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Isolation and susceptibility patterns of bacteria associated with lung diseases

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Abstract

Lung diseases have been a worldwide health problem,with high endemicity in developing countries. Bronchopneumonia remains the main disease of the lung. Despite advances in modern medicine, there is no-effective drug available that stimulate lung function, offer protection to the lung from damage or help to regenerate pulmonary cells destroyed by disseminated bacteria. There is an urgent need for effective drugs to replace/supplement those in current use. This study was embarked upon to evaluate the effect of *Asplenium bulbiferum* leaf extracts on nosily infected mice with S*treptococcus pneumoniae* isolate of suspension containing $5x10^6$ cells/*ul*. *Streptococcus pneumoniae* was obtained from human specimen, identified through cultural, morphological and biochemical examination, in addition, polymerase chain reaction (PCR) technique was performed using universal primers to support the identification process. The antibacterial activity was carried out using agar well diffusion technique. Tube dilution technique was used to determine the minimum inhibition concentration (MIC) and minimum bactericidal concentration (MBC) using double fold serial dilution at concentrations of 400mg/ml to 25mg/ml. The effect of the leaf extract was evaluated by treating *mus musculus*_mice (WBC count $4.5x10^{3)}$ with sub-MIC of *A. bulbiferum* extracts after being nosily infected with *S. pneumoniae* and appearance of disease septum with $11.3x10^3$ WBC count after 5days from infection. The photochemical analysis revealed the presence of alkaloids; phenolics, tannins, saponins, flavonoids, steroids and glycosides. The ethanolic extract exhibited more activity than the aqueous extract against *S.aureus* most followed by *S. pneumoniae, H. influenza* and *K. pneumoniae* was the least. After treating the infected mice with the plant extract for 6 days from infection, the WBC count reduced to $5.1x10^3$µ*l* which is normal range and the mice were healthy with good physiological behaviour. For the infected untreated mice, the WBC and other immunological parameters remain high even after 16 days from infection. The study showed that *Asplenium bulbiferum* possessed antibacterial properties and could serve as alternative therapy for ameliorating lung infections.

Keywords: Lung disease; *Stretococcus pneumoniae; Asplenium bulbiferium;* Antibacterial activity

1. Introduction

Lung is one of the most vital organs in the body. It plays a pivotal role in life supportive respiration, that is inhalation and exhalation of air. It is the principal organ for transporting atmospheric oxygen into the blood and releasing carbon dioxide back into the atmosphere [1] In fact, human health is dependent on the individual health of the various internal systems. The natural environment within each lung is moist. This condition makes the organ most susceptible to bacterial and viral infection [2] The role played by the lung is one of the most vital organs in the body. It plays a pivotal role in life supportive respiration, that is inhalation and exhalation of air. It is the principal organ for transporting atmospheric oxygen into the blood and releasing carbon dioxide back into the atmosphere. It is this process of inhalation and exhalation that makes it more prone to challenges of foreign compounds culminating in lung dysfunction [3]. Bacteria are the most common cause of community-acquired pneumonia (CAP), with *Streptococcus pneumoniae* isolated in nearly 50% of lung disease cases [4]. Other commonly isolated bacteria include *Haemophilus influenzae* in 20%

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Chlamydophila pneumonia in 13%, and *Mycoplasma pneumoniae* in 3% of cases [5].*Saphylococcus aureus, Moraxella catarrhalis, Legionella pneumonia* and other gram- negative bacilli [5].

Molecular techniques are major tools for the characterization of bacteria, from food and other biological substances [5]. Polymerase chain reaction (PCR) is a biochemical technique in molecular biology used to amplify a single copy of DNA or a few copies of DNA across serial order of magnitude, generally thousands to million copies of a particular DNA sequence [6]. The polymerase chain reaction (PCR) is now a common and often indispensable technique used in medical and biological research laboratories for a variety of applications. These include DNA cloning for sequencing, DNA based phylogeny, or functional analysis of genes; the diagnosis of hereditary diseases; the identification of genetic fingerprints (used in forensic sciences and paternity testing); and the detection and diagnosis of infectious diseases [6].

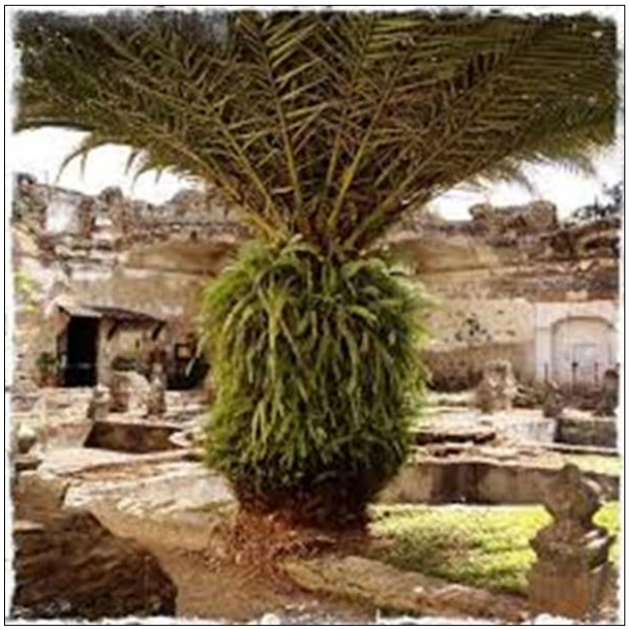


Figure 1 Fern plant on palm tree

Figure 2 Fern plant

Lung diseases remain one of the major threats to public health and are worldwide problem [7]. They are mainly caused by bacterial and viruses, chemicals, fumes, organic and inorganic substances, radiation cancer treatment, administration of chemotherapy drugs, autoimmune disorder and infections [2] and [8]. Infection due to bacteria is the main cause of a number of respiratory illness. Lung inflammations or infections include common cold and cough, chronic obstructive pulmonary disease; cystic fibrosis (C.F.); asthma; pneumonia, pleurisy, etc [9] and [8]. Bacteria and viruses that are inhaled thrive most in the lung environment if the immune system is not strong enough to fight initial contact. This makes infection inevitable, adversely affecting lung capacity. The inflammation also affects the original maximum

capacity of the lungs to hold a particular volume of air and facilitate maximum inhalation [9]. Lung diseases and infections can also be triggered by occupation and environmental factors that leads to a long-term exposure to pollutants and toxins [10]. This is commonly the case with people working in the presence of asbestos and silica dust.

