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Evaluation of Antimicrobial Activity, Synergistic Efficacy, Qualitative and Quantitative Phytochemical Determination of Alstonia Boonei Leaf and Stem Bark on Selected Clinical Isolates

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ABSTRACT

This study evaluated the antimicrobial activity, of Alstonia boonei leaf, stem bark and the synergistic efficacy of A. boonei leaf plus stem bark on seven bacteria (Gram positive and Gram negative bacteria) which are strains of Escherichia coli, Staphylococcus aureus, Bacillus subtilis, Pseudomonas aeruginosa, Proteus viridans, Salmonella typhi and Klebsiella pneumoniae, and three fungal isolates which are Candida albicans, Aspergillus flavus and Trichophyton rubrum. This study also evaluates the quantitative and qualitative phytochemical properties of A. boonei leaf and stem bark, as well as the minimum inhibitory concentration of A. boonei leaf and stem bark crude extracts on the selected clinical isolates. A. boonei was collected from ore in Odigbo local government of Ondo state. Crude extraction of air dried leaf and stem bark was carried out by soaking the plant in dichloromethane, standard agar good diffusion method was used for sensitivity testing and the minimum inhibitory concentration values are obtained by agar dilution method. The crude extract from both plant parts shows high antimicrobial property, while the synergistic effect of the leaf and stem bark shows comparatively higher antimicrobial property to the individual tissue. Sensitivity test revealed that the highest zone of inhibition was observed from the synergy of A. boonei leaf and stem bark against E. coli with 25mm at 100mg/ml, while the least zone of inhibition is observed from the stem bark extract against K. pneumoniae with 2mm at 6.25mg/ml. the highest antifungal activity is from the synergistic effect against C. albicans with 20mm zone of inhibition at 100mg/ml, and the least antifungal activity is observed against T. rubrum with 11mm zone of inhibition at 100mg/ml to the leaf extract of A. boonei. The qualitative and quantitative phytochemical analysis reveals the presence of phytochemicals such as Alkaloid, cardiac glycoside, steroids, tannin, saponin, flavonoid, anthraquinone, phenol and reducing sugars in the different tissues and it supports their antimicrobial activities.

Keywords: Alstonia boonei leaf, stem bark Antibacterial, Synergistic efficacy and Phytochemical activity

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INTRODUCTION

Alstonia boonei, a large evergreen tree 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 (Opoku and Akoto, 2015). Almost all plant parts

Figure 1. *Alstonia boonei* leaf with stem bark

viz. leaves, stem bark; root and inflorescences have been used and are further under investigative study. 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. The stem bark of Alstonia 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. It is also used in treating painful micturition and rheumatic conditions (Opoku and Akoto, 2015).

Alstonia boonei is a very large, deciduous, tropical forest tree belonging to the dogbane family Apocynaceae. It is native to tropical West Africa, with a range extending to Ethiopia and Tanzania. Its common name in the English timber trade is cheese wood, pattern wood, or stool wood, while its common name in the French timbe trade is 'emiem'. The wood is fine-grained, lending itself to detailed carving; the tree also finds many uses in folk medicine. Like many other members of the apocynaceae (a family rich in toxic and medicinal species), A. boonei contains alkaloids and yields latex (Fakae et al., 200).

Alstonia boonei is a tall forest tree, which can reach 45 metres (148 ft) in height and 3m (9.8ft) in girth, the bole being cylindrical and up to 27m (89 ft) in height with high, narrow, deep-fluted buttresses. The leaves are borne in whorls at the nodes, the leaf shape is oblanceolate, with the apex rounded to acuminate and the lateral veins prominent and almost at right angles to the mid rib. The flowers are yellowish-white and borne in lax terminal cymes. The fruits are pendulous, paired, slender follicles up to 16 centimetres (6.3 in) long, containing seeds bearing a tuft of silky, brown floss at either end to allow dispersal by the wind, the latex is white and abundant.

Alstonia grows into a giant tree in most of the evergreen rain forests of tropical West Africa. The plant thrives very well in damp riverbanks. It is well known to all the traditional healers practicing along the west coast of Africa. It occurs in deciduous and fringing forest of Nigeria (Gosse et al., 1999).

Alstonia boonei is a deciduous tree up to 35meters high (Figure1). It buttresses deep-fluted high and narrow. Its white latexes are copious. The leaves are in whorls at nodes, oblanceolate, apex rounded to acuminate, lateral vein prominent almost at right angle to midrib. The flowers are white with lax terminal cymes. The fruits are paired with slender follicle up to 16 cm long with brown floss at each end.

The bark of Alstonia tree is one of the effective analgesic herbs available in nature. All the parts of the plant are very useful but the thick bark cut from the matured tree is the part that is most commonly used for therapeutic purposes. The bark of the tree is highly effective when it is used in its fresh form; however, the dried one could equally be used. Therapeutically, the bark has been found to possess antirheumatic (Abbiw, 1990), anti-inflammatory, analgesic/pain-killing, antimalaria/antipyretic, antidiabetic (mild hypoglycaemic), antihelminthic, antimicrobial and antibiotic properties (Haidi and Bremner, 2001).

A decoction could be sweetened with pure honey and be taken up to 4 times daily as an effective painkiller for the following conditions; Painful menstruation (dysmenorrhoea), when associated with uterine fibroid or ovarian cysts in women; lower abdominal and pelvic congestion associated with gynaecological problems such as pelvic inflammatory diseases; to relieve the painful urethritis common with gonococcus or other microbial infections in men. Alstonia decoction also exerts a mild antibacterial effect in this case, relieving the aches and pains associated with malaria fever. Alstonia is taken in the form of preparations that exhibits antipyrexia and anti-malaria effects, to combat rheumatic and arthritic pains. The decoction of Alstonia bark could be taken alone as an effective pain-killing agent. A cold infusion made from the fresh or dried bark of Alstonia taken orally two to three times daily exerts a mild hypoglycaemic effect on diabetic patients. The cold infusion is also administered orally for expelling around worms, threadworms, and other intestinal parasites in children (Fakae et al., 2000).

The fresh bark of Alstonia could be used in preparing herbal tinctures; it is particularly useful as an effective antidote against snake, rat, or scorpion poison. It is also useful in expelling retained products of conception and afterbirth when given to women. Asthma can be treated with a drink prepared from parts of Trema orientalis and decoction of the bark of Alstonia boonei mixed with the roots and bark of cola and fruits of Xylopia parviflora with hard potash. The bark decoction of Alstonia boonei is used with other preparations in the treatment of fractures or dislocation, jaundice, and for inducing breast milk. Its latex is taken as a purgative. The hardened latex is used for the treatment of yaws (Odugbemi et al., 2007).

