

Antimicrobial and Phytochemical Properties of *Alstonia Boonei* Extracts

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Abstract

Alstonia boonei De wild is a major spice crop grown abundantly in Ghana and other tropical countries. In this study we have carried out phytochemical analysis and antimicrobial investigation of ethanol and aqueous extracts of the root of *Alstonia boonei* against a panel of clinically significant bacterial and fungal strains. Four Gram positive and Gram negative bacteria namely *Escherichia coli*, *Bacillus subtilis*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* were subjected to test the antimicrobial activity along with one fungi namely, *Candida albicans*. The ethanol and aqueous extracts of root of *Alstonia boonei* were subjected to microbial susceptibility assays using agar well diffusion method. Phytochemical screening was done to test the presence of phytochemicals responsible for the antimicrobial potential of roots of *Alstonia boonei*. The results of the phytochemical studies revealed the presence alkaloids, cyanogenetic glycosides, flavonoids, terpenoids and steroids and saponins. Susceptibility testing by disc diffusion assay revealed significant antimicrobial activity of methanol and aqueous extracts of the roots against the pathogens tested. The minimum inhibitory concentrations (MIC) of the various extracts by Agar Dilution method ranged from 3.0 to 10.0 mg/ml. The ethanol extracts exhibited better antimicrobial activity than aqueous extract. The study findings provide supportive evidence for the use of *Alstonia boonei* in traditional medicines.

Keywords: *Alstonia boonei*; Antimicrobial activity; Agar well diffusion; Phytochemical

Introduction

Medicinal plant is defined as any plant with one or more of its organs containing substance that can be used for therapeutic purpose or which can be used as precursors for the synthesis of antimicrobial drugs [1]. Plants are presently the sources of medicines for many people of different age in many country of the world, where diseases are treated primarily with traditional medicines obtained from plants. The modern pharmaceutical industry itself still relies largely on the diversity of secondary metabolites in plants and secondary metabolites of which at least 12,000 have been isolated; a number estimated to be less than 10% of the total [2]. In plants, the synthesized aromatic substances (metabolites) are used as defensive weapons against predation by microorganisms, insects and herbivores.

Alstonia boonei, a large evergreen tree belonging to the family Apocynaceae is one of the widely used medicinal plants in Africa and beyond. The important plants of genus *Alstonia* includes *Alstonia scholaris*, *Alstonia boonei*, *Alstonia congensis* and *Alstonia macrophylla* which have proved to be useful in various diseases. Almost all plant parts viz. leaves, stem bark; root and inflorescences have been used and are further under investigative study. It is distributed throughout the tropics and the rain forest of west and Central Africa [3,4]. It is known by different names in different cultures and tribal settings. It is not edible as food but possess roots, stems, barks, leaves fruits, seeds, flowers, and latex which are claimed to have medicinal properties in some cultures.

Various pharmacological studies have been carried out on this plant products which showed that the extracts possess antimalarial, antipyretic, analgesic and anti-inflammatory properties [4,5], anthelmintic [6], diuretic, spasmolytic and hypotensive properties [7], Immuno-stimulant property [8], antipsychotic and anxiolytic effect [9], reversible antifertility effect [10].

The stem bark of *A. boonei* is used in traditional medicine to treat fever, painful micturition, insomnia, malaria and chronic diarrhea, rheumatic pains, as anti-venom for snake bites and in the treatment of

arrow poisoning [11]. It is also used in treating painful micturition and rheumatic conditions [11].

An infusion of the root and stem bark was taken as a remedy for asthma liquid made from the stem bark and leaves is drunk to treat impotence. In Ghana, it is given to assuage toothache and, after child delivery, to aid in expelling the placenta.

In Africa and elsewhere, plant extracts are still widely used in the treatment of malaria and other ailments, and up to 80% of the African population use traditional medicines for primary health care [12]. Since little scientific data exist to validate antimicrobial properties of medicinal plants, it is important that their claimed antimicrobial properties are investigated, in order to establish their efficacy and determine their potential as sources of new antimicrobial drugs.

In this study, the phytochemical and antimicrobial activity of extract fractions of *A. boonei* was carried out with a view to provide scientific basis on the claim by traditional healers of its uses in traditional medicine in the context of the continued search for active therapeutic agents from plants.