In spite of the tremendous advance in modern medicine, there is no effective drug available that stimulates lung function, offer protection to the lungs from damage or help to regenerate thoracic cells due to multiple drug resistance by microbes (superburg) such as methicillin resistance *Staphylococcus aureus* (MRSA). It is therefore, necessary to search for alternative drugs for the treatment of lung diseases to replace currently used drugs of doubtful efficacy and safety. Medicinal plants play a key role in human health care [11]. About 80% population of the world rely on the use of traditional medicine, which is predominantly based on plant material (WHO, 2003). Scientific researches available on medicinal plants indicated that promising phytochemicals can be developed for many health problems [12]. Reports on ethno- botanical records indicated a general consensus on the use of antimicrobial active medicinal plants to provide cheaper drugs [11]. There is need to search for new and potent compounds of natural origin to complement the existing synthetic drugs that are gradually becoming less potent against pathogenic organisms. The use of natural remedies for the treatment of lung diseases has a long history and medicinal plants and their derivatives are still used all over the world in one form or another for this purpose. Lung protective plants contain a variety of chemical constituents like phenols, coumarins, monoterpenes, glycosides, alkaloids, tannins and xanthenes. Different plant parts and components (roots, leaves, stem barks, flowers or their combinations, essential oils) have been employed in the treatment of infectious pathologies in the respiratory system, urinary tract, gastrointestinal [13]. *Asplenium bulbiferum* (fern) commonly known as "*Ibu*" in South Eastern Nigeria belongs to the family pteridophytes, and a native in India, North America, Europe, Iran, Northern Austeralia, Malaysia, Papua New Guinea, and other parts of Asia. *Asplenium bulbiferum* grows on a variety of trees particularly palm trees, mango, and lime across Nigeria [14]. *Asplenium bulbiferum* is an evergreen parasitic plant growing on the branches of trees where it forms bushes 3 to 7 feet in diameter.

Asplenium bulbiferum contains glycoside, saponin, alkaloids, flavonoids, tannins, phenols, and steroids [15] and [16]. *Asplenium bulbiferum* (fern) is said to be derived from the celtic word" all heal". This correlates with its historical use for every ailment [8]. Some studies have investigated *Asplenium bulbiferum* scientifically and it was found to possess a number of notable pharmacological effects on lung diseases [12] , immune system [13] and lung infection induced by bacteria such as *Streptococcus pneumoniae* as well as secondary infections caused by chemicals.

1.1. Statement of problem

In spite of the tremendous advances in modern medicine, there is no effective drug available that stimulates lung function, offer protection to the lungs from damage or help to regenerate thoracic cells due to multiple drug resistance by microbes (superburg) such as methicillin resistance *Staphylococcus aureus* (MRSA).

Aim

To evaluate the effect of leaf extract of *Asplenium bulbiferum* on bacterial induced-lungs disease.

Objectives of study

- To isolate and characterize microorganisms associated with lung infection;
- To conduct molecular characterization of the isolates
- To conduct phytochemical analysis of the fern plant;
- To carryout susceptibility testing of the isolates using *Asplenium bulbiferum* leaf extract;
- To conduct pathogenicity, toxicology tests and histopathology analysis of the isolates on immunocompetent and immunocompromised laboratory animals, and
- To compare antibacterial effect of the *Asplenium bulbiferum* extract with that of the commercially available antibacterial agents using disc diffusion and agar- well diffusion methods.

2. Material and methods

2.1. Sample collection and classification

In this study, sputum samples were collected from patients suffering from lung diseases at General Hospital, Onitsha., St. Charles Borromeo Hospital, Onitsha and Nnamdi Azikwe University Teaching Hospital, Nnewi. All the hospitals are in Anambra State, Nigeria. Each sample was classified according to names, genders and age of the patients.

2.2. Collection of sputum sample was as follows

One hundred and fifty samples were collected from each of the above mentioned hospitals for analyses. Each patient was given two containers labeled specimen A and B respectively, the containers were cleaned, dried, wide-necked, and leak-proof. Then the patients were requested to cough deeply to produce sputum specimen into the container [17].

2.3. Collection of Plant Materials

2.3.1. Collection, authentication and processing of plant materials

The fresh leaves of *Asplenium bulbiferum* were collected from Umuoma, Uli, Ihiala Local Government Area, Anambra State, Nigeria. The plant materials were identified and authenticated by a Botanist at the Biological Science Department of Nnamdi Azikwe University, Awka, Nigeria. Confirmation of taxonomic identity of the plants was achieved by comparison with voucher specimens kept at the Herbarium of the Department of Biological Sciences, UNIZIK and use of documented literature [18]. The plant materials were air-dried in the laboratory for four weeks and then ground into powdered form, weighed and kept ready for extraction of active ingredients. It was then stored according to methods of [18].

2.4. Sterilization of Glass wares

The glass wares were washed then sterilized using electric oven. These were air dried and placed inverted inside the oven at 160°C for 3 h.

2.4.1. Isolation and Identification of Test Organism

The test organisms used in this study were isolated from the sputum of the infected patients. The purulent part of the sputum was washed in about 5ml of sterile physiological saline. The samples were plated on blood agar and chocolate agar plates then incubated aerobically at 37°C for 24 h. The organisms obtained were aseptically subcultured on nutrient agar plate and incubated at 37°C for 24 h. The pure cultures of the test organism were identified using morphological characteristics, gram staining and biochemical reactions [19].

2.4.2. Gram staining

This was carried out using the modified method of [1]. In this process, a thin smear of the culture was prepared on a clean grease free slide, air dried and heat fixed. The smear was flooded with crystal violet solution for 60 seconds and rinsed with water. It was then covered with Gram iodine for 60 seconds and rinsed with water. Alcohol (95% w/w ethanol) was used to decolorize the slide content for 10 seconds and rinsed with water. The smear was then counter stained with safranin solution for 60 seconds, rinsed with water and air-dried. A drop of oil immersion was applied on each stained smear, then they were observed under the light microscope using oil immersion objective lens.

2.4.3. Motility test

This was carried out using the modified method of [1]. The medium used was semi-solid agar. It was prepared by adding 4 g of bacteriological agar to 15 g of nutrient broth in 1 litre of deionized water. Heat was applied to dissolve the agar and 10 ml amount were dispensed into test tubes and sterilized by autoclaving. The test tubes were allowed to set in a vertical position. Inoculation was done by making a single stab down the center of the test tube to about half the depth of the medium using a sterile stabbing wire. The test tubes were incubated at room temperature and growth examined after about 6 hours. Motile bacteria swarm gave a diffused spreading growth that was visible to the naked eye.

2.4.4. Oxidase test

This was carried out using the modified method of [1]. Oxidase reagent containing 1.0% (w/v) tetramethyl-p-phenylenedimine dihydrochloride was prepared by dissolving 0.1 g of this compound in 10 ml of deionized water. Strip of filter paper was soaked with this reagent, smears of the isolate from the pure cultures were made on the oxidase paper strips and observed for color change from gray to purple or violet color between 1-5 seconds for oxidase positive organisms. For oxidase-negative bacteria, there was no color change. The change of color was due to the possession of cytochrome.