Alstonia boonei is regarded as one of few herbs with potential anti-HIV indicators. In some African countries Alstonia boonei is considered a sacred tree and worshiped in the forest and hence human beings in those countries do not eat its parts (Amole and Ilori, 2010).

MATERIALS AND METHODS

Plant sample

Source and collection of plant samples: The stem bark and leaves of Alstonia boonei used in this study were collected from fresh water swamp forest in ore, Odigbo local government area of Ondo State, Nigeria.

Authentication of plant samples: The plants were authenticated at the Department of Plant Science and Biotechnology, Adekunle Ajasin University, Akungba-Akoko, Ondo state, Nigeria.

Preparation of plant samples: The stem barks and leaves of A. boonei after collection were first washed thoroughly with sterile distilled water and appropriately air dried at room temperature for two weeks to ensure the samples lose most of their moisture content. The stem bark of Alstonia boonei was powdered, after which the sample was then macerated using electronic blender. The leaves of A. boonei after being air dried, was powdered and milled at the department of Microbiology, Adekunle Ajasin University, Akungba-Akoko, Ondo State, Nigeria.

Extraction of plants: For the extraction of each plant part, 500g of each dried and powdered plant sample was weighed separately into corked containers containing 1500ml each of dichloromethane, the mixtures were initially shaken rigorously and left for 7 days. All mixtures were filtered using sterile whatman filter papers, and the filtrates were collected directly into sterile crucibles. The filtrate was extracted using rotary evaporator, and the residues obtained were kept at room temperature (Osuntokun, 2015).

Standardization of plants extracts: At aseptic condition, the extracts are reconstituted by adding 1g of each extract to 2.5ml of Dimethylsulphoxide (DMSO) and 7.5ml of sterile distilled water, making it 100mg/ml. For each extract, 5ml of distilled water is measured into four sterile bijou bottles. In bijou bottle A, 5ml from the 100mg/ml bijou bottle was drawn and added, making it 50mg/ml. The serial concentration was prepared to get concentrations of 50mg/ml, 25mg/ml, 12.5mg/ml and 6.25mg/ml respectively (Osuntokun, 2014).

Test Organisms

The test organisms used were standard strains of pathogenic bacteria and clinical fungal isolate. They include strains of Staphylococcus aureus (ATCC 55620), Salmonella typhi Escherichia coli (ATCC 23922), Klebsiella pneumoniae (ATCC 15380), Pseudomonas aeruginosa (ATCC 27853) and Proteus mirabilis (ATCC 13325). The fungi isolates include strains of Aspergillus flavus, Trichophyton rubrum and Candida albicans, as identified via macroscopic and microscopic observations. The bacterial isolates were cultured in slanted Mueller Hinton agar in bijou bottles and transported at a low temperature to microbiology laboratory Adekunle Ajasin University, Akungba Akoko (AAUA), ondo state and incubated in an incubator for reactivation of the bacteria. The fungal isolates used were cultured on a slanted Saboraud dextrose agar in bijou bottles and transported at low temperature to microbiology laboratory A.A.U.A and incubated in an incubator for reactivation. They were then sub-cultured and stored -4^{0}C prior to bioassay of the

extracts. (Osuntokun and Olajubu, 2014).

Source of test organism: The test organisms were obtained from the stock culture of organisms at the College of medicine, University of Lagos, and transported at 37^0C to Microbiology laboratory, Adekunle Ajasin University, Akungba-Akoko ondo state.

Standardization of test organisms: Slants of the various organisms were reconstituted at aseptic condition, using a sterile wire loop, approximately one isolated colony of each pure culture was transferred into 5ml of sterile nutrient broth and incubated for 24 hours. After incubation, 0.1ml of the isolated colony was transferred into 9.9ml of sterile distilled water contained in each test tube using a sterile needle and syringe, and then mixed properly. The liquid now serve as a source of inoculum containing approximately 10^6cfu/ml of bacterial suspension (Osuntokun and Olajubu, 2014).

Antimicrobial screening of the extracts

Standard agar good diffusion method was employed for the antimicrobial testing.

Antibacterial and antifungal screening of the extract: All the test bacteria, were sub-cultured onto sterile Mueller Hinton agar plates, and incubated at 37^0C for 18-24 hours. Five distinct colonies for each organism were inoculated onto sterile Mueller Hinton broth and incubated for 3-4hours. All inocula were standardized accordingly to match the 0.5 McFarland standards, and this standard was used for all susceptibility tests. All the extracts were reconstituted accordingly into the following concentrations; 100, 50, 25, 12.5, 6.25mg/ml, using Dimethylsulphoxide (DMSO). The susceptibility testing was investigated by the agar good diffusion method. A 0.1ml of 1: 10,000 dilution (equivalent to 10^6cfu/ml) of fresh overnight culture of the clinical isolates grown in Mueller Hinton agar and Sabouraud dextrose agar was seeded into 40ml of Mueller Hinton agar, and properly mixed in universal bottles.

The mixture was aseptically poured into sterile Petri dishes and allowed to set. Using a sterile Cork borer of 6mm diameter, equidistant wells were made in the agar. Drops of the re suspended, (2ml per well) extracts with concentrations between 100mg/ml to 6.25mg/ml were introduced into the wells till it was filled. Levofloxacin 50mg/ml was used as the control experiment for bacteria, while fluconazole 50mg/ml was used as the positive control for fungi. The plates could stand on the bench for an hour, to allow pre-diffusion of the extracts before incubation at 37^0C for 24 hours for the bacterial isolates and 24^0C for 48 hours for the fungal isolates.

The zones of inhibition were measured to the nearest millimeter (mm) using a standard transparent meter rule. All experiments were performed in duplicates (Osuntokun and Oladele, 2014).

Determination of minimum inhibitory concentration

Minimum inhibitory concentration (MIC) was also measured. Dilution law was used to determine the concentration per plate. The working concentration was 0.25mg/ml, 0.5mg/ml, 1mg/ml, 2mg/ml, 4mg/ml, 8mg/ml, and 16mg/ml. The dilution law;

$C_1*V_1 = C_2*V_2$. While C_1 is 100mg/ml and V_2 is 20mls was employed to determine the quantity of the extracts to be added to the agar. The mixture of the agar and extract could solidify and the standardized organisms were inoculated on the plates each. For this procedure, extracts were tested on the test bacterial and fungal isolates to determine the minimum concentration of inhibition. The Petri dish containing the agar and extract mix strip with the organism was incubated at 37^0C for bacteria and 24^0c for fungi, and examined after 24 and 48 hours respectively. The lowest concentration of the extract at which there is inhibition of the organism growth is taken as the minimum inhibitory concentration (MIC) (Osuntokun, 2014).