Materials and Methods

Collection of plant material

The plant material was collected from a farm at Mfensi in the Atwima Nwabiagya south district of Ashanti region, Ghana. The plants were authenticated at the Department of Biology. The samples were

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washed thoroughly to remove dirt particles present on the surface. The samples were then sun dried and cut into small pieces. The sample was then milled at the Department of Pharmacy, Kwame Nkrumah University of Science and technology, Kumasi.

Preparation of methanol extract

Twenty five grams of the material was soaked in 100 ml of methanol and allowed to stand for 24 hrs followed by boiling until the volume was reduced to one-third. The crude extracts were obtained by filtration and stored in a refrigerator at 4°C [13].

Preparation of aqueous extract

Extracts were prepared using the modified method of Case [14]. 50 g of pulverized root material was infused in 100 ml of hot (~95°C) distilled water and left overnight under refrigeration (4°C). After 24 h, the extracts were kept in rotary shaker at 100 rpm for 1h, filtered with Whatman No.1 filter paper and subsequently lyophilized at -47.5°C. The frozen extract was then freeze-dried to a powder and stored at 4°C until further use.

Antimicrobial test

Preparation of micro-organism: The organisms used in this study were *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *candida albicans* and *Bacillus subtilis*. The strains were maintained on nutrient agar slants at 40°C. A loopful of each bacterial strain was inoculated into 50 ml of sterile nutrient broth in 100 ml conical flask. The flask was incubated on a rotary shaker for 24 hr to activate the strain. Mueller Hinton Agar medium was used as bacterial culture medium in the antibacterial assay.

Testing for antimicrobial activity: Antibacterial activity of ethanol and aqueous root extract were tested using agar well diffusion method. 200 µl of bacteria were aseptically introduced and spread using cotton swabs on surface of gelled sterile Muller Hilton agar plates. A well of about 6.0 mm diameter with sterile cork borer was aseptically punched on each agar plate. 50 µl of the extracts were introduced into the wells in the plates. A negative control well was too made with 50 µl of the extracting solvent (DMSO). A positive control was made by placing antibiotic disc (Ciprofloxacin for bacteria) on agar plate. Plates were kept in laminar flow for 30 minutes for pre diffusion of extract to occur and then incubated at 37°C for 24 hours. The diameter of the zone of inhibition around each well was taken as a measure of antibacterial activity.

Minimum Inhibitory Concentration (MIC): The MIC was defined as the lowest concentration that completely inhibited the growth of microorganisms for 24 hours. The MIC of the extracts was done using the agar well diffusion technique. Two fold dilution series of each extract was prepared to achieve a decreasing concentration range of 2.5 to 10 mg/ml. A 0.5 ml volume of each solution was added aseptically into the wells of Mueller Hinton agar plates that were already inoculated with standardized bacterial isolates (106 cfu/ml). The plates were incubated at 37°C for 24/hours. MIC was calculated as the lowest concentration at which a clear zone of inhibition was observed.

Screening of phytochemicals: The phytochemical screening of the sample was carried out as described by Swadhini et al. [15] and Pathak et al. [16]. The samples were screened for the following components.

Test for saponins: To 1 ml of plant extract, 5-10 ml of distilled water was added and shaken in a graduated cylinder for 15 minutes

lengthwise. Formation of 1cm layer of foam indicates the presence of saponins.

Test for flavonoids: A fraction of the extract was treated with concentrated sulphuric acid and observed for the formation of orange color.

Test for alkaloids: To 2 ml of plant extract, 2 ml of concentrated hydrochloric acid was added. Then few drops of Mayer's reagent were added. Presence of green color or white precipitate indicates alkaloids.

Test for glycosides: To 2 ml of plant extract, 1 ml of glacial acetic acid and 5 % ferric chloride was added. Then few drops of concentrated sulphuric acid were added. Presence of greenish blue color indicates glycosides.

Test for terpenoids and steroids: A fraction of the extract was dissolved in chloroform. A few drops of acetic anhydride was added followed by two drops of conc. H₂SO₄. Reddish –pink colouration indicates terpenoids and steroids.

Test for carotenoids: To about 2ml of the extract, 3ml of antimony trichloride was added. Dark-blue colouration is indicative of carotenoids.