2.4.5. Methyl-Red Voges-Proskauer (MR-VP) test

Biochemical method was carried out according to [1] , 7.5 g of MRVP medium (oxoid) was dissolved in 500 ml of deionized water. Ten millitres (10 ml) of the medium were taken and dispensed into test tubes. The medium was then sterilized and cooled. The test tube was divided into two equal sets, one for methyl red (MR) test and the other for voges

proskauer (VP) test. Isolates from the pure stock culture were then inoculated into different sets of test tubes and labeled. The test tubes were incubated at 37°C for 24 h. For methyl red, 2 ml of MR reagent was added to the test tube of the culture and color changes was observed. From yellow to red indicated positive result while the persistence of the yellow color indicated a negative result. Six drops of Barritt reagent (x-naphthol solution) was added to the broth tubes, followed by the addition of 5 drops of 40% (w/v) potassium hydroxide solution. The presence of pink- red coloration showed a positive result while absence of color change was regarded as VP-negative.

2.4.6. Indole test

Biochemical method was carried out according to [17] , Indole is a nitrogen-containing compound formed when the amino acid tryptophan is hydrolyzed by bacteria that have the enzyme tryptophanase. This was detected by using Kovac's reagent. For this test, isolates were grown in peptone water (oxoid) in 500 ml of deionized water. This was dispensed into test tubes in 10 ml amounts and sterilized. The medium was then inoculated with the isolates and incubated at 37°C for 24 h. Five drops of Kovac's reagent were carefully layered onto the top of the 24 hours old culture. The presence of indole was revealed by the formation of red layer colouration on top of the broth culture.

2.4.7. Sugar fermentation

Biochemical method was carried out according to [17] , this test was carried out to know the ability of the isolates to metabolize some sugars (glucose, sucrose, lactose, manitol etc.) with the resultant production of acid and gas or either. Into 1 litre of 1% (w/v) peptone water was added to 3 ml of 0.2% (w/v) bromocresol purple and dispensed in 9 ml amounts, into tests that contained inverted Durham tubes. The medium was then sterilized by autoclaving. The sugar solutions (glucose and sucrose) were each prepared at 10% (w/v) and sterilized. One millitre (1 ml) amount of each sugar was dispensed aseptically into the test tubes. The medium was then inoculated with the appropriate isolates and the cultures incubated at 37°C for 48 h and was examined for the production of acid and gas. A change in color from purple to yellow indicated acid formation while gas production was assessed by the burbles in the inverted Durham tubes.

2.4.8. Coagulase test

Biochemical method was carried out according to [17] , this test was used to identify those organisms that can produce the enzyme coagulase. A drop of distilled water was placed on each end of a slide. This was emulsified by a colony of the test organism. A drop of plasma was added to one of the suspensions and mixed gently. Clumping of the organism within 10 seconds indicated a positive result.

2.4.9. Bile solubility test

This helps to differentiate *S. pneumoniae,* which is soluble in bile and bile salts, from other alpha-hemolytic *streptococci* (viridians streptococci which are insoluble. This was done by tube method.

A heavy inoculums of the test organism was emulsified in physiological saline and the bile salt sodium deoxychocolate was added. This dissolves *S. Pneumoniae* as shown by clearing the turbidity within 10- 15 minutes. Viridians and other streptococci were not dissolved and therefore, there is no clearing of the turbidity (Monica Cheesbrough *et al.,* 2005).

2.5. Molecular characterization of the isolates using PCR

This stage of the study was carried out at Faculty of Pharmacy Nnamdi Azikiwe University, Agulu in Department of Microbiology.

The method of polymerase chain reaction (PCR) was fully adopted [6]. This is a biochemical technique in molecular biology used to amplify a single copy of DNA or a few copies of DNA across serial order of magnitude, generating thousands to millions copies of a particular DNA sequence. The purpose of PCR is to make a huge number of copies of gene. This is necessary to have enough starting template for sequencing. There are three major steps in a PCR, which are repeated for 30 or 40 cycles. This is done in an automated cycler which can heat and cool the tubes with the reaction mixture in a very short time. These include denaturation, Annealing and extension /Elongation [6].

2.5.1. Procedure

The following protocol is designed for up to 200μl of biological liquid sample including colony stimulating factor (CSF), buffy coat, body fluid (semen), and cell suspensions containing less than $5x10^6$ cells.

- 800µl Genomic Lysis buffer was added to 200µl liquid sample of the isolates. Mixed briefly by vortexing, then stood at room temperature for 5 minutes. 1m1 of the supernatant was transferred to the zymo-spin™ column.
- The zymo-spinTM column was transferred in a collection tube and centrifuged at 10,000xg for one minute then the collection tubes with the flow through were discarded.
- The zymo- spin™ column was transferred to new collection tubes. 200µl of DNA pre-wash buffer was added to the spin- column centrifuged at 10,000xg for one minute.
- 500µl of g- DNA wash buffer was added to the spin columns, then centrifuged at 10,000xg for one minute.
- The spin column was transferred to clean microcentrifuge tubes. 50µl DNA elution buffer was added to the spin column. Incubated for 4 minutes at room temperature and then centrifuged at top speed for 30 seconds to elute the DNA. The eluted DNA was used immediately for the following molecular analysis based on the PCR amplifications of 16s rRNA gene. The above DNA extracted from the isolates were subjected to a single PCR essay targeting species specifically 16s rRNA gene were carried using the following universal primers.

(F:5'-GCGCAATCGCGTCAGGTAATG-3')
(R:5 GCTAAGAAGAAGCAGCCTATGTCC-3')

Both primers and template DNA were added into PCR kit (Gotaq, promega, USA) following the manufacture's guide. 30 cycles were performed in a themocycler, each cycle followed, has three steps of denaturation (94^0C FOR 1 min), annealing (55^0C for 1 min), extension (72^0C for 3min) and final extension of 72^0C for 5 minutes. The amplified products were detected by electrophoresis which was later viewed under UV light and shown positive results by bands that correspond to the control model bands (figure 3 and 4).

2.6. Preparation of Plant Extract

The powdered plant material (20g) leaf was percolated in ethanol (200ml) in 250ml conical flask, stoppered and kept for two weeks intermittent shaking. The percolates were filtered with Whatman's No. 1 filter paper. The extracts were concentrated at 40^0C under reduced pressure using rotary evaporator (R1-10). The same quantity of plant material was again percolated with distilled water for one week after filtration, the aqueous extract was concentrated in hot oven at 40^0C [18]. The concentrated extracts were labelled ALE (*Asplenium bulbiferum* ethanol leaf extract) and ALA (*Asplenium bulbiferum* aqueous leaf extract).

2.7. Phytochemical Analysis

Phytochemical analysis for qualitative detection of alkaloids, flavonoids, tannins and saponins was performed on the extracts as described by [15] and [16].