Phytochemical analysis of medicinal plant

Qualitative phytochemical analysis

Test for Reducing Sugars**:** One milliliter of the plant filtrate was mixed with Fehling A and Fehling B separately; a brown colour with Fehling B and a green colour with Fehling A indicate the presence of reducing sugars.

Test for Alkanol

TLC method 1: Solvent system: Chloroform: methanol: 25% ammonia (8:2:0.5). Spots can be detected after spraying with Dragendorff reagent. Presence of orange spot shows it's a positive result (Tona et al., 1998).

TLC method 2**:** The powdered test samples were wet with a half diluted NH_4OH and lixiviated with EtOAc for 24hr at room temperature. The organic phase was separated from the acidified filtrate and basify with NH4OH (pH 11-12). It was then extracted with chloroform (3X), condense by evaporation and use for chromatography. The alkaloid spots were separated using the solvent mixture chloroform and methanol (15:1). The spots were sprayed with Dragendorff's reagent. The presence of orange spot shows it's a positive result (Mallikharjuna et al., 2007).

Test for Anthraquinone

Borntrager's test: Heat about 50mg of extract with 1ml 10% ferric chloride solution and 1ml of concentrated hydrochloric acid. Cool the extract and filter. Shake the filtrate with equal amount of diethyl ether. Further extract the ether extract with strong ammonia. Pink or deep red coloration of aqueous layer (Kumar et al., 2007).

Borntrager's test: 1 ml of dilute (10 %) ammonia was added to 2 ml of chloroform extract. A pink-red color in the ammoniacal (lower) layer (Onwukaeme et al., 2007).

Test for Cardiac glycosides

Kellar- Kiliani test: 50mg of methanolic extract was

dissolved in 2 ml of chloroform. H_2SO_4 was added to form a layer. Brown ring at interphase shows it's a positive result (Onwukaeme et al., 2007).

TLC method - The powdered test samples were extracted with 70% EtOH on rotary shaker (180 thaws/min) for 10hr. 70% lead acetate was added to the filtrate and centrifuged at 5000rpm/10 min. The supernatant was further centrifuged by adding 6.3% Na2CO3 at 10000 rpm/10min.The supernatant was retained and re-dissolved in chloroform and use for chromatography. The glycosides were separated using EtOAc-MeOH-H2O (80:10:10) solvent mixture. The colour and hRf values of these spots were recorded under ultraviolet (UV254 nm) light (Mallikharjuna et al., 2007).

Test for Flavonoid

Shinoda test: To 2-3ml of methanolic extract, a piece of magnesium ribbon was added and 1ml of concentrated hydrochloric acid. Pink red or red coloration of the solution shows it's a positive result (Kumar et al., 2007).

TLC method: 1g powdered test samples was extracted with 10ml methanol on water bath (60°C/ 5min). The filtrate was condensed by evaporation, and a mixture of water and EtOAc (10:1 mL) was added, and mix thoroughly. the EtOAc phase was retained and used for chromatography. The flavonoid spots were separated using chloroform and methanol (19:1) solvent mixture. The colour and hRf values of these spots were recorded under ultraviolet (UV254nm) light (Mallikharjuna et al., 2007).

Test for Phenol

Phenol test: The extract was spotted on a filter paper. A drop of phoshomolybdic acid reagent was added and expose to ammonia vapours. Blue coloration of the spot shows it's a positive result (Kumar et al., 2007).

Test for Saponin

Frothing test / Foam test: 0.5ml of filtrate was added with 5ml of distilled water and shook well. Persistence of frothing shows it's a positive result (Parekh and Chanda, 2007).

TLC method: Two grams of powdered test samples were extracted with 10 ml 70% EtOH by refluxing for 10 min. The filtrate was condensed, enriched with saturated n-BuOH, and mix thoroughly. The butanol was retained, condensed and used for chromatography. The saponins were separated using chloroform, glacial acetic acid, methanol and water (64:34:12:8) solvent mixture. The chromatogram was exposed to the iodine vapours. The colour (yellow) and hRf values of these spots were recorded by exposing chromatogram to the iodine vapours (Mallikharjuna et al., 2007).

Test for Steroid

TLC method: Two grams of powdered test samples were extracted with 10ml methanol in water bath (80°C/15 min). The condensed filtrate was used for chromatography. The sterols were separated using chloroform, glacial acetic acid, methanol and water (64:34:12:8) solvent mixture. The color and hRf values of these spots was recorded under visible light after spraying the plates with anisaldehyde-sulphuric acid reagent and heating (100°C/6 min). The colour (Greenish black to Pinkish black) and hRf values of these spots were recorded under visible light (Mallikharjuna et al., 2007).

Test for Tannin

Braemer's test: 10% alcoholic ferric chloride was added to 2-3ml of methanolic extract (1:1). Dark blue or greenish grey coloration of the solution shows it's a positive result (Kumar et al., 2007); (Parekh and Chanda, 2007)

Quantitative Method of Analysis

Determination of Saponins: About 20grams each of dried plant samples were ground and, put into a conical flask after which 100 ml of 20 % aqueous ethanol were added. The mixtures were heated using a hot water bath. At about 55^{O}C, for 4 hours with continuous stirring, after which the mixture was filtered and the residue re-extracted with a further 200 ml of 20% ethanol. The combined extracts were reduced to 40 ml over a water bath at about 90°C. The concentrate was transferred into a 250ml separatory funnel and 20rnl of diethyl ether was added and then shaken vigorously. The aqueous layer was recovered while the ether layer was discarded. The purification process was repeated three times. 60rnl of n-butanol was added. The combined n-butanol extracts were washed twice with 10m1 of 5% aqueous sodium chloride. The remaining solutions were heated in a water bath. After evaporation, the samples were dried in the oven to a constant weight; the saponin content was calculated as percentage of the starting material

Determination of Flavonoids: About 10 g of the plant sample was extracted repeatedly with 100 ml of 80% aqueous methanol, at room temperature. The whole solutions were filtered through Whatman filter paper No 42. The filtrate was later transferred into a crucible and evaporated into dryness over a water bath; the dry contents were weighed to a constant weigh.

Determination of Tannins: About 500 mg of the plant sample were weighed into a 50-ml plastic bottle. 50 ml of distilled water was added and shaken for 1 hour on a mechanical shaker. This was filtered into a 50-ml volumetric flask and made up to the marked level. Then, 5 ml of the filtrate was transferred into a test tube and mixed with 2 ml of 0.1 M FeCl in 0.1 M Hcl and 0.008 M potassium ferrocyanide. The absorbance was measured at 120 nm within 10 minutes. The tannins content was calculated using a standard curve of extract.

