



## Phytochemical Composition, In vitro Antioxidant and Antimicrobial Activities of Methanol extract of Cheese Wood (*Alstonia boonei*) Leaves collected from Benin City, Nigeria

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**ABSTRACT:** The Cheese wood (*Alstonia boonei*) tree, known as Egbu-ora in Igbo, Ahun in Yoruba, Ukpukunu in Urhobo and Ukhu in Edo, is a widely distributed plant in the lowlands and rain-forest areas of Nigeria. It has been listed in the African Pharmacopoeia as an antimalarial drug and a traditional medicine for the treatment of fever, painful micturition, insomnia, chronic diarrhoea, and rheumatic pains as antivenom for snake bites and in the treatment of arrow poisoning. This work aims to investigate the phytochemical composition, *in vitro* antioxidant, and antimicrobial activities of methanol extract of cheese Wood (*Alstonia boonei*) leaves collected from Benin City, Nigeria. This study investigated the phytochemical composition, antioxidants, and antimicrobial activities of methanol extract of the *Alstonia boonei* leaves using standard methods. The phytochemicals present in *Alstonia boonei* are flavonoids, phenols, saponins, terpenoids, tannins, alkaloids, proanthocyanidins, glycosides, steroids and phytosterols, the *in-vitro* antioxidant was accessed using DPPH radical scavenging assay, reducing power assay, ferric reducing antioxidant potential and Nitric Oxide inhibition. The antimicrobial activity was evaluated using the disk diffusion method. The total phenolic, proanthocyanidin, flavonoids and tannins were determined using standard methods. The methanolic extract of *A. boonei* was shown to be highest in total phenol, flavonoid, and tannin and showed moderate consideration in proanthocyanidins. The methanolic leaves extract of *A. boonei* has 50% inhibition concentration IC<sub>50</sub> values for the radical scavenging activity of (64.47µg/mL), significantly higher than that of ascorbic acid (8.10 µg/mL). The reducing power assay showed that the standard ascorbic acid has better-reducing power when compared with *A. boonei*. However, *A. boonei* showed a marked increase at higher concentrations of (800 and 1000 µg/mL), and it also showed that *A. boonei* is a better nitric oxide inhibitor when compared with the standard gallic acid. The antimicrobial assay showed that *A. boonei* has better antifungal activity when compared to its antibacterial activity.

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In recent years, the utilization of natural plant resources for therapeutic and medicinal purposes has garnered significant attention owing to their rich bioactive constituents. Among these, *Alstonia boonei*, a well-known plant in traditional medicine systems, has attracted interest due to its potential health benefits (Alamed *et al.*, 2009). The phytochemical composition of plant extracts is of paramount importance, as it

directly influences their medicinal properties. The leaves of *Alstonia boonei*, a tree native to various parts of Africa, have been used traditionally to treat a range of ailments (Olajide *et al.*, 2000). Researchers and scientists have extensively studied the phytochemical constituents of this plant, as well as its *in vitro* antioxidant and antimicrobial activities, to unearth its potential applications in modern medicine.

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Phytochemicals, which include alkaloids, flavonoids, terpenoids, and phenolic compounds, are natural bioactive compounds present in various plant species. These compounds have been linked to a myriad of health benefits, including antioxidants and antimicrobial properties (Scalbert *et al.*, 2005). *Alstonia boonei*, commonly referred to as "God's Tree," is rich in these phytochemicals, making it a valuable resource for the development of therapeutic agents (Kris-Etherton *et al.*, 2002). Antioxidants play a vital role in protecting the body from oxidative stress caused by free radicals, which is implicated in various chronic diseases such as cardiovascular disorders, cancer, and neurodegenerative conditions. The *in vitro* antioxidant potential of *Alstonia boonei* leaves' methanol extract provides insights into the plant's ability to neutralize harmful free radicals. This property not only contributes to its traditional use as a remedy but also sparks curiosity for its potential application in modern medicine. Free radicals are inevitably produced in biological systems and also encountered exogenously when in excess, they have damaging effects on cells. (Das *et al.*, 2014; Kedare and Singh, 2014). Furthermore, the antimicrobial activities of plant extracts are of immense importance in combating microbial infections. *Alstonia boonei*'s leaves have been investigated for their ability to inhibit the growth of various pathogenic microorganisms. With the rise of antibiotic resistance, natural products like those derived from *Alstonia boonei* could offer a promising source for the development of new antimicrobial agents.