2.7.1. Quantitative determination of the presence of phytochemicals

2.7.2. Alkaloids

Five milliliters (5 ml) of the sample was mixed with 96% ethanol-20% tetraoxosulphate (vi) ac**2** id (1:1). One milliliter (1ml) of the filtrate from the mixture was added to 5 ml of 60% H_2SO_4 and allowed to stand for 5 minutes. Then, 5 ml of 0.5% formaldehyde was added and allowed to stand for 3 h. The reading was taken at absorbance of 565 nm.

2.7.3. Glycosides

This was carried out using Buljet's reagent. One gram (1g) of the fine powder of the sample was soaked in 10 ml of 70% alcohol for 2 h and then filtered. The extract was then purified using lead acetate and disodium hydrogen tetraoxosulphate (vi), ($Na_2H_2SO_4$) solution before the addition of freshly prepared Buljet's reagent. The absorbance was taken at 550 nm.

2.7.4. Flavonoids

5ml of the extract was mixed with 5 ml of dilute hydrochloric acid (HCL) and boiled for 30 minutes. The boiled extract was allowed to cool and then filtered. One (1 ml) of the filtrate was added to 5 ml of ethyl acetate and 5 ml of 1% ammonia solution. The absorbance was taken at 420 nm.

2.7.5. Phenolics

Ten (10 *ml)* of the sample was boiled with 50ml acetone for 15 minutes. Five millitres of the solution was pipette into a 50 ml flask. Then, 10 ml of distilled water was added. This was followed by the addition of 2 M NH_4OH and 5ml of concentrated amyl alcohol. The mixture was left for 30 minutes and absorbance was taken at 505 nm.

2.7.6. Tannins

Ten (10 ml) of the sample was pipette into 50 ml plastic bottle containing 50 ml of distilled water. This was shaked for 1 h on a mechanical shaker. The solution was filtered and 5 ml of the filtrate was mixed with 2 ml of $FeCl_3$ in O.INHCL. The absorbance was read at 120 nm.

2.7.7. Steroids

The extract was eluted with normal NH_4OH solution. Two millitres (2 ml) of the eluate was mixed 2 ml of chloroform in a test tube. Three (3 ml) of ice cold acetic anhydride was added to the mixture and two drops of concentrated H_2SO_4 was continuously added to the mixture and allowed to cool. The absorbance was taken at 420 nm.

2.7.8. Saponins

Five millitres (5 ml) of the sample was dissolved in aqueous methanol. Then, 0.25 ml taken for spectrophotometric determination for total saponins at 544 nm.

2.7.9. Determination of Extractive Value

The concentration of the extracts were determined by evaporating 1.0 ml of the extracts in evaporating dish of known weight in an oven to dryness and weighed. The dish containing the residue was allowed to cool and then weighed. The weight of the residue was obtained by subtracting the weight of the empty dish from the weight of the dish and residue. The above process was repeated in duplicate [18].

2.8. Maintenance of Test Organisms

The isolated test organisms were used for the antibacterial sensitivity testing. Prior to the test, the organisms were subcultured on nutrient agar plate at 37°C for 24 h. Then the 24 h cultures were transferred into nutrient broth and incubated aerobically at 37°C for 24 h [17].

2.8.1. Standardization of Inoculum

The inocula were prepared from the stock cultures, which were maintained on nutrient agar slant at 4^0C and subcultured onto nutrient broth using a sterilized wire loop. The density of suspension inoculated onto the media for susceptibility test was determined by comparison with 0.5 McFarland standard of Barium sulphate solution [17].

2.8.2. Test Organisms

Staphylococcus aureus, Streptococcus pneumoniae, Streptococcus klebsiella and *Haemophilus pneumoniae* were isolated from sputum samples. This was followed by washing with physiological saline and streaking sputum samples on-appropriate media for isolation. Cultural and morphological identification as well as biochemical characterization of isolates using protocol described by [17].was carried out. Pure cultures of the isolates were maintained in appropriate media in slant for future use.

2.8.3. Susceptibility of the extract

In this study, concentrations of 400 mg/ml of the extracts were used to screen for the antimicrobial activity. This was done by using the modified methods of [1] and [17]. Here, 2.5 g of the extract was dissolved in each of the extracting solvents.

2.9. Antimicrobial disc preparation

Conventional antibiotics using disc diffusion method. Here, a growth medium, usually Mueller- Hinton agar was first evenly seeded throughout with the isolates that has been standardized using McFarland standard concentration (0.5 colony forming units per ml). Commercially prepared discs, each of which are pre-impregnated with a standard concentration of a particular antibiotic, are then evenly dispensed and lightly pressed onto the agar surface. The test antibiotic begins to diffuse outward from the disc, creating a gradient of antibiotic concentration, in the agar such that the highest concentration is found close to the disk with decreasing concentration further away from the disc. After an

overnight incubation, the bacterial growth around each disc is observed. If the test isolate is susceptible to a particular antibiotic, a clear area of ''No growth'' will be observed around that particular disc.

The zone around an antibiotic disc that has no growth is referred to as the zone of inhibition since approximates the minimum antibiotic concentration sufficient to prevent growth of the isolate. This zone is then measured in mm and compared to a standard interpretation chart used to categorize the isolate as susceptible, intermediately susceptible or resistant [17].

2.9.1. Antibacterial Assay

Antibacterial sensitivity testing was determined by using the agar well diffusion method. Stock solution of 400mg/ml each of the plant extracts were diluted separately, five- fold serially in 1m1 of dimethyl sulphoxide (DMSO) to obtain different concentrations (200mg/ml, 100mg/ml, 50mg/ml and 25mg/ml). A 0.1m1 of standized broth culture of bacteria isolates were seeded into a concentrations using a sterile cork borer of 6mm. Approximately 0.1ml of plant extracts were introduced into each of the well respectively. The set up was allowed to stand for 1 h for pre-diffusion before incubation at 37^0C for 24 h. At end of the incubation period, the antimicrobial activity of the isolated compounds was evaluated by measuring the inhibition zone diameter (IZD) against 0.05% cephalosporin was used as positive control [20].

2.9.2. Determination of minimum inhibitory concentration (MIC)

The minimum inhibitory concentration of bacterial isolates was carried out using tube dilution technique. Here, 0.5ml equivalent to $1x10^6$CFU stock solution of 400mg/ml which gave various concentrations of (200mg/ml, 100mg/ml, 50mg/ml and 25mg/ml), that were made from the original solution of 400mg/ml was assayed against the test bacterial. The MIC was defined as the lowest concentration able to inhibit any visible bacterial growth. This was determined and recorded [20].

2.9.3. Determination of minimum bacterial concentration (MBC)

Here, equal volumes of various concentrations of those tubes that did not produce any growth from MIC was plated on fresh sterile poure plate and incubated aerobically at 37^0C for 24 h. The lowest concentration of the extracts that killed the test bacterial was taken as the MBC [20].