Determination of Alkaloids: Five grams of the plant sample were weighed into a 250ml beaker and 200ml of 10% acetic acid in ethanol was then be added, the reaction mixture was covered and allowed to stand for 4 hour. This was filtered and the extract was concentrated on a water bath to one-quarter of the original volume. Concentrated

ammonium hydroxide was added drop-wise to the extract until the precipitation is complete. The whole solution could settle and the precipitate was collected, washed with dilute ammonium hydroxide and then filtered; the residue being the alkaloid, which was dried and weighed to a constant mass**.**

RESULTS

Table 1. shows the diameter (in mm) of the zones of inhibition of bacterial growth at different concentrations (100mg/ml, 50mg/ml, 25mg/ml, 12.5mg/ml and 6.25mg/ml) of Alstonia boonei leaf extract. The zones of inhibition were recorded as mean of two replicates. Bacillus subtilis was shown to be the most susceptible organism out of all the test organisms, showing its highest susceptibility at a concentration of 100mg/ml with 24mm in diameter and its lowest susceptibility at a concentration of 6.25mg/ml with 10mm in diameter. S. typhi and P. aeruginosa also showed intermediate susceptibility to the leaf extract with 19mm and 21mm zones of inhibition respectively at 100mg/ml and 6mm and 8mm diameter respectively at 6.25mg/ml. from this study, K. pneumoniae was observed to be the least susceptible organism to the Alstonia boonei leaf extract among the test organisms used, with 14mm in diameter zone of inhibition at 100mg/ml and 3mm in diameter at 6.25mg/ml. it was also observed from this table that Candida albican showed the highest susceptibility to the leaf extract, with 18mm zone of inhibition at a concentration of 100mg/ml and 6mm at a concentration of 6.25mg/ml. T. rubrum was the least susceptible organism with 11mm at 100mg/ml and 4mm at 25mg/ml.

Table 2. Showed the antimicrobial activity of dichloromethane extract of Alstonia boonei stem bark against all the test organisms. E. coli was observed to have the highest susceptibility to the stem bark extract with 21mm in diameter zone of inhibition at 100mg/ml and 9mm in diameter zone of inhibition at 6mg/ml. S. aureus showed relatively good susceptibility to this extract having 20mm zone of inhibition at 100mg/ml, and 9mm zone of inhibition at 6mg/ml. From this study, K. pneumoniae was also observed to have the lowest susceptibility to the stem bark extract of the plant, with 13mm at 100mg/ml and 2mm at 6mg/ml. the antifungal activities of A. boonei stem bark against the test fungal isolates was also recorded in this table. Candida albican also showed the highest susceptibility to the stem bark extract with 18mm at 100mg/ml and 4mm at 6mg/ml. A. flavus and T. rubrum showed almost the same level of susceptibility to this extract, with 14mm and 11mm respectively at 100mg/ml concentration, and 4 and 3mm at 12mg/ml respectively.

Table 3. shows the antibacterial activity of the synergistic effect of Alstonia boonei stem bark and leaf (stem bark extract + leaf, ratio 1:1). From this table, E. coli was shown to have the highest susceptibility with 25mm zone of inhibition at 100mg/ml concentration, and 10mm zone of inhibition at a concentration of 6.25mg/ml. It was also observed from this table that S. aureus and P. aeruginosa have good susceptibility with 21mm and 23mm zones of inhibition respectively at 100mg/ml and 9mm and 10mm zones of inhibition respectively at 6.25mg/ml. K. pneumoniae has the least susceptibility to this extract, having zones of inhibition to be 15 and 3mm in diameter at concentrations of 100mg/ml and 6.25mg/ml respectively. the antifungal activity of the synergistic effect of the stem bark + leaf of A. boonei as shown on this table, also shows that C. albican was the most susceptible organism out of the test fungal isolates used in the study, with 20mm zone of inhibition at 100mg/ml and 5mm zone of inhibition at a concentration of 6.25mg/ml.

Table 4 shows the minimum inhibitory concentration (MIC) value of the dichloromethane extract of the stem bark against the test organisms used in the study. From this table, S. aureus and E. coli were the bacteria with the least MIC values, having their MIC values at 0.5mg/ml respectively, while K. pneumoniae was the test bacteria with the highest MIC value, having its MIC value at 8mg/ml. Candida albican and T. rubrum were observed to have their MIC values at 8mg/ml and A. flavus has the highest MIC value among the test fungal isolates with its MIC value at approximately 16mg/ml.

Table 5 shows the MIC value of the dichloromethane extract of A. boonei leaf against the test organisms (bacteria and fungi). From this table, B. subtilis, P. mirabilis and P. aeruginosa were observed to be the bacteria with the least MIC values, having their MIC values at 0.5mg/ml, while E. coli and K. pneumoniae are the test bacteria with the highest MIC values, having their MIC values at 4mg/ml respectively. It was observed from this study and as shown in the table that C. albican is the least fungal isolate with the least MIC value, having its MIC value at 4mg/ml and T. rubrum with the highest MIC value, having its MIC value at 16mg/ml.

Table 6 showed the qualitative phytochemical analysis of Alstonia boonei stem barks and leaves using methanol. From this table, it was observed that alkaloid, cardiac glycoside, steroids, phenol, tannins, saponin and flavonoid were all present in the stem bark, anthraquinone was not detected and reducing sugar was negative. In the leaf extract, all the screened phytoconstituents were all present.

Table 7 showed the qualitative phytochemical analysis of Alstonia boonei stem barks and leaves usng ethyl acetate, it was also seen from this table that alkaloid, anthraquinone and reducing sugar were negative in the stem bark, flavonoid was not detected and other phytoconstituents screened for were positive. While in the leaf, only cardiac glycoside was found to be negative, and other phytoconstituents were all positive.