In conclusion, *Alstonia boonei* leaves hold promise as a valuable source of phytochemicals with potential antioxidant and antimicrobial activities. By elucidating the composition and effects of its methanol extract, this research contributes to the understanding of its therapeutic potential and opens doors for the development of novel natural remedies. As we continue to bridge the gap between traditional knowledge and modern scientific investigation, *Alstonia boonei* emerges as a captivating subject for further exploration in the realm of natural medicine. Hence, this study investigated the phytochemical composition, in vitro antioxidant, and antimicrobial activities of methanol extract of cheese Wood (*Alstonia boonei*) leaves collected from Benin City, Nigeria

## MATERIALS AND METHODS

**Plant materials:** The leaves of *Alstonia boonei* used in this study were collected from several locations within Nigeria. The leaves were identified by a Botanist in the Department of Plant Biology and Biotechnology of the University of Benin, Benin City, Nigeria.

**Preparation of Extract:** The leaves of *Alstonia boonei* were air-dried for 3 days, they were then pulverized to their powdered form and stored in an air-tight container. The sample was then weighed using a sensitive weighing balance. A fraction of *Alstonia boonei* leaves powder (600 g) was extracted in 2000 ml of 70% methanol at room temperature for 72 hours. The solution was filtered using Whatman No. 1 filter paper. The resulting solution was then concentrated using a rotary evaporator. The extract obtained was used for phytochemical analysis, antimicrobial assay, and antioxidant activity.

**Chemicals and Reagents:** Methanol, 1,1-Diphenyl 1-2-picrylhydrazyl (DPPH), Chloroform, Gallic acid, Folin Ciocalteu, phenol reagent and potassium acetate were products of Sigma-Aldrich Chemical Company Ltd (St. Louis, U.S.A) while ascorbic acid, aluminium chloride hydrate and ethanol were from JDH; China.

**Qualitative determination:** The qualitative screening was carried out using established protocols as described by Sofowora (Sofowora, 1993), and Trease and Evans (1989)

**Total Phenolic Content:** The phenolic content of methanol extract of *Alstonia boonei* leaves was determined by the method described by Roy *et al.*, (2018). The phenolic content was estimated using Folin-Ciocalteu reagent (FCR) method. Firstly, FCR was prepared by adding 10 ml of FCR to 90 ml of distilled water. Then 5% sodium carbonate was prepared by dissolving 5 g of sodium carbonate in 100 ml of distilled water. In a test tube, 200 µl of extract/standard was added to 1.5 ml of the prepared FCR reagent and incubated for 5 mins in the dark at room temperature. After which 1.5 ml of the prepared sodium carbonate was added and mixed properly. This was again incubated for 2 hrs in the dark and the absorbance read at 750nm. The standard curve was plotted using 1mg/ml Gallic acid dissolved in methanol at different concentrations of 5, 10, 25, 50, 75, 100, 150 and 200 µg/ml. The phenolic content was expressed as milligrams Gallic acid equivalent (GAE) per gram of dried extracts.

**Total Flavonoid Content:** The flavonoid content of methanol extract of *Alstonia boonei* leaves was determined by the modified method described by Roy *et al.*, (2018). The flavonoid content was determined using the aluminium chloride colorimetric method. Sodium nitrite (5%) was first prepared by dissolving 5g of sodium nitrite in 100 ml of distilled water. The aluminium chloride (10%) was then prepared by adding 10 ml of aluminum chloride to 90 ml of

distilled water. Quercetin (1mg/ml) was used as standard and was prepared at different concentrations of 10, 50, 75, 100, 150, 200, 250 and 300 µg/ml. To 1 ml of extract/standard, 0.3 ml of 5% sodium nitrite was added and the solution incubated for 5 mins at room temp. After which 0.3 ml of 10% aluminium chloride was added. This was again incubated for 5 mins at room temperature. Two millilitres of 1M NaOH was then added and thereafter, incubated for 10 mins at room temperature. The absorbance was read at 510nm. The total flavonoid content obtained was expressed in terms of mg of Quercetin equivalent per gram of extract.