2.9.4. Acute toxicity studies

This was carried out using the modified method of [21]. The animals (Mice) were grouped into ten (10) and each group comprised of three mice.

The average weight of each group was taken using electric weighing balance. Group 1 and 2 were administered orally with 1.0ml of the extracts (400mg/ml) these mice were left for 72 h and result were recorded.

2.10. Pathogenicity

In this study, pathogenicity of the isolate was carried out using the methods of [21].The test animals used for this study were mice (breed in animal house) Edem- Ani Road, Nsukka, Enugu State. Both male and female laboratory *mus musculus* (white albino) weighing 30-35g, 10-12 weeks in age. They were kept singly in metabolic cages under room temperature 35-37^0C. The mice were randomly divided into 2 groups, each containing 7 and 3 mice respectively. A total number of 10 mice were used for this study. The above two (2) groups were called immunocompromised and immunocompetent. The immunocopromised include (A ,B, C, E, F and G) while the immunocompetent include (H,I and J). The immunocopromised were starved for two days while the immunocopetent were fed very well with 20g of mice chow and water. The mice were infected with *Streptococcus pneumoniae* isolate suspension containing $5x10^6$ cells ml [22] by exposing them to the aerosol coming out from the prepared isolate put in a special container intranasally (i.n.). The mice were observed for clinical manifestation for 4-16days to detect changes such as noticeable labored breathing, dyspnoea, heavy mucus on their nostrils, unsual snizzing, etc. When the symptoms of disease appeared, animals were isolated and placed in a separate cages with the recommended food and water. Among the immunocopromised group, mice A, C, D and E. Note, mouse B showed no symptoms of disease after exposure to the isolate. Mice A, C, D and E were administered the sub-MIC of plant extract twice daily orally for 6 consecutive days using different concentrations of the extracts (200,100,50 and 25mg/ml). Then mouse F was administered the cephalosporin (positive control) and the last (mouse G) was left untreated. The remaining three mice were exposed to distilled water, cephalosporin and vanconmycin respectively. The distilled water served as negative control while cephalosporin and vancomycin were used as positive controls.

2.11. Histopathological study

This study was carried out using the modified method of [22] at University Teaching Hospital Enugu, in the Department of histopathology. After 4 weeks, the mice were autopsied. The lungs were removed, portion of this organ were washed with PBS and stored in formalin solution for histopathological examination.

2.12. Examination of protected mice

The protected mice carefully observed for the clinical manifestation of the inoculated organisms for period of 2 weeks, the protection rates of the inhibitory substances were determined, and the mice were sacrificed and gross examination of the morphologies of the lungs was carried out. Also the lungs were harvested and some portions of this organ was cultured on TCBS agar, and incubated at 37^0C for 48 H. The counts were taken and the colonies were identified morphologically and biochemically [17]. The remaining portion of the organ was subjected to hispathological examination [22].

2.13. Toxicological study

Total WBC count: Blood samples were obtained from the lung of anesthetized mice as described above and put in heparinised tubes, mixed well, then the WBC was counted using culture instrument and classical way [23].

In vivo effect of *Asplenium bulbiferum* leaf extract on the above animals used for pathogenicity testing were adapted and used for this study.

In vivo antimicrobial activity: nosely infected mice with *Streptococcus pneumoniae* S6B isolate of suspension containing $5x10^6$ cells/mL [22]. The mice were randomly divide into 3 groups the first group include mouse H (disease control) received distilled water only, the second group include (A,C,D, and E) showed clinical manifestation after exposing them to the challenges of bacterial suspension and also received the sub-MIC of plant extract; then the third group which includes (mouse G was left untreated while mouse F received cephalosporin antibiotics.

Enumeration of isolate cells in the blood was performed according to [24] during 0,4,8 and 12 days from infection. The frequencies of WBC and deferential WBC were evaluated [23] for 4 consecutive days and the animals were observed for 12 days from the day on which the disease was induced and the death rate was recorded.

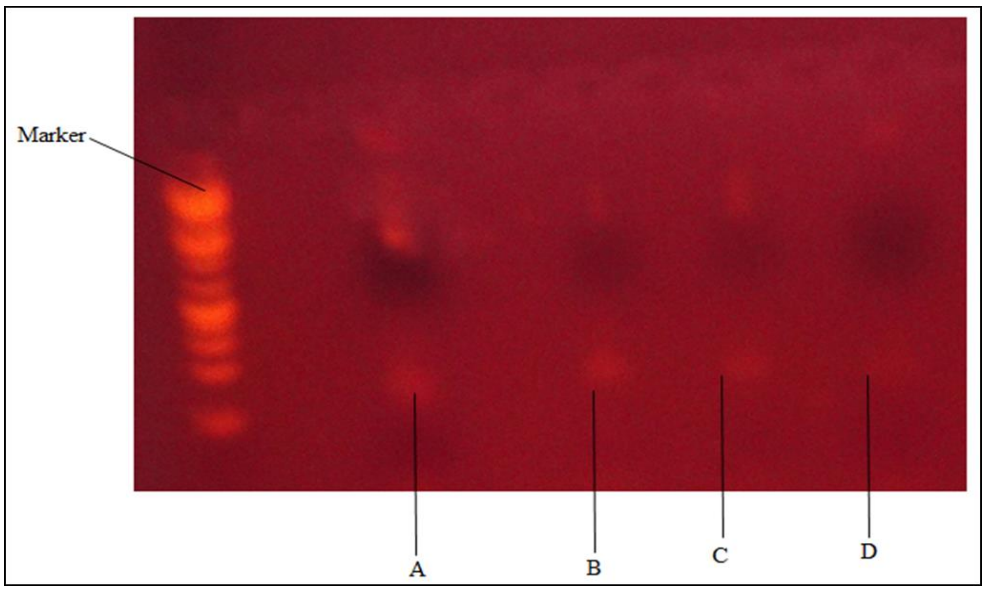
3. Results

Four isolates (*S. pneumoniae, S. aureus, H. influenzae* and *K. pneumoniae*) were obtained from sputum samples of patients with one complaint of chest infection and the other at University Teaching Hospital, Nnewi (NUATH), Onitsha General Hospital and Borromeo Hospital all in Anambra State. These isolates were identified culturally, morphologically and biochemically in addition to PCR molecular characterization technique (Table 1).