Test for coumarins: A small quantity of plant extract was taken into a test-tube. The test-tube was then covered with a piece of filter paper moistened with dil. NaOH solution and placed in a hot water bath. After about 15 minutes, the paper was removed and exposed to U.V light. Yellow-green fluorescence indicates the presence of coumarins.

Test for anthraquinones: A small amount of the extract was boiled with 25 ml of 0.5M KOH and 4 ml of H₂O₂. The mixture was then cooled and acidifies with a few drops of acetic acid. The acidulated mixture was extracted with a small amount of benzene (15ml). The benzene extract was shaken with a small amount of NH₄OH. Red colouration indicates anthraquinones. Colourless alkaline layer indicates absence of anthraquinones.

Test for anthraquinone glycosides: To about 2ml of the extract, 20 ml of dilute H₂SO₄ was added and boiled. The mixture was filtered hot and a portion of the cooled filtrate was shaken with an equal volume of benzene. The benzene layer was separated and shaken with about half its volume of dilute NH₃ solution. A colourless ammoniacal layer indicates the absence of anthraquinone glycosides.

Test for cyanogenetic glycosides: To about 2ml of the extract was taken into a test-tube. A few drops of chloroform was then added and a piece of moist sodium picrate paper was inserted into the test-tube, taking care that it does not come into contact is kept warmed at 35°C for about 3 hours. The presence of red colour of the sodium picrate paper after the 3 hours indicates cyanogenetic glycosides.

Results and Discussion

The main goal of this work was to investigate the potential antimicrobial properties of *Alstonia boonei* used in traditional medicine, mostly in Ghana, against malaria and/ or fever, and providing scientific validation for their use. Two extracts from the plants species, was screened for their potential antibacterial properties against *Staphylococcus aureus*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Candida albicans*. The extracts were prepared by sequentially extracting the plant material with water and ethanol.

Phytochemical constituents such as alkaloids, flavonoids, tannins, phenols, saponins, and several other aromatic compounds are

secondary metabolites of plants that serve a defence mechanism against predation by many microorganisms, insects and other herbivores [17]. The present study carried out on the plant samples revealed the presence of medicinally active constituents. The phytochemical constituents of the selected plants investigated are summarized in (Table 1).

The preliminary phytochemical screening of the root extract of *A. boonei* revealed the presence of medicinally active constituents such as alkaloids, cyanogenetic glycosides, flavonoids, terpenoids and steroids and saponins (Table 1) some of which have been previously associated with antibacterial activity [18]. These biologically active constituents are known to act by different mechanism and exert antimicrobial action [19]. Alkaloids are medicinally useful, possessing analgesic, antispasmodic and bactericidal effects. Flavonoids are hydroxylated phenolic substance known to be synthesized by plants in response to microbial infection and it should not be surprising that they have been found *in vitro* to be effective antimicrobial substances against a wide array of microorganisms. Their activity is probably due to their ability to complex with extracellular and soluble proteins and to complex with bacterial cell walls [20]. Coumarins are also known act against gram positive bacteria and it is produced in carrots in response to fungal infection which could be attributed to its antimicrobial activity [21]. Antimicrobial property of saponin is due to its ability to cause leakage of proteins and certain enzymes from the cell [22]. Steroids have been reported to have antibacterial properties, the correlation between membrane lipids and sensitivity for steroidal compound indicates the mechanism in which steroids specifically associate with membrane lipid and exerts its action by causing leakages from liposomes [23].

Antimicrobial activity of ethanol and aqueous extracts of roots of *A. boonei* were analysed against five clinically significant organisms using agar well diffusion method (Tables 2 and 3). MIC values were determined for ethanol, and aqueous extracts (Table 4). Both extracts tested showed varying degree of antibacterial activities at a concentration of 100 µg/disc which were compared with negative control DMSO that showed no activity with any of the extracts. The activity of ethanol extracts (inhibition zone 5-10 mm) were found to be more pronounced than the aqueous extract (inhibition zone 5-8 mm) against all the organisms tested. The highest inhibition was observed in ethanol over *S. aureus* (10 mm) and the least by aqueous on *B. subtilis* (3 mm). The results were highly significant for all pathogens. An overall agreement was seen between the zone levels and MIC values.