**Total Tannin Content:** The tannin content of extracts *Alstonia bonnei* leaves was determined by the modified method described by Roy *et al.*, (2018). The tannin content was estimated by Folin-Ciocalteu method. 1mg/ml gallic acid was prepared at different concentrations of 10, 50, 100, 200, 300, 400, 500 and 600 µg/ml. In a test tube, 100 µl of extract/standard was added to 7.5 ml distilled water, followed by 0.5 ml of the 10% FCR and 1ml of the 35% Sodium carbonate salt. This was mixed well and incubated for 10 mins at room temperature. The absorbance of the extract and Gallic acid standards were read at 725 nm. The tannin content obtained was expressed as milligram of Gallic acid equivalent (GAE) per gram of extract.

**Total Alkaloid Content:** The total alkaloid content of *Alstonia bonnei* extract leaves was determined by the modified method described by Ajanal *et al.* (2012). Atropine standard solution (0.5 mg/ml) was prepared in distilled water at different concentrations of 10, 50, 100, 200, 300, 400 and 500 µg/ml. For the plant extract, 100 mg of the extract was first dissolved in 10 ml of 2N HCl and filtered to extract the alkaloid. Then 1ml of the filtrate and 1ml of standard (at different concentrations) were added to 5 ml of phosphate buffer and 5 ml of bromocresol green solution in a separating funnel. This was well mixed, and 4 ml of chloroform added and shaken vigorously. A BCG-Chloroform complex was formed, and the chloroform fraction collected into a test tube. The absorbance was read at 470nm. The total alkaloid content was expressed as mg atropine equivalent per gram of extract (mgAE/g).

**Total Proanthocyanidin Content:** Determination of proanthocyanidin was based on the procedure defined by Sun *et al.* (1998). A volume of 0.5 ml of 1 mg/ml of extract of *Alstonia bonnei* was mixed with 1ml of 4% vanillin methanol solution and 0.75 ml hydrochloric acid. The mixture was left undisturbed for 15 mins after which the absorbance was read at 500

nm. The concentrations used were 10, 25, 30, 75, 100 and 150 µg/ml. The total proanthocyanidin content was expressed as mg ascorbic acid equivalent per gram of extract (mgAA/g).

**DPPH Radical Scavenging Assay:** DPPH scavenging activity was determined by the modified method described by Roy *et al.* (2018). DPPH (2, 2-diphenyl-1-picrylhydrazyl) is a molecule with free radical. In the presence of an antioxidant which donates an electron to DPPH, the purple colour of the DPPH radical turns yellow. The hydrogen atom donating ability of the plant extract is determined by the decolourisation of the DPPH solution from purple to yellow, and the change in absorbance measured at 517nm. The extract/standard ascorbic acid stock solutions (1 mg/ml) were prepared into different concentrations of 10, 25, 50, 100, 200, 300, 400 and 500 µg/ml. The DPPH solution was prepared by dissolving 0.3 mM DPPH in methanol. 1 ml of the extract or standard was added at different concentrations to 2 ml of the prepared 0.3 mM DPPH solution. These were shaken vigorously and incubated for 30 mins in darkness. Absorbance was then read at 517nm. The assays were performed in triplicates.

**Nitric Oxide Radical Scavenging Assay:** Nitric oxide radical scavenging activity was determined by the slightly modified method described by Boora *et al.* (2014). The procedure is based on the principle that sodium nitroprusside in aqueous solution generates nitric oxide which reacts with oxygen to produce nitrite ions (radicals) that can be measured using Griess reagents. The antioxidants in the extracts then donate protons to the nitrite ions. 1mg/ml of the extract and standards (Gallic acid) were prepared and serially diluted with distilled water into different concentrations of 12.5, 25, 50, 100, 200, 400, 800 and 1000 µg/ml. 0.1% Griess reagent and 10 mM sodium nitroprusside were also freshly prepared in distilled water immediately before use. To 1 ml of the extract or standard at different concentrations were added 0.5 ml of the freshly prepared sodium nitroprusside. This was then incubated for 2 hrs in the dark at room temperature. After incubation, 1.5 ml of the freshly prepared Griess reagent was added and again incubated for 30min at room temperature. The absorbance was read at 546nm.