Table 8 showed the result of the qualitative phytochemical analysis of Alstonia boonei stem barks and leaves using dichloromethane (DCM), from this table, it was observed that only cardiac glycoside was negative (i.e. not present) in the stem barks, while other phytoconstituents screened for were present. From the leaf

Table 1. Antimicrobial activities of dichloromethane extract of *Alstonia boonei* leaf

Test Organisms	Dichloromethane extract of *Alstonia boonei* leaf Concentration (mg/ml)						
	100	**50**	**25**	**12.5**	**6.25**	**Positive control**	**Negative control**
Salmonella typhi	19	17	14	10	6	27	0.0
Staphylococcus aureus (ATCC 25923)	18.	15	12	9	6	28	0.0
Pseudomonas aeruginosa (ATCC 27853)	21	18	14	11	8	0	0.0
Bacillus subtilis	24	21	18	14	10	25	0.0
Klebsiella pneumoniae (ATCC 13883)	14	10	7	5	3	0	0.0
Proteus mirabilis (ATCC 7002)	16	13	10	8	5	19	0.0
Escherichia coli (ATCC 25922)	15	12	9	6	4	24	0.0
Candida albican	18	14	12	10	6	19	0.0
Aspergillus flavus	13	10	7	3	0	0	0.0
Trichophyton rubrum	11	7	4	0	0	14	0.0

Data are presented as mean of zone of inhibition of two replicates measured in mm. Zone of inhibition does not include the diameter of the cork borer (6mm). Positive control for bacteria is 50mg/ml Levofloxacin, and for fungi is 50mg/ml fluconazole. Negative control is 25%DMSO.

Table 3.2: Antimicrobial activity of dichloromethane extract of *Alstonia boonei* stems bark

Test Organisms	Dichloromethane extract of *A. boonei* stem bark Concentration (mg/ml)				
	100	**50**	**25**	**12.5**	**6.25**
Salmonella typhi	17	15	11	9	6
Staphylococcus aureus (ATCC 25923)	20	17	15	12	9
Pseudomonas aeruginosa (ATCC 27853)	17	14	11	8	5
Bacillus subtilis	16	14	11	9	5
Klebsiella pneumoniae (ATCC 13883)	13	11	6	4	2
Proteus mirabilis (ATCC 7002)	17	15	11	8	5
Escherichia coli (ATCC 25922)	21	18	16	2	9
Candida albican	18	15	11	6	4
Aspergillus flavus	14	10	7	4	0
Trichophyton rubrum	11	7	5	3	0

Data are presented as mean of measurement of zone of inhibition of two replicates measured in mm. Zone of inhibition does not include the diameter of the cork borer (6mm). NI = No Inhibition

extract, using this same solvent, alkaloid, anthraquinone, and reducing sugar were negative, as other phytoconstituents were present.

Table 9 also showed the qualitative phytochemical analysis of Alstonia boonei stem barks and leaves using N-Hexane as the solvent. It was observed from this table that alkaloid, anthraquinone and reducing sugar were negative from this in the stem barks, while phenol, tannin, saponin, flavonoid, cardiac glycosides and steroid were all detected. It was also observed from this table that alkaloid, steroid and reducing sugar were negative from the leaf extract, flavonoid was not detected, and all other phytoconstituents screened for were all present.

Table 10 showed the quantitative phytochemical analysis of Alstonia boonei stem barks and leaves using methanol. It was observed from this table that flavonoid is the most abundant phytochemical in the stem bark, with 4.55, and others such as alkaloid, oxalate, phytate, phenol, tannin and saponin ranged between 2.37 and 2.10, with oxalate being the least with 2.10. It was observed from the

Table 3. The antimicrobial activity of the synergistic effect of dichloromethane extract of *Alstoni a boonei* leaf plus stem bark

Test Organisms	Dichloromethane extract of *A. boonei* leaf plus stem bark Concentration (mg/ml)				
	100	**50**	**25**	**12.5**	**6.25**
Salmonella typhi	20	17	14	11	8
Staphylococcus aureus (ATCC 25923)	21	18	16	13	9
Pseudomonas aeruginosa (ATCC 27853)	23	20	16	13	10
Bacillus subtilis	17	15	12	10	6
Klebsiella pneumoniae (ATCC 13883)	15	12	9	6	3
Proteus mirabilis (ATCC 7002)	18	15	12	9	6
Escherichia coli (ATCC 25922)	25	22	18	14	10
Candida albican	20	17	14	11	8
Aspergillus flavus	16	13	10	7	4
Trichophyton rubrum	14	11	8	4	NI

Data are presented as mean of measurement of zone of inhibition of two replicates measured in mm. Zone of inhibition does not include the diameter of the cork borer (6mm). NI = No Inhibition

Table 4. Minimum inhibitory concentration of dichloromethane extracts of *Alstonia boonei* stem bark

	0.25mg/ml	**0.5mg/ml**	**1mg/ml**	**2mg/ml**	**4mg/ml**	**8mg/ml**	**16mg/ml**
S. aureus	+	-	-	-	-	-	-
S. typhi	+	+	-	-	-	-	-
B. subtilis	+	+	+	-	-	-	-
E. coli	+	-	-	-	-	-	-
P. mirabilis	+	+	+	-	-	-	-
P. aeruginosa	+	+	+	-	-	-	-
k. pneumoniae	+	+	+	+	+	-	-
C. albican	+	+	+	+	+	-	-
A. flavus	+	+	+	+	+	+	-
T. rubrum	+	+	+	+	+	-	-

Key
+ = growth was observed
- = no growth observed

Table 5. Minimum inhibitory concentration of dichloromethane extract of *Alstonia boonei* leaf

	0.25mg/ml	**0.5mg/ml**	**1mg/ml**	**2mg/ml**	**4mg/ml**	**8mg/ml**	**16mg/ml**
S. aureus	+	+	-	-	-	-	-
S. typhi	+	+	-	-	-	-	-
B. subtilis	+	-	-	-	-	-	-
E. coli	+	+	+	+	-	-	-
P. mirabilis	+	-	-	-	-	-	-
P. aeruginosa	+	-	-	-	-	-	-
k.pneumoniae	+	+	+	+	-	-	-
C. albican	+	+	+	+	-	-	-
A. flavus	+	+	+	+	+	-	-
T. rubrum	+	+	+	+	+	+	-

Table 6. Qualitative phytochemical analysis of *Alstonia boonei* (Methanol

Sample	Alkaloid	Cardiac Glycoside	Steroids	Anthraquinone	Phenol	Tannins	Saponin	Flavonoids	Reducing sugar
A	+ ve	+ ve	+ ve	ND	+ ve	+ ve	+ ve	+ ve	-ve
B	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve	+ve

A = *Alstonia boonei* bark -Ve = Negative
B = *Alstonia boonei* leaf ND = Not detected

Table 7. Qualitative phytochemical analysis of *Alstonia boonei* (Ethyl acetate)

Sample	Alkaloid	Cardiac Glycoside	Steroids	Anthraquinone	Phenol	Tannins	Saponin	Flavonoids	Reducing sugar
A	- ve	+ ve	+ ve	- ve	+ ve	+ ve	+ ve	ND	- ve
B	+ ve	- ve	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve

A = *Alstonia boonei* bark +Ve = Positive , ND = Not detected
B = *Alstonia boonei* leaf -Ve = Negative

Table 8. Qualitative phytochemical analysis of *Alstonia boonei* (Dichloromethane)

Sample	Alkaloid	Cardiac Glycoside	Steroids	Anthraquinone	Phenol	Tannins	Saponin	Flavonoids	Reducing sugar
A	+ ve	- ve	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve
B	- ve	+ ve	+ ve	- ve	+ ve	+ ve	+ ve	+ve	- ve

A = *Alstonia boonei* bark +Ve = Positive , ND = Not detected
B = *Alstonia boonei* leaf , -Ve = Negative

Table 9. Qualitative phytochemical analysis of *Alstonia boonei* (N- hexane)

Sample	Alkaloid	Cardiac Glycoside	Steroids	Anthraquinone	Phenol	Tannins	Saponin	Flavonoids	Reducing sugar
A	- ve	+ ve	+ ve	- ve	+ ve	+ ve	+ ve	+VE	- ve
B	- ve	+ ve	- ve	- ve	+ ve	+ ve	+ ve	ND	- ve

A = *Alstonia boonei* bark -Ve = Negative ,
B = *Alstonia boonei* leaf ND = Not detected +Ve = Positive

Table 10. Quantitative phytochemical analysis of *Alstonia boonei* (METHANOL)

Sample	Alkaloid	Oxalate	Phytate	Phenol	Tannins	Saponin	Flavonoids
A	2.20	2.10	2.32	2.37	2.30	2.25	4.55
B	2.50	3.57	2.56	2.49	3.49	3.45	5.87

A = *Alstonia boonei* bark , B = *Alstonia boonei* leaf

Table 11. Quantitative phytochemical analysis of medicinal plant (ETHYL ACETATE)

Sample	Alkaloid	Oxalate	Phytate	Phenol	Tannins	Saponin	Flavonoids
A	24.03	30.14	32.31	26.09	28.09	6.70	6.42
B	20.34	24.34	20.72	23.18	19.82	5.95	2.35

A = *Alstonia boonei* bark , B = *Alstonia boonei* leaf

Table 12. Quantitative phytochemical analysis of medicinal plant (N-Hexane)

Sample	Alkaloid	Oxalate	Phytate	Phenol	Tannins	Saponin	Flavonoids
A	21.03	20.14	21.31	20.09	21.09	16.70	16.42
B	20.34	24.34	20.72	20.18	10.82	5.95	2.35

