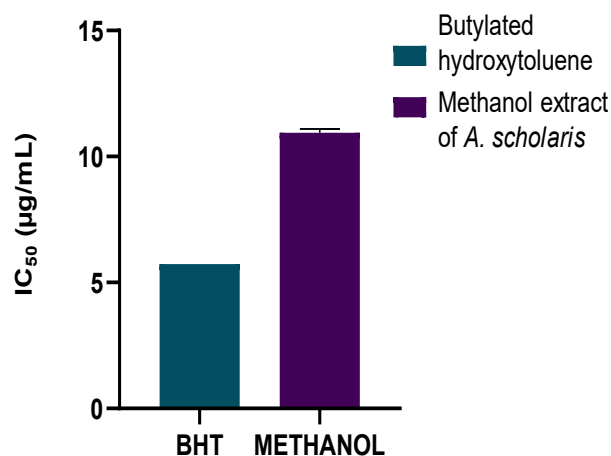


Fig. 6. IC_{50} comparison between *A. scholaris* bark extract and BHT. Data are presented as means \pm standard error of means ($n = 3$).

Ferric reducing antioxidant power assay

The potassium ferricyanide reducing power of *A. scholaris* bark extract is depicted in Figure 7. The plant extract and standard ascorbic acid both showed increasing antioxidant activity corresponding to increased concentrations. However, the plant extract was less effective than ascorbic acid at each concentration tested.

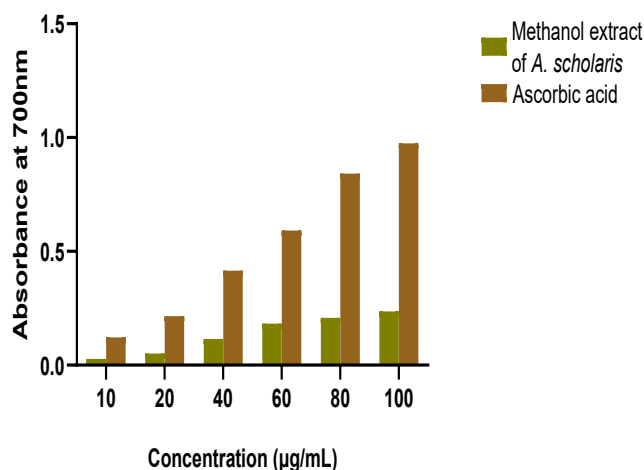


Fig. 7. Ferric reducing antioxidant power of *A. scholaris* bark extract and ascorbic acid. Values are expressed as means \pm standard error of means ($n = 3$).

Antibacterial activity

The antibacterial activity of the methanol extracts of *A. scholaris* bark at 50, 100, and 200 mg/mL and ciprofloxacin is shown in Table 2. All bacteria tested responded well to the antibiotic used. The most notable observation was that the plant extract showed inhibitory action against both Gram-positive and Gram-negative bacteria. The least effectivity was noted on *E. coli*, against which inhibition (0.89 ± 0.25 mm) was only at 50 mg/mL. In terms of inhibition zones, the plant extract was most effective against *S. typhi*. Statistical comparison between the different concentration of the plant extract and ciprofloxacin is given in Figure 8.

Table 2. Inhibition zones of *A. scholaris* bark extract and ciprofloxacin against different bacteria.

Bacteria	Gram stain	<i>A. scholaris</i> bark extract (mg/mL)			Ciprofloxacin
		50	100	200	20 µg/mL
<i>Escherichia coli</i>	Negative	0.89±0.25	-	-	33.16±0.25
<i>Salmonella typhi</i>	Negative	4.40±0.25	7.20±0.76	-	25.43±0.40
<i>Bacillus cereus</i>	Positive	2.10±0.99	2.60±1.00	1.02±0.01	31.90±0.16
<i>Enterococcus faecalis</i>	Positive	3.71±0.60	2.55±0.28	1.84±0.62	15.43±0.00
<i>Staphylococcus aureus</i>	Positive	1.41±0.53	3.76±0.31	-	23.04±0.12