**Ferric Reducing Antioxidant Power Assay:** The assay is based on the ability to reduce ferric ions ( $Fe^{3+}$ ) to ferrous ions ( $Fe^{2+}$ ) according to the method of Benzie and Strain (1996). The methanol extract of *Alstonia boonei* leaves and the standard (ascorbic acid) with concentrations of 1 mg/ml were serially diluted into 12.5, 25, 50, 100, 200, 400, 800 and 1000 µg/ml. Frap

reagent was prepared by mixing 300 mM Sodium acetate buffer (pH 3.6), 10mM TPTZ (Tripyridyl triazine) solution and 20 mM FeCl<sub>3</sub>.6H<sub>2</sub>O solution in a ratio 10:1:1. To 1 ml of the serially diluted extract and standard was added 3 ml of Frap reagent. The reaction mixture was then incubated for 30 mins at 37°C. The increase in absorbance was then measured at 593nm.

**Minimum inhibitory concentration:** Minimum inhibitory concentration (MIC) is defined as the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation. (Andrews, 2001). Minimum inhibitory concentration (MIC) is the lowest concentration (expressed as mg/L) of an antimicrobial that inhibits the visible growth of microorganisms *in vitro*. The MIC test is used to determine the antimicrobial activity of specific bacterial and fungi species. Agar disk diffusion is one of many methods used to determine MIC values. The antimicrobial's lowest concentration (highest diffusion) preventing the appearance of turbidity (growth) is considered MIC. At this dilution the antimicrobial is bacteriostatic.

**The Disk Diffusion Method:** The disk diffusion method (DDM) also known as an agar diffusion method (ADM). The methanol extract in the agar diffuses from its reservoir through the agar medium seeded with the test microorganism. Generally, the reservoir is a filter paper disc, which is placed on top of an agar surface. If the plant extract is active, an inhibition zone develops around the filter paper disk after incubation. The diameter of the inhibition zone properly describes the antimicrobial potency of the plant extract. The assay is used to determine the susceptibility of isolated microorganisms (bacteria and fungi).

The experiment is performed by inoculating the agar surface in the agar plates with a standard inoculum of the test microorganisms (bacteria and fungi), the filter paper disc (6mm in diameter) containing the test compound (plant extract) at a desired concentration are placed on the agar surface. To obtain uniform growth the agar plate is streaked again in one direction rotated 120° and streaked again, rotated another 120° degree and streaked again. The petri dishes are incubated under suitable conditions.

Generally antimicrobial agents diffuse into the agar and inhibit germination and growth of the test microorganisms and then the diameter of the zone of inhibition growth are measured. The zone of inhibition is a disc shaped area on all sides of the spot of antibiotic in which the bacteria and fungi colonies do not grow.

## RESULTS AND DISCUSSION

The exploration of natural compounds for their potential therapeutic properties has gained significant attention in recent years, with plant extracts being a promising source. *Alstonia boonei* is a medicinal plant native to tropical Africa that has been used in traditional medicine to treat a variety of diseases such as malaria, diarrhoea, fever, coughs, and rheumatism. The methanolic extract of the leaves of *Alstonia boonei* was investigated to determine its phytochemical composition and antimicrobial properties. The qualitative screening of the phytochemical composition of the methanol extract of the leaves of *Alstonia boonei* reported in Table 1 revealed the presence of the phytochemicals-saponins, alkaloids, tannins, terpenoids, phenols, flavonoids, glycosides, steroids, and anthocyanin in varying concentrations in the extract.

**Table 1:** Qualitative Screening of Phytochemical Constituents of Methanol Extracts of *Alstonia boonei* leaf

Phytochemicals	Methanol extract of AB
Saponins	+++
Alkaloids	+++
Tannins	+++
Terpenoids	+++
Phenols	+++
Flavonoids	+++
Glycosides	++
Steroids	++
Phytosterols	+
Anthocyanins	++

– Absent; + Present in low concentration; ++ Present in moderate concentration, +++ Present in very high concentrations.