A = *Alstonia boonei* bark , B = *Alstonia boonei* leaf

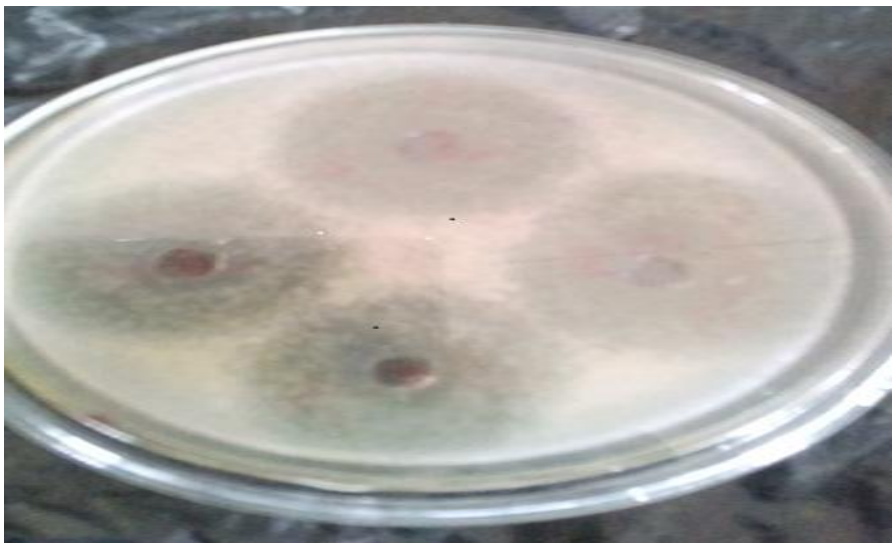

Plate 1. Antibacterial activity of *Alstonia boonei* stem bark against *E. coli*

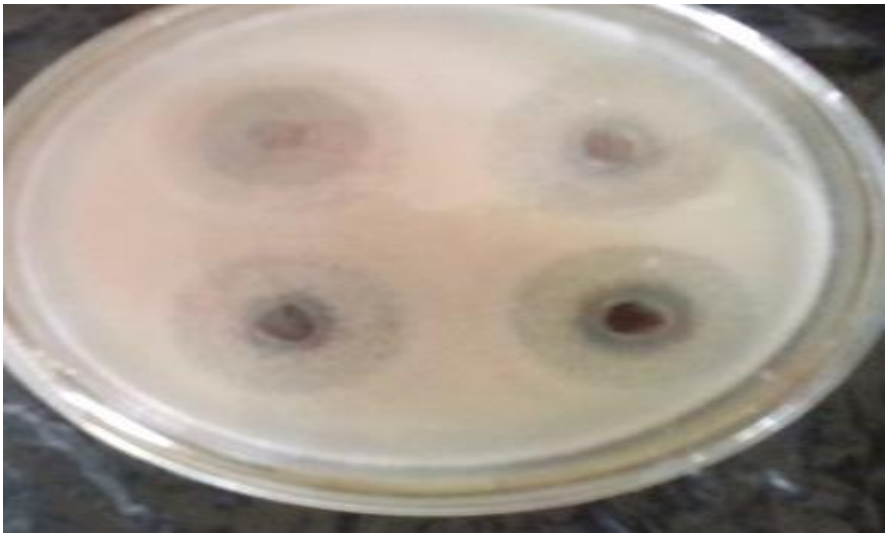

Plate 2. Antibacterial activity of *Alstonia boonei* leaf against *S. typhi*

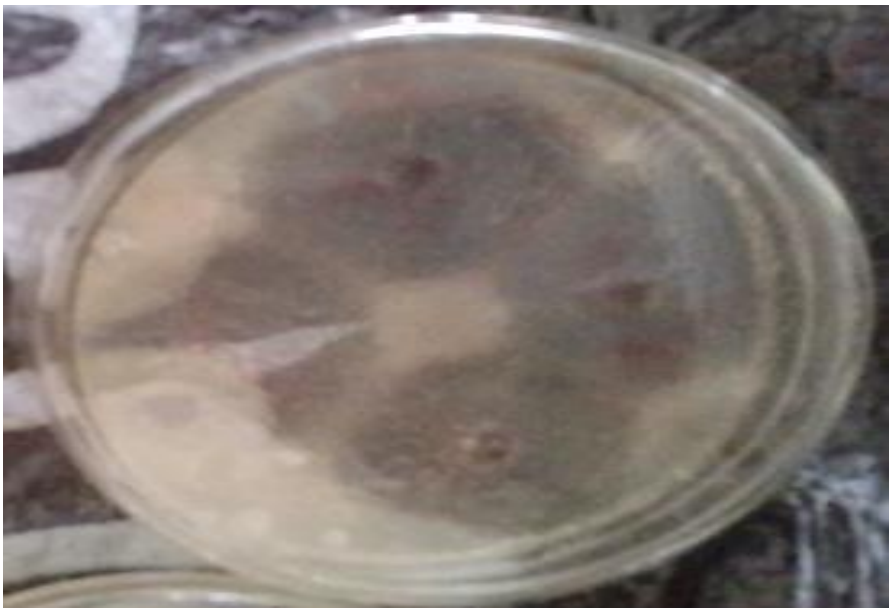

Plate 3. Synergistic effect of *Alstonia boonei* stem bark plus leaf against *E. coli*

leaf that flavonoid was also the most abundant compound with 5.87, while oxalate, tannin and saponin have 3.57, 3.49 and 3.45 respectively. Alkaloid was the least abundant with 2.50

Table 11 showed the quantitative phytochemical analysis of Alstonia boonei stem barks and leaves using ethyl acetate. As observed from the analysis of the stem bark, phytate and oxalate were the two most abundant compounds, at 32.31 and 30.14 respectively. Tannin, phenol and alkaloid have intermediate values of 28.09, 26.09 and 24.03 respectively. Flavonoid and saponin are the least abundant compounds at 6.42 and 6.70 respectively. For the leaf extract, it was observed that oxalate was the most abundant compound, having a value of 24.34, phenol with 23.18 and flavonoid being the least abundant with a value of 2.35.

Table 12 showed the quantitative phytochemical analysis of Alstonia boonei stem barks and leaves using N-Hexane. For the stem barks, Phytate, tannin and alkaloid were the 3 most abundant compounds, having values of 21.34, 21.09 and 21.03 respectively. Phenol and oxalate with intermediate values of 20.09 and 20.14 respectively, and saponin and flavonoid detected to be the least abundant compounds with values at 16.72 and 16.42 respectively. It was observed for the leaf extract that oxalate was the most abundant compound with 24.34,

while Phytate, alkaloid and phenol have 20.72, 2034 and 20.18 respectively. Flavonoid was observed to be the least abundant compound with a value of 2.35.

DISCUSSION

For centuries, medicinal plants have been the main source for drugs in many countries, and it is estimated that at least 25% of modern medicine are derived either directly or indirectly from medicinal plants (Prasannabalaji et al., 2012).

The screening of plant extracts for antimicrobial activity has shown that higher plants represent a potential source of novel antibiotics (Afolayan, 2003). A. boonei is commonly found in the rain forest region of Nigeria, along with other plants with good medicinal properties. The main goal of this work was to investigate the antimicrobial properties that might be present in Alstonia boonei leaf and stem bark extract, using dichloromethane as the extracting solvent, to detect whether the bioactive properties possessed by the dichloromethane extract of the leaf and stem bark differ from the bioactive properties present in the plants by extracting with other solvents, to compare the phytochemical composition of the dichloromethane extract of the plant's leaf and stem bark to that possessed by using other solvents, to test the efficacy of the synergistic effect of the stem bark and leaf, as compared to the activities of the leaf, and stem bark against some selected clinical isolates and to provide scientific validation for their use.

In this study, two different parts (leaf and stem bark) of A. boonei plant were extracted using dichloromethane, and were tested for their antibacterial properties against Salmonella typhi, Staphylococcus aureus, Klebsiella pneumoniae, Proteus mirabilis, Escherichia coli, Pseudomonas aeruginosa, and Bacillus subtilis, and antifungal properties against Candida albican, Aspergillus flavus and Trichophyton rubrum.

The crude plants extract tested in this study showed antimicrobial activities against all the test bacterial and fungal isolates. However, differences were observed between their antimicrobial activities. These differences could be attributed to the differences in their chemical composition and amount of the bioactive compounds extracted by the solvent. These compounds usually accumulate in different parts of the plants (Raquel, 2007, and such secondary metabolites have been found to produce many effects including antibacterial and antiviral properties, this is in line with the observation of Dwivedi et al., 2015.

The zones of inhibition for the dichloromethane extract of A. boonei leaf range from 24mm – 14mm at a concentration of 100mg/ml, and 10mm- 3mm at 6.25mg/ml. Baillus subtilis was discovered from this study to be the most susceptible organism to the dichloromethane extract of A. boonei leaf, while Klebsiella pneumoniae was seen to be the least susceptible to this extract. Other test organisms showed very good susceptibility to the leaf extract. The dichloromethane extract of A. boonei leaf, as observed from this study, can be a source of a novel antimicrobial agent, especially with good activities against organisms like P. aeruginosa and K. pneumoniae, which are resistant to the antibiotic Levofloxacin, and are seen to be susceptible to this extract. Alstonia boonei leaf was also very active against some fungal isolates used in this study, with Candida albicans being the most susceptible fungal isolate among the tested isolates, having 18mm zone of inhibition at 100mg/ml and 6mm zone of inhibition at 6.25mg/ml. The most glaring aspect is the antifungal activity of dichloromethane extract of Alstonia boonei leaf having relatively good antifungal activity against Trichophyton rubrum at a concentration of 25mg/ml, a fungal isolate that was resistant to 50mg/ml fluconazole, this observation goes in line with the finding of (Raquel,2007).

The dichloromethane extract of Alstonia boonei stem bark, also showed comparatively great antimicrobial activity against the test organisms (bacteria and fungi) used in the study. E. coli was the most susceptible bacterial isolate with 21mm zone of inhibition at 100mg/ml and 9mm zone of inhibition at 6.25mg/ml, while C. albicans was the most susceptible fungi isolate with 18mm and 4mm at 100mg/ml and 6.25mg/ml respectively. Dichloromethane extract of A. boonei stem bark also exhibited good inhibitory activities against P. aeruginosa and K. pneumoniae, which are bacterial resistant to Levofloxacin, an antibiotic used in the study for positive control. Its activity was also novel on Trichophyton rubrum, with comparatively better zone of inhibition against the organism (11mm at 100mg/ml and 3 at 12.5mg/ml) as shown in table 4.2 compared to the leaf extract, and the organism was resistant fluconazole at 50mg/ml the antifungal agent used in the study, this goes in line with the finding of Prsannabalaji et al., 2012.