Table 1 Characteristics and identities of the tested organisms

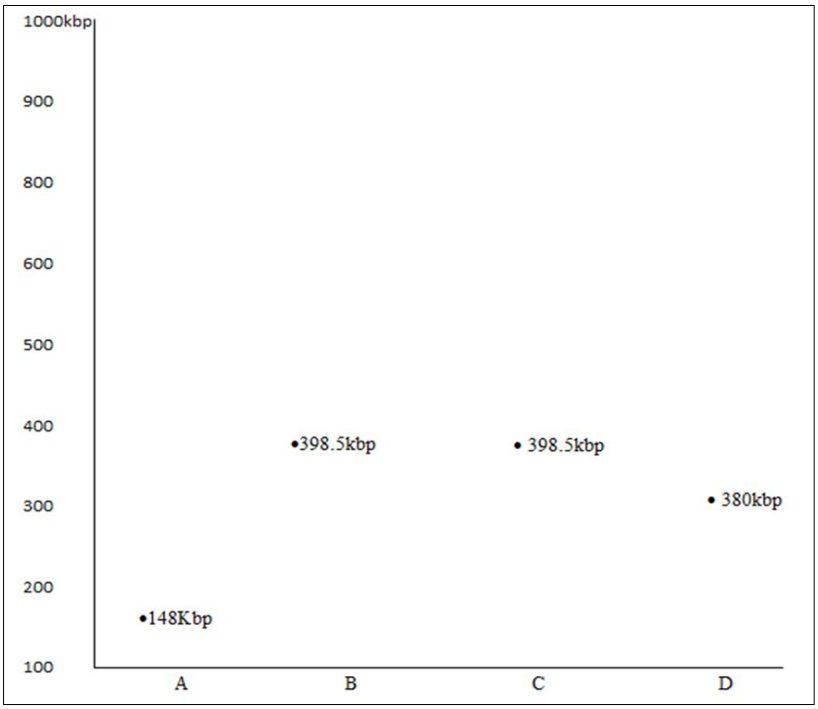
Parameter	*S. pneumoniae*	*S. aureus*	*K. pneumoniae*	*H. influenzae*
Appearance blood agar	Greenish	Milkish	Redish	Milkish
Margin	Entire	Entire	Entire	Horizon
Gram reaction	+	+	-	-
Catalase	-	+	-	-
Oxidase	-	-	-	+
Motility	-	-	-	+
Coagulase	+	+	+	-
Indole	+	-	-	+
MR test	-	-	-	-
Glucose	+	-	+	+
Maltose	A/G	A/G	A/G	-

The extraction and amplification of DNA of the four isolates of bacterial genera by PCR using universal primer (*S. pueumoniae, S. aureus*, *K. pneumoniae* and *H. influenzae*) (figure 1 and 2) revealed that *S. pneumoniae* was found between 100 and 200kbps which was measured exactly 148kbp against the marker, *K. pneumoniae* and *H. influenzae* were traced almost at 400 against the marker and measured to be 398.5kbps and *S. aureus* was traced at exactly 380kbp against the marker used as the experimental control.



A = *Streptococcus pneumoniae*; *B = Streptococcus klebsiellae; C = Haemophilus influenza; D = Staphylococcus aureus*

Figure 2 The picture of bacterial genera characterized using universal PCR primers



A = *Streptococcus pneumonia*; B = *Streptococcus klebsiellae*; C = *Haemophilus influenza*; D = *Staphylococcus aureus*

Figure 3 Molecular characterization of the isolated bacterial genera using universal PCR primers

The quantitative phytochemical analysis of the leaves of *Asplenium bulbiferum* were shown in Table 2. The result revealed the presence of alkaloids, saponins, flavonoids, phenolics, tannins and glycosides. These phytochemical constituents may be responsible for the activity of the leaf extracts of *Asplenium bulbiferum*.

Table 2 Quantitative phytochemical constituents of *Asplenium Bulbiferum* (fern plant) leaf extracts

Phytochemicals	ALA	ALE/RESULTS
Alkaloids	++	-
Flavonoids	+	+
Saponins	++	+
Tannins	+	-
Phenolics	++	+
Resins	+	-
Steroids	+	++
Glycosides	+	+

Key: ALA = *Asplenium* leaf Aqueous, ALE = *A. bulbiferum* leaf ethanol, + = present, - absent.

The diameter of zones of inhibition of the aqueous extracts of *Asplenium bulbiferum* at different concentrations as shown in Table 3 revealed that as the concentration of the extracts increase showing more antibacterial activity. Also the extracts inhibited *S. aureus* most followed by *S. Pneumoniae, H. Influenzae* and *K. Pneumoniae* as the least.

Table 3 Mean diameter zones of inhibition of aqueous extracts against the tested organisms (mm)

Concentration of extract (mg/ml)	*S. aureus*	*S. pneumoniae*	*H. influenzae*	*K. pneumoniae*
400	11.00	10.10	9.60	8.10
200	9.00	8.60	7.70	6.80
100	7.00	6.30	5.60	4.00
50	-	-	-	-
0.05%CEP	21.00	17.00	14.50	12.80

The mean zones diameter of inhibition of the ethanol extracts of *Asplenium bulbiferum* at different concentrations as shown in Table 4. revealed that as the concentration of the extracts increases, the antibacterial activity increase as more zone of inhibitions were produced. The extracts inhibited *S .aureus* most followed by *S. pneumoniae*, *H. influenzae* and *K. pneumoniae* was the least.

Table 4 Mean diameter zones of inhibition of ethanol leaf extracts against the tested organisms (mm)

Concentration of extract (mg/ml)	*S. aureus*	*S. pneumoniae*	*H. influenzae*	*K. pneumoniae*
400	14.00	12.50	11.80	10.60
200	12.00	10.00	9.30	8.40
100	9.00	8.00	7.20	6.80
50	7.00	6.20	5.10	-
0.05%CEP	21.00	17.00	14.50	12.80

The MIC of the ethanol and aqueous extracts of *Asplenium bulbiferum* as shown in Table 4. The results revealed that the ethanol extracts exhibited more activity than the aqueous extract and the extracts were more potent to *S. aureus* and *S. pneumoniae* than *H. influenzae* and *K. Pneumoniae* in each study.

Table 5 Minimum inhibitory concentration (MIC) of the test extracts (mg/ml)

Extracts	*S. aureus*	*S. pneumoniae*	*H. influenzae*	*K. pneumoniae*
Ethanol	50	50	100	100
Aqueous	100	100	200	200

The MBC of the ethanol and aqueous extracts of *Asplenium bulbiferum* shown inTable 5. the results revealed that the ethanol extracts exhibited more activity than the aqueous extract and the extracts were more potent to *S. aureus* and *K. pneumoniae* in each study.

Table 6 Minimum Bactericidal concentration (MBC) of the test extracts (mg/ml)

Extaracts	*S. aureus*	*S. pneumoniae*	*H. influenzae*	*K. pneumoniae*
Ethanol	100	100	200	200
Aqueous	200	200	400	400

The acute toxicity study of the leaf extracts was shown in table. The result revealed that no death was recorded after 72 h, all other immunological parameters remain still after exposure to the animals to the *Asplenium bulbiferum* leaf extracts.