The quantitative phytochemical screening showed a significant concentration of total phenolic, flavonoid, tannin, and proanthocyanidin content in the methanol extract of the leaves of *Alstonia boonei* -Total Phenol Content-111.14±1.70 (mg GAE/g extract), Total Flavonoid Content-225.50±15.00 (mg QE/g extract), Total Tannin Content- 175.01±1.00 (mg TAE/g extract), and Proanthocyanidin- 84.50±2.19 (mg AAE/g extract) (Table 2). These phytochemicals are secondary metabolites that have been reported to exhibit diverse pharmacological activities including antioxidant and antimicrobial effects (Nweze *et al.*, 2000). These biologically active constituents are known to act by a different mechanism and exert antimicrobial action (Oliver, 1960). The phenol and flavonoid families of phytonutrients, in particular, are thought to have a variety of *in vivo* and *in vitro* roles that serve as radical scavengers (Igbinsosa *et al.*, 2009). Numerous classes of phytochemical substances found in plants are known to exist, and new classes are routinely discovered. Studies have revealed that these substances possess a variety of therapeutic properties. For instance, flavonoids are well-known as antibacterial agents that are effective against a variety

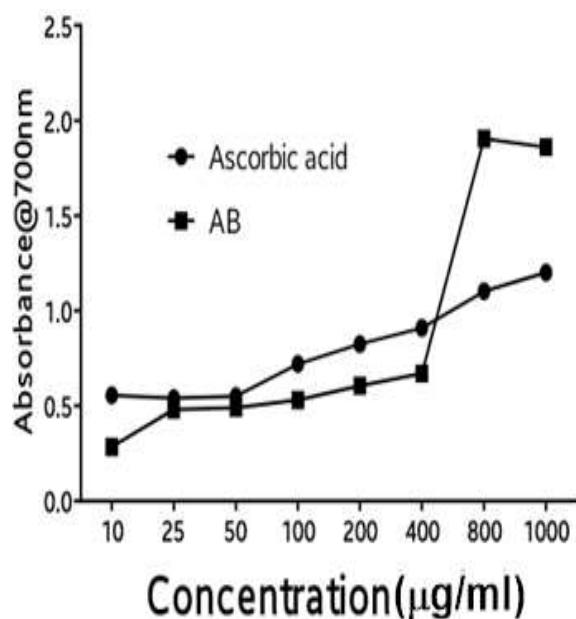
of pathogenic microbes (Farhan *et al.*, 2019). Phenols have antibacterial, anti-inflammatory, and antimutagenic properties, among several other health benefits (Chandrasekara *et al.*, 2016). Triterpenes have been described as anti-inflammatory, antiviral, antimicrobial, and anti-tumoral agents and as immunomodulator compounds.

**Table 2:** Quantitative Estimation of Phytochemical Constituents of Methanol Extracts of *Alstonia boonei* leaf

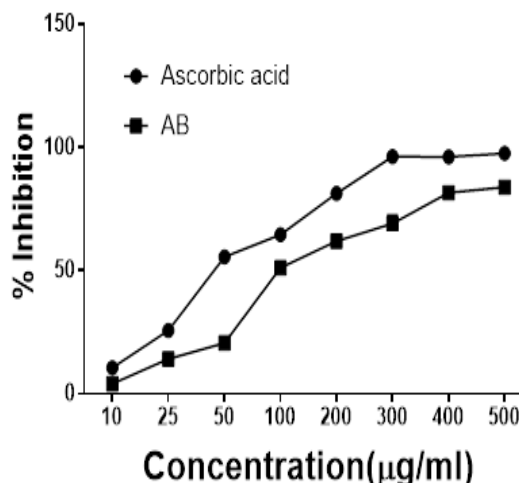
Phytochemical	Concentration
Total Phenol Content (mg GAE/g extract)	111.14±1.70
Total Flavonoid Content (mg QE/g extract)	225.50±15.00
Total Tannin Content (mg TAE/g extract)	175.01±1.00
Proanthocyanidin (mg AAE/g extract)	84.50±2.19

Values are expressed as mean ± SEM (n = 3/group). Values in a column with superscripts are not significantly different (P < 0.05)