From the study, the antibacterial and antifungal activities of the synergistic effect of the DCM extract of A. boonei leaf plus stem bark was also shown in table 4.3. E. coli was observed to be the most susceptible organism to this extracts, having zones of inhibition to be 25mm and 10mm at 100mg/ml and 6.25mg/ml respectively. K. pneumoniae was the least susceptible with 15mm and 3mm at 100mg/ml and 6.25mg/ml respectively as shown in table 4.3. The antifungal activity of these extracts as seen in this table also showed that C. albicans was the most susceptible fungi out of the test fungal isolates. The antimicrobial activities of the synergistic effect of A. boonei stem bark plus leaf was observed to be more potent than other extracts used in the study. Although, it was observed that, the leaf has more inhibitory effect than the stem bark plus leaf on some organisms, but on a larger term, with its effect on all the organisms used in this study combined, stem bark plus leaf was observed to be very potent, especially due to its activity on P. aeruginosa and K. pneumoniae which are resistant to Levofloxacin, and its activity on T. rubrum which is resistant to fluconazole.

The minimum inhibitory concentration (MIC) value of the stem bark was shown in table 4.4, and it was shown that the dichloromethane extract of A. boonei stem bark showed different MIC values against the test bacterial and fungal isolates. S. aureus and E. coli were shown to have the least MIC values with their growth inhibited at 0.5mg/ml respectively, while K. pneumoniae was the bacterial isolate with the highest MIC value, having its growth inhibited at 8mg/ml. P. mirabilis and P. aeruginosa also have relatively low MIC values and are at 2mg/ml respectively. Candida albican and T. Rubrum were the fungal isolates with the least MIC values from this table, and were inhibited at 8mg/ml respectively, while A. flavus was the highest with MIC value at 16mg/ml.

Table 5 above. showed the MIC values of DCM extract of A. boonei leaves against the test bacterial and fungal isolates and B. subtilis, P. mirabilis and P. aeruginosa were the bacteria with the least MIC values, having their growth inhibited at 0.5mg/ml respectively, and K. pneumoniae and E. coli with the highest values at 4mg/ml respectively. The fungal isolates showed rather higher MIC values, with C. albicans having the lowest MIC value among the test fungal isolates and the MIC value is at 4mg/ml. T. rubrum was the fungal isolate with the highest MIC value, with its growth inhibited at a concentration of 8mg/ml.

The positive control and negative controls used in the study are 50mg/ml of Levofloxacin and fluconazole for bacteria and fungi respectively, and 25%DMSO for negative control, have their data presented in table 4.1. The different zones of inhibition at 50mg/ml concentration for the test organisms were also recorded. From this study, it was observed that S. aureus was the most susceptible bacteria to Levofloxacin, with 28mm zone of inhibition, and P. mirabilis with the least susceptibility with 19mm zone of inhibition. K. pneumoniae and P. aeruginosa were not susceptible to Levofloxacin at the concentration used in the study. C. albican and A. flavus have 19mm and 14mm zones of inhibition to fluconazole respectively, while T. rubrum was shown to be resistant. 25%DMSO was used for the negative control, and from the study, it was observed that the 25%DMSO which was used for reconstituting was not inhibitory to the growth of any organisms. Therefore, DMSO was not responsible for any antimicrobial activities observed in the study which also goes in line with the observation of Prasannabalaji et al., 2012.

The qualitative phytochemical screening of Alstonia boonei stem bark (dichloromethane) revealed the presence of medicinally active constituent such as alkaloid, flavonoid, tannins, saponin, reducing sugars, steroid, phenol and anthraquinone (table 8 above), while for leaf revealed various active constituents such as cardiac glycoside, steroids, phenol, tannins, saponin, and flavonoid, some of which have been previously associated with antibacterial activity as observed by Nweze et al., 2004.

These biologically active constituent is known to act by different mechanism and exert antimicrobial action (Oliver, 2000). 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 (Marjorie, 2009). The antimicrobial property of saponin is due to its ability to cause leakage of proteins and certain enzymes from the cell (Zablotowicz et al., 2006). 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 (Raquel, 2007).

The quantitative phytochemical screening of A. boonei stem bark, and leaf using different solvents, showed the presence of different phytoconstituents in different quantities. For stem bark using methanol, flavonoid was shown to be present in the largest quantity with 4.55, and oxalate was found to be the least abundantly present with 2.10. For the leaf, using the same solvent, flavonoid was also found to be the most abundantly present at 5.87 and phenol being the least abundantly present with 2.49 as shown in table 4.14. The quantitative phytochemical screening of A. boonei stem bark and leaf using ethyl acetate, showed the presence of these phytoconstituents in a quite larger abundance. For the stem bark, phytate was found to be the most abundant with 32.31, followed by oxalate with 30.14, and phenol and alkaloid having 26.09 and 24.03 respectively, while for the leaf, oxalate was mostly abundant with 24.34, followed by phenol at 23.78, and phytate and alkaloid at 20.72 and 20.34 respectively as shown in table 4.15. The greater abundance of the ethyl acetate extract of A. boonei stem bark and leaf could indicate that, the crude ethyl acetate extract will possess antimicrobial properties than the methanol extract of this same plant.

CONCLUSION

Plants are found in nearly all the regions of the world. The prevailing climatic, soil and environmental conditions often play a vital role in determining the type of plant species that could be found in such region. Due to the challenges associated with drug resistance, which have made scientists to search for effective and sustainable means of managing the problem. Plants have emerged as an alternative to synthetic antibiotics which is prone to reoccurring drug resistance. The result of antimicrobial susceptibility assay showed promising evidence for the antimicrobial effects of A. boonei against bacterial (P. aeruginosa, K. pneumoniae, B. subtilis, P. mirabilis, E. coli, S. aureus and S. typhi), and fungal (C. albican, T. rubrum, and A. flavus) isolates used in this study. The MIC value of

different organisms are verified, and thus, MIC are assays capable of verifying that the compound has antimicrobial activities, and that it gives reliable indication of the concentration of medicine required to inhibit the growth of microorganisms. Phytochemical analysis is 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.

RECOMMENDATION

From the result obtained from the antimicrobial activity of this plant, I recommend that Alstonia boonei should be used as antimicrobial agent against infections caused by susceptible organisms. I also recommend that Alstonia boonei stem bark and leaf should be used synergistically, because of the great antimicrobial property it exhibited in this study.

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