Table 7 Acute toxicity studies of the *Asplenium bulbiferum* leaf extracts

Extract	Number of animals	Death after	Member of surviving animals
		24 h 48 h 72 h	
Ethanol extract	2	- - -	2
Aqueous extracts	2	- - -	2
Cephalosporin	2	- - -	2

The effect of *Asplenium bulbiferum* leaf extract on *S. Pneumoniae in vivo* after 5 days, 8 days, 12 days and 16days from infection (Table 7) showed the appearance of symptoms of disease. Total WBCs count was $9.7x10^3$cell/ml and $11.3x10^3$ for infected only and infected then treated with plant extract respectively comparing with $4.6x10^3$/ml for control treatment. After 8 days from infection the WBC raised to 14.4 for infected mice and the value of WBC decreased from 11.3 to 9.5 for infected and treated with plant extract, but the control remains 4.6 $x10^3$ cell/ ml. After 12 days from infection the value of total WBC for infected untreated raised to $16.3x10^3$, while for infected and treated mice decreased to 7.3, then to 5.1 after 16 days from infection and the mice became healthy.

Table 8 The effect of *Asplenium bulbiferum* leaf extract on *S. Pneumoniae in vivo*

Treatments	T. WBC SX10^3	NEX10^3	Lyx10^3	MOX10^3	EOX10^3
After zero time from infection A	4.60	3.05	1.17	0.37	0.00
B	4.30	2.79	1.07	0.34	0.00
AB	4.50	3.05	1.17	0.37	0.00
After 5 days from infection A	4.60	3.05	1.17	0.37	0.00
B	9.70	7.96	1.02	0.72	0.00
AB	11.30	10.05	1.18	0.10	0.00
After 8 days from infection A	4.60	3.05	1.17	0.37	0.00
B	14.40	7.13	0.61	0.69	0.00

AB	9.50	7.96	1.02	0.72	0.00
After 12 days from infection A	4.60	3.05	1.17	0.37	0.00
B	16.30	13.84	1.32	0.94	0.00
AB	7.30	6.31	0.09	0.58	0.00
After 16 days from infection A	4.60	3.05	1.17	0.37	0.00
B	13.60	11.85	1.63	0.21	0.00
AB	5.10	5.58	0.98	0.51	0.00

A = Control; B = infected mice with *S. Pneumoniae*; AB = infected mice with *S. Pneumonia* and treated with plant extract; TWBCs = total white blood cells; Ne= Neutrophil; Ly = Lymphocyte; Mo= monocyte; Eo = Eosinophil.

4. Discussion

Apslenium bulbiferum phytochemical screening revealed presence of flavonoids, saponins, Alkaloids, Phenolics, tannins, Resins, Steroids and glycocides in both ethanol and aqueous extracts (Table 1) in agreement with report by [25] reported that plants occur in varying habitats, a great magnitude of variation in the concentration and composition of phytochemical ingredients in different parts of such plant is expected. Moreover, [20] reported that phytochemical are produced in response to perceived threats by the plants, therefore variation exist in the production of these phytochemicals depending on the type and amount of threat encountered by the plant.

The study revealed that the leaf extracts exhibited pronounced activity against the tested organisms. The ethanolic leaf extract inhibited more isolates than the aqueous leaf extract. This could be due to the fact that ethanol is an organic and polar solvent hence, dissolved more of the phytochemicals which are mainly organic in nature. The extracts inhibited *S. aureus* most (14.00) followed by *S. Pneumoniae* (12.50) *H. Influezae* (11.80) and *K. Pneumoniae* (10.60) was the least (Table 4). This means that extracts could be easily used to manage enteric infections or any infection associated with *S. aureus or* S. *Pneumoniae.*

The result revealed different symptoms of diseases in laboratory mice (*Mus musculus*) that were infected nosily with 5 x 10^6 CFU/ML suspension of *S. pneunoniae* (S6B) isolate after 5 days of infected mice, such as swallowing, lung raised to out as was previously demonstrated by [22] the body and they become weak and the total WBCs count raised from 4.5x10^3 cells to 11.3x10^3 cells/µl as shown in (Table 8). The type of leukocytes also elevated, neutrophil raise from 3.054 x10^3 cells/µl to 10.05x10^3 cell/µl. The WBCs used as an immunological parameters to determine the case of infection [26] , while normal range of total WBCs were 4.2X10^3 cells/µl [26] , because the main type of phagocytic cells which is required to participate in the phagocytosis in the ingestion of foreign bodies (like bacterial cells) are neutrophil and macrophage [27] ; [28] ; [29] , so during infection with bacteria, the range of neutrophils increase comparing with control. While basophile and eosinophil are role model in immunity, eosinopil increasing in cancer and parasitic infections [30]. High level of basophil generally corresponds to an active allergic response.

After three days of administration, all the infected mice were examined to number the total WBCs and differential leukocyte count to check the effects of aqueous extracts at 400mg/ml, the total WBCs of infected mice were treated with sub- MIC only was 5.1x10^3 cells µl and differential leukocyte count decreased when compared with control group.

At the end of the experiment, when the amounts were examined for TWBCs and other immunological parameters, the result indicated that for infected mice the total WBC raised to 16.3x 10^3, due to the effect of *A. bulbiferum* extract.

During infection, all the infected mice and the control examined to total bacteria in the blood (Table 8) represent count of viable bacteria presented in the blood.

After 5 days from infection, viable bacteria increased to 166 and 333 for infected with bacteria only and infected mice treated with plant extract respectively.

After 8 days from infection in the extract treated mice, bacterial number decreased considerably to 166, then to zero up to the end of experiment, while 5 x 10^2 and then increased to 1x10^3 cells/ml, after 12 days, then decreased to 5x10^2 at the end of the experiment. These findings have clearly demonstrated that the clearance of *S. Pneumoniae* from the blood of infected mice by aqueous extract was not zero, as compared with the infected untreated mice even after 16 days from infection the number was 5x10^2 cells/µl. Furthermore, it was more effective than other treatments.

The effect of *A. Bulbiferum* extract may be due to that *Ibu* is rich in tannin and other components and the antimicrobial activity of tannin is well documented [31] ; [32] ; [33]. The aqueous extract displayed broad spectrum of activity, i.e. G+ and G^- bacteria were inhibited with *A. bulbiferum* extracts [32].

The results of MIC and MBC of the leaf extracts showed that the ethanolic and aqueous leaf extracts of *A. Bulbiferum* possess antibacterial activity against *S. pneumoniae, S. aureus, H. Pneumoniae* and *K. Pneumoniae*.

5. Conclusion

This study has shown that the phytochemical constituents of *Asplenium bulbiferum* can influence and restore cellular functions as well as structural integrity of the lungs. The results of the present study support the folkloric usage of the plant and suggests that medicinal plants may be a potential source of natural, safe and cheap regimen for the treatment of pneumonia infection and infections caused by pathogenic microorganisms.