The antioxidant activity of the extract was evaluated using various in vitro assays, including the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay, ferric reducing antioxidant power (FRAP) assay, nitric oxide inhibitory assay, and reducing power assay. The reducing power assay showed that the standard ascorbic acid has a better reducing power when compared to methanol extract of *A. boonei* leaves although the extract showed a significant increase at higher concentrations of 800 and 1000 µg/mL (Figure 1). The DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay showed the methanolic leaves extract of *A. boonei* has 50% inhibition concentration IC<sub>50</sub> values for the radical scavenging activity of (64.47 µg/mL) which was significantly higher than that of ascorbic acid (8.10 µg/mL) (Figure 2; Table 3).



**Fig 1.** Reducing power activity of different concentrations of ascorbic acid, and methanol extract of *A. boonei* leaf. Values are expressed as mean ± SEM, n = 3/group

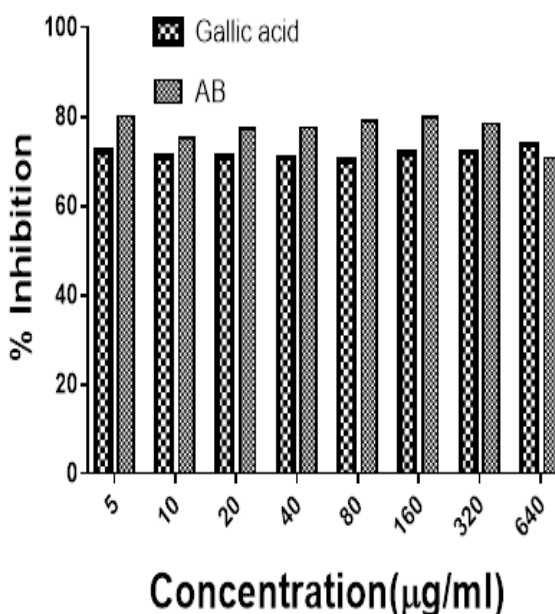


**Fig 2.** DPPH Radical Scavenging Activity of different concentrations of ascorbic acid and methanol extract of *A. boonei* leaf. Values are expressed as mean ± SEM, n = 3/group

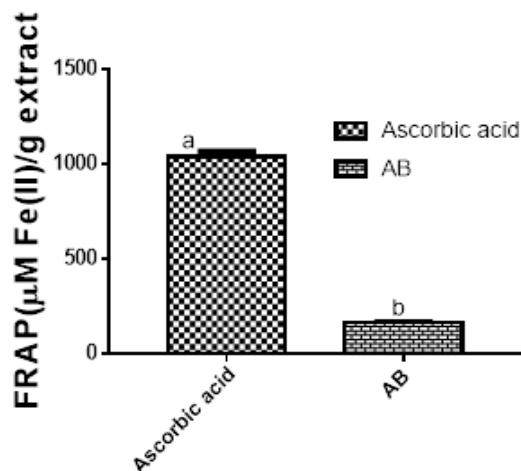
**Table 3;** DPPH Radical Scavenging and Nitric Oxide Inhibitory Activities IC<sub>50</sub> Values of Methanol Extract of *Alstonia boonei* leaf

Sample	DPPH, IC <sub>50</sub> µg/mL	NO, IC <sub>50</sub> µg/mL
Ascorbic acid	8.10	-
Gallic acid	-	73.40
AB	64.47	76.53

Figure 3 shows the nitric oxide inhibition activity of the methanol extract of *Alstonia boonei* which shows that *Alstonia boonei* is a good nitric oxide inhibitor at the different concentrations when compared against the standard Gallic acid, but at 640 µg/ml it was able to inhibit nitric oxide formation when compared to the standard Gallic acid.



**Fig 3:** Nitric oxide inhibitory activities of different concentrations of ascorbic acid and methanol extract of *A. boonei* leaf. Values are expressed as mean ± SEM, n = 3/group



**Fig 4:** Ferric Reducing Antioxidant Potential of different concentrations of ascorbic acid and methanol extracts of *A. boonei* leaf. Values are expressed as mean  $\pm$  SEM, n = 3/group. AB- *Alstonia boonei*

**Table 4:** Antibacterial activities of various concentrations of *Alstonia boonei* extract against some bacterial and fungi isolates tested by disc diffusion assay.