In comparison the aqueous extract showed less pronounced antimicrobial activity. The poor activity of the aqueous extract against most bacterial strains investigated in this study is in agreement with previous reports [24,25]. This could be due to the insolubility of the active compounds in water or the hot water could have caused denaturation of the active compounds. It is also observed from the results that the ethanol extract of root had wide antibacterial activity against both gram positive and gram negative (Table 4).

Growth of *Candida* was inhibited at a MIC value of 0.39 mg/ml followed by *S. aureus*, *E. coli*, and *P. aeruginosa*, and *B. subtilis* showed highest MIC value of 4.42 mg/ml for the ethanol extract. Antifungal activities against *Candida albican* was shown in disc diffusion testing (5-9 mm) but MIC values were found to be >3 mg/ml. The aqueous extract gave a MIC value of 3.12 mg/ml for *P. aeruginosa*, *S. aureus* (3.64 mg/ml), *B. subtilis* (8.84 mg/ml), *Candida* (2.68 mg/ml), and *E. coli* (3.12 g/ml). Both extracts showed appreciable antimicrobial activity against some of the strains tested. Ethanol extracts showed better antimicrobial activity compared to aqueous extracts. The results of this study reflect the potent antimicrobial phytochemicals present

in solvent extracts of the roots of the plant. The ability of the extracts to inhibit the growth of several bacterial species is an indication of the broad spectrum antimicrobial potential of *A. boonei* which makes the plant a candidate for bio-prospecting for antimicrobial drugs. The antimicrobial activities of plant extracts could be attributed to the presence of different bioactive compounds [24]. The wider zones of inhibition of ethanol extracts of *A. boonei* (Table 2) exhibit a better antimicrobial activity compared to aqueous extracts at the tested concentration.

Conclusion

The result of antimicrobial susceptibility assay showed promising evidence for the antimicrobial effects of *A. boonei* against bacterial (*B. subtilis*, *S. aureus*, *P. aeruginosa*, *E. coli*) and fungal (*C. albicans*) pathogens. Ethanolic extract showed maximum inhibition 10 mm against *S. aureus* than other aqueous extract. Thus MICs assay are capable of verifying that the compound has antimicrobial activity and that it gives reliable indication of the concentration of medicine required to inhibit the growth of microorganisms. Phytochemical analysis are responsible for the identification of components which are responsible for antimicrobial activity of plant, thus these traditional species can be used as a potential source of medicine against various diseases.

Secondary Metabolite	Water extract	Ethanol extract
Saponins	-	+
General Glycosides	+	+
Flavonoids	+	+
Terpenoids and steroids	+	+
Carotenoids	-	-
Coumarins	-	-
Alkaloids	+	+
Antraquinones	-	-
Antraquinones Glycosides	-	-
Cyanogenetic Glycosides	-	-

Table 1: Phytochemical analysis of ethanolic and aqueous root extracts of *A. boonei*

	10 mg/ml	5 mg/ml	2.5 mg/ml	Ciprofloxacin
<i>Staphylococcus aureus</i>	10	9	8	40
<i>Bacillus subtilis</i>	8	7	6	23
<i>Candida</i>	9	7	5	N.A.
<i>Pseudomonas aeruginosa</i>	8	6	5	35
<i>Escherichia coli</i>	8	7	5	37

Table 2: Antimicrobial activity of ethanol root extract of *Alstonia boonei*

	10 mg/ml	5 mg/ml	2.5 mg/ml	Ciprofloxacin
<i>Staphylococcus aureus</i>	7	6	4	40
<i>Bacillus subtilis</i>	7	5	3	23
<i>Candida</i>	8	6	5	N.A.
<i>Pseudomonas aeruginosa</i>	5	4	7	35
<i>Escherichia coli</i>	5	4	7	37

Table 3: Antimicrobial activity of aqueous root extract of *Alstonia boonei*

	Ethanol extract (mg/ml)	Aqueous extract (mg/ml)
<i>Staphylococcus aureus</i>	0.98	3.64
<i>Bacillus subtilis</i>	4.42	8.84
<i>Candida</i>	0.39	2.68
<i>Pseudomonas aeruginosa</i>	2.68	3.12
<i>Escherichia coli</i>	2.29	3.12

Table 4: Minimum Inhibitory Concentration (MIC) of root extract of *A. boonei*

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