Recommendation

This study recommend that further research be carried out on this plant to elucidate the particular plant ingredient that has actually carried out the antibacterial activities, *in vivo* assays is to establish the relationship between the MICs and MBCs obtained in this research, and effective dozes that should be administered in ethnomedical practice should be elucidated.

Compliance with ethical standards

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Disclosure of conflict of interest

The authors are in agreement, there is no conflict of interest.

Statement of ethical approval

The present research work does not contain any studies performed on animals/humans subjects by any of the authors.

References

[1] Abu-shanab BG, Adwan D, AbuSafiya K, Adwan C, M Abu-shanab. Antibacterial activity of *Aplenium bulbiferum* extracts growing in Palestine (*Journal*., *Islamic University Gaza).* 2005; 13(2): 147–153.

[2] Asha J, Push paradan. The encyclopedia of infections diseases (3rd ed). 2008; 242–10.

[3] Bain GA, Flower CD. Pulmonary eosinophilia. *European Journal of Radiology.* 2006; 23(1): 3-8.

[4] Bodakh M, Ram B. *In vitro* and *In vivo* anti bacterial studies of Clerodendrum serratuminn. *International journal of ferm plant.* 2007; 20(2): 745–752.

[5] Cheesbrough M. Medical Laboratory manual for tropical countries. 5th ed. pp 223–392. *Tropical health technology publications,* U.K. 2002.

[6] Chung KT, Wong CT, Wei Huang YW, Lin Y. Tannins and human health: *A critical review of Medical science Drug*. 2008; 38–421.

[7] Eddy O. Community Acquired Pneumonia. From common pathogens to Emerging Resistance. *Emergency Medicine Practice.* 2005; 7 (12): 30–45.

[8] Ernst JD, O Stendahl. Phagocytosis of Bacteria and Bacterial Pathogenicity. Cambridge University Press, Cambridge. 2006.

[9] Farooq A, Sajid L, Muhammad A, Anwarul Hassan G. *Asplenium bulbiferum*: a food plant with multiple medicinal uses. *Phytotherapy Research*. 2007; 21(3): 17 –25.

[10] Gina N, Anitha J, Emilin RR. Isolation, analysis and identification of phytochemicals of antimicrobial activity of *Asplenium bulbiferum*. *Current Biotica. 2009;* 3(1): 33–37.

[11] Goden B, Touito E, Rubenstein A, Athamna M. A new approach for a treatment of deep akin infection by an ethosomal antibiotic preparation: An *invivo* study. *Journal of Antimicrobial Chemotherapy*. 2005; 55(1): 989–994.

[12] Gulmez M, N Oral, L Vatansever. The effect of water extract of *A. bulbiferum*(*ibu*) and lactic acid on decontamination and treatment of bacterial infections. Advance Medicine and Sciences. 2006; 85(3): 1466–1471.

[13] Gupta A. Diagnosis and management of pneumonia and other respiratory infections (2nd edn). Professional Communications. Caddo. 2004; 28-29.

[14] Henderson B, Oyston PCF. Bacterial Evasion of Host Immune Respones: *Advance Molecular and Cellular Microbiology*. pp. 22-39 Cambridge University Press, United Kingdom. 2003.

[15] Hoffman R, Benz EJ, Shatill SJ, Furie B, Cohn HJ, Siberstein LE, McGlave P. *Hematology: Basic principles and Practice*. 4th (edn). 2000; 88-100.

[16] Khare PB. Ferns and ferm allies, they are significant and fantastic. *In applied Botany abstracts*. 1996; 16: 18–30.

[17] Khare PB, Adekunle AS. Preliminary assessment of antimicrobial properties of aqueous extract of plants against infections diseases. *Biology and Medicines.* 2009; 1(3): 20–30.

[18] Kumar D, Prassad DN, Parkash M, Bhat N. Antibacterial activity of ethanolic extracts of aerva alanatal, in pharmacology online. *Journal of Ferm Plant*. 2009; 22(3): 1075–1081.

[19] Martines A. Community acquired bacterial pneumonia. *Expert Opinion Pharmacotherapy.* 2007; 11(3): 336–341.

[20] Mullis K, Kelly C, Harryz C. The unusual origin of the polymerase chain reaction. *Scientific American Journal.* 2009; 262(4): 56–61.

[21] Nester NC, Roberts TO, Anderson R, Nester KO. Definition, characteristics and origin of lung infectious disease. *A human perspective Microbiology* 2nd edition. 2000; 130–144.

[22] Nwobu AW, Ezike AC, Nwafor SV, Okoli CO, Enwerem NM. Evaluation of the antibacterial property of *Asytasia gangetica* leaf extracts. *International Journal of Ethnopharmacology*. 2010; 59(8): 284–295.

[23] Provan D, CRJ Singer, T Baglin, Lilleyman J. *Oxford Handbook of Clinical Hematology*. New York. Oxford University Press. Inc. 2004; 163–290.

[24] Reynold J. Laboratory Procedure Manual.on isolation of bacteria and virus. 14th ed. 2003; 191-200.

[25] Robert AN, Peigning Y, Alison DP, Keith IB. Cardiac glycosides as noval anticancer therapeutic agents. *Molecular Inventions.* 2009; 88(6): 36 –59.

[26] Scheuttpels B, Stev O, Kate M. Characteristics of some medicinal plants. *Journal of Ethnopharmacology.* 2007; 56(4): 7–30.

[27] Shama SA, Mayler G, Esehun U. Radiological imaging in *pneumoniae* recent innovations. *Current Opinion in Pulmonary Medicinal.* 2007; 13(3): 159–69.

[28] Sofowora EA. Medicinal and Traditional Medicine in Africa. London. John Wiley and Sons. 2009; 249.

[29] Song Z, Johansen V, Faber C, Moser A, Harazm U. Ginseng treatment reduces bacterial load and lung pathology in chronic *pseudomonas aeruginosa* pneumonia in rats. *Antimicrob. Agents.* 2007; 41(5): 961–964.

[30] Thmel H, Diem H, Haferlach T. Color atlas of hematology practical microscopic and clinical diagnostics. 2nd Ed. 2004; 181.

[31] Tortora MV, Okubo S, Oshnishi R, Shimamura I. Effect of *Ageratum conyzoides* linnon clonidine and haloperidol induced catalepsy in mice. *Pharmacology Online.* 2005; 31(2): 186-–94.

[32] Trease GE, Evans WC. Pharmacognosy. 15th edition. 1996; 13-21.

[33] Virgii MC, Henry O. Antibacterial activities of some folklore medicinal plants used by tribals in Western Ghats of Inida. *Journal of Pharmacolog.* 2013; 51(1): 63–91

[34] Wikipedia the free Encyclopedia. Basophilia from Wikipedia, the free encyclopedia. 2010.

[35] World Health Organization. Epidemiology. Global tuberculosis control strategy. A *Journal of Diseases Control*. 2012; 66(3): 333–62.