Isolate	Concentration Of Extract (mg/ml)			
	200	100	50	25
	Zone of inhibition (mm)			
<i>Escherichia coli</i>	13	8	-	-
<i>Serratia</i>	8	6	-	-
<i>Pseudomonas aeruginosa</i>	10	8	-	-
<i>Bacillus cereus</i>	14	-	-	-
<i>Staphylococcus aureus</i>	12	10	-	-
<i>Aspergillus niger</i>	11	10	-	-
<i>Mucor spp.</i>	-	-	-	-
<i>Aspergillus flavus</i>	14	11	10	-
<i>Penicillium chrysogenum</i>	14	12	-	-

Ferric reducing antioxidant potential of ascorbic acid and methanol leaves of *Alstonia boonei* is shown in Figure 4. It was observed that *Alstonia boonei* has a low ferric reducing antioxidant potential when compared to the standard ascorbic acid ( $164.5 \pm 10 \mu\text{M Fe(II)/g extract}$ ). This agreed with Xie *et al.* (2015) which showed that extract from plants reduced  $\text{Fe}^{3+}$  to  $\text{Fe}^{2+}$ . The antimicrobial activity of the methanol extracts of *A. boonei* leaves was analyzed against nine clinically significant micro-organisms (the bacterial isolates- *Bacillus cereus*, *Staphylococcus aureus*, *Escherichia coli*, *Serratia*, and *Pseudomonas aeruginosa*; the fungal isolates- *Aspergillus niger*, *Aspergillus flavus*, *Penicillium chrysogenum*, and *Mucor*) using the agar disc diffusion method (Table 4). The methanol extract tested showed varying degrees of antibacterial and antifungal activities at various concentrations. The inhibition pattern of the extract against the isolates varied across the different concentrations. The inhibition and concentration showed a direct relationship, that is, an increase in the concentration of the extract led to a corresponding increase in the inhibition of the various isolates. All

the bacterial isolates showed high inhibition at higher concentrations which indicates the susceptibility of these isolates to the extract. Therefore, the methanol extract of the leaves had wide antibacterial activity against both gram-positive and gram-negative bacteria. The inhibitory effects of the extract on the fungal isolates also varied. *Mucor* was not inhibited by the various concentrations of the extract which indicates that these fungal isolates are resistant to the extract. *Penicillium chrysogenum*, *Aspergillus niger* and *Aspergillus flavus* were inhibited by the extract at concentrations of 200 mg/ml and 100 mg/ml while growth was observed at concentrations of 50 mg/ml and 25 mg/ml. This result indicates that the organisms are susceptible to the extract at higher concentrations and resistant to the extract at lower concentrations. The results of this study indicate the presence of potent antibacterial phytochemicals in the methanol extracts of the plant's leaves. *A. boonei* has a broad-spectrum antibacterial potential, which makes it a candidate for bioprospecting for antimicrobial medications. This potential is demonstrated by the extracts' capacity to suppress the growth of several bacterial species. The presence of various bioactive compounds in plant extracts may be responsible for their antimicrobial properties (Huda-Faujan *et al.*, 2009). Flavonoids, which are hydroxylated phenolic compounds that plants produce in response to microbial infection, have been shown to be efficient antibacterial compounds in vitro against a variety of pathogens. Its capacity to interact with extracellular and soluble proteins as well as bacterial cell walls is likely what causes them to be active (Marjorie, 1999). The capacity of saponin to release proteins and specific enzymes from cells is what gives it its antibacterial properties (Zablotowicz *et al.*, 1996). The link between membrane lipids and steroid sensitivity suggests that steroids particularly bind with membrane lipids and exert their action by generating leakages from liposomes. Steroids have been demonstrated to have antimicrobial characteristics (Raquel, 2007).

**Conclusion:** In recent scientific investigations, there has been a growing emphasis on the exploration of natural compounds, specifically those derived from plants, to uncover their potential therapeutic advantages. The leaves of *Alstonia boonei* have been demonstrated to possess an abundance of phytochemicals, which may be responsible for the plant's notable antioxidant and antimicrobial properties.

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