± shows standard errors of means of three replicates

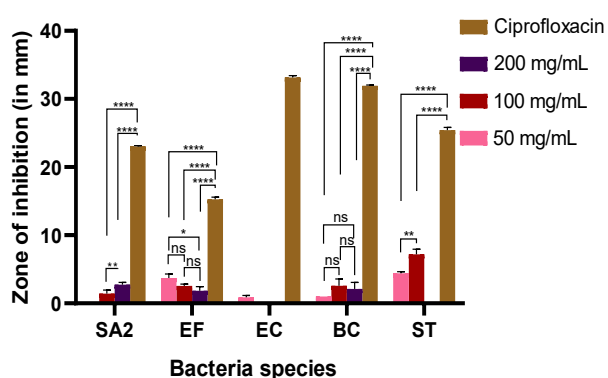


Fig. 8. Comparison of the antibacterial activity of *A. scholaris* bark extract (ASE) at different concentrations and ciprofloxacin (standard) against *Staphylococcus aureus* (SA2), *Enterococcus faecalis* (EF), *Escherichia coli* (EC), *Bacillus cereus* (BC), and *Salmonella typhi* (ST). Values are in means ± standard error of means (n = 3); **** $p \leq 0.0001$, *** $p \leq 0.002$, ns = not significant.

Discussion

Plants, constituting our primary diet, serve as the direct and principal sources of nutraceuticals, fulfilling essential nutritional requirements and therapeutic needs. Their chemical constituents encompass molecules vital for normal cellular metabolisms, offering external sources to sustain cellular activities, especially those crucial in various cellular disorders (27, 28). Our study revealed the presence of biologically important phytochemicals, including alkaloids, carbohydrates, flavonoids, glycosides, phytosterols, saponins, tannins, and reducing sugars present in the methanol extract of *Alstonia scholaris* bark. These diverse groups of compounds, notably phenols, alkaloids, flavonoids, saponins, steroids, and tannins, are plant-derived elements from which various medicinal molecules, including pharmaceutical drugs and nutritional supplements, have been isolated and produced (29, 30). Consequently, our data adds *A. scholaris* to the list of medicinal plants, established it as a source of important bioactive molecules and nutraceutical compounds.

Free radicals or reactive oxygen species (ROS) are normal biochemical products from cellular metabolic reactions. However, they become problematic when they accumulate in the cells, transforming into highly toxic substances that cause damages to fundamental cellular components such as DNA, RNA, proteins, and lipids (31). These cumulative damages can lead to the development of chronic diseases such as cancer, diabetes, and heart diseases (32). Balancing these effects is crucial, and external antioxidants play a significant role. Plants,

forming a major part of our diet, serve as the primary source of these antioxidants (33). In our study, we observed appreciable amounts of phenols, flavonoids and total antioxidants in *A. scholaris*, indicating the plant's overall antioxidant value. Reliable tests for evaluating antioxidant activity include the DPPH and FRAP assays (34, 35). Our finding demonstrated that the bark extract of *A. scholaris* exhibited strong antioxidant activity in both DPPH and FRAP assays.

The development and spread of drug resistance in microbes have spurred research into new and alternative medications, with plants emerging as one of the most promising sources of leads (36). While many plants and their secondary metabolites have been investigated as potential antibacterial agents, the translation of this research into medical application has not yet reached a significant level (37). Most plants, even if they exhibit antibacterial activity, often show limited prospects due to their poor and narrow effectiveness against the most important bacteria (38). Our data indicate that *A. scholaris* possesses broad-spectrum antibacterial activity against both Gram-negative and Gram-positive species. The lack of activity or diminished effectiveness of plant extracts or any drug at higher concentrations, particularly at 200 mg/mL in our study, can be attributed to the phenomenon of hormesis or the ceiling effect commonly encountered in microbes, where they become unresponsiveness beyond certain threshold doses (39, 40). Therefore, the general susceptibility trend observed in different bacteria suggests that *A. scholaris* holds promise as a source of antibacterial molecule.

Conclusion

The bark extract of *Alstonia scholaris* was found to contain important bioactive compounds, including alkaloids, carbohydrates, flavonoids, glycosides, phytosterols, saponins, tannins, and reducing sugars. The presence of these compounds indicates that the plant possesses natural molecules with health benefits. The plant extract was determined to be a source of antioxidant, as evidenced by the phenolic, flavonoid and total antioxidant assays. Its ability to scavenge free radicals was validated through the DPPH and FRAP assays. An additional interesting observation was its broad-spectrum antibacterial activity against both Gram-negative and Gram-positive species. Therefore, this study highlights the need for further investigations into the possible bioactive principle and their exact biological actions.

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Authors' contributions

KLC and LND designed the concept. LNM, LBT, LLP and PBL carried out the experimental works. LNM, LND and PBL prepared the first draft. KLC finalised data interpretation and the manuscript. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: The authors do not have any conflict of interests to declare.

Ethical issues: None.

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