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Isolation and In-Vitro Activities of *Asplenum Bulbiferum* Leaf Extracts on 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 medicines, 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 embacked upon to evaluate the *in-vitro* effect of *Asplenium bulbiferum* leaf extracts on isolates from patient's sputum. *Streptococcus pneumoniae* was obtained from human specimen, identified through cultural, morphological and biochemical examination. 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 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. The study showed that *Asplenium bulbiferum* possessed antibacterial properties and should serve as alternative therapy for ameliorating lung infections.

KEYWORDS: *Leaf, Lung, Asplenium bulbiferum, In-vitro*

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INTRODUCTION

Lung diseases remain one of the major threats to public health and are worldwide problem (Asha and Pushparadan, 2018). 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 (Robert *et al.,* 2019; Tortora *et al.,* 2015 and Khare *et al.,* 2016). 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 (Gina, 2019, Khare *et al.,* 2019*; Robert et al.,*2019).

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) (Chattopadhyay, 2013).

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 (Scheuttpels *et al.,* 2017). *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 Trease and Evans (2016), Sofowora (2019). *Asplenium bulbiferum* (fern) is said to be derived

from the celtic word" all heal". This correlates with its historical use for every ailment (Khare, 2016). Some studies have investigated *Asplenium bulbiferum* scientifically and it was found to possess a number of notable pharmacological effects including lung diseases (Gupta, 2014), men and women's sterility, nasal polyps, kidney infections, gonorrhoea, rheumatism, headache, asthma, wound healing and so many others (Vasudev, 2019), Wang e*t al.,* 2015; Khare and Adekunle, 2019). Rudoff steiner (1861-1925) created what is known as anthroposophical medicine. This mystical system used a variety of unsual remedies including special extracts of *Asplenium bulbiferum* for infection.

Despite these medicinal roles played by *Asplenium bulbiferum* in human body, no work has been published on its ability to alleviate lung infection induced by bacteria such as *streptococcus pneumoniae* as well as secondary infections caused by chemicals thus; this work was undertaking to evaluate the antibacterial activities of *Asplenium bulbiferum* leaf extracts to alleviate lung infection induced by bacteria such as *streptococcus pneumoniae*.

Materials and Methods

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 and Nnamdi Azikwe University Teaching Hospital, Nnewi. Each was classified according to names, genders and age of the patients.

Collection of sputum sample was as follows:

Two hundred (200) samples were collected from each of the above mentioned hospitals for analyses.

Each patient was given two containers labelled specimen A and B respectively, the container was dried, clean, wide- necked, and leak-proof. Then the patient was requested to cough deeply to produce sputum specimen into the container (Monica Cheesbrough *et al.,* 2015).

Collection of leaf samples

Collection, authentication and processing of plant materials

The fresh leaf of *Asplenium bulbiferum* was 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, 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 from Dalziel (2016). 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 using electric grinder and stored for future use (Nwobu *et al.,* 2016).

Preparations of Media for Isolation

The media used for this study included: Nutrient agar (oxoid), blood agar, chocolate agar (oxoid), Mueller Hinton agar (oxoid) and Nutrient broth (oxoid). The media were prepared according to the manufacturer's instruction and sterilized by autoclaving at I21°C, 15psi for 15 minutes.

Isolation and Identification of Test Organism

The test organisms used in this work 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 and incubated anaerobically by using gas pak at 37°C for 24 h. The organisms obtained were aseptically sub-cultured on nutrient agar plate and incubated at 37°C for 24 h. The pure culture of the test organism was identified using morphological characteristics, gram staining and biochemical reactions (Reynold, 2015).

Extraction

The powdered plant material (20g) leaf was percolated in ethanol (200ml) in 11 capacity conical flasks, stoppered and kept for two weeks with 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 (Rl-10). The same quantity of plant material was again percolated with distilled water for one week and after filtration, the aqueous extract was concentrated in hot oven at 40"C (Nwobu *et al.,* 2016). The concentrated extracts were labelled ALE (*Asplenium bulbiferum* ethanol leaf extract) and ALE (*Asplenium bulbiferum* aqueous leaf extract).

Phytochemical Analysis

Phytochemical analysis for qualitative detection of alkaloids, flavonoids, tannins and saponins was performed on the extracts as described by Trease and Evans (2019), Sofowora (2018).

Quantitative determination of the presence of phytochemicals

Alkaloids

Five milliliters (5 ml) of the sample was mixed with 96% ethanol-20% tetraoxosulphate (vi) acid (1:1). One milliliter (1 ml) of the filtrate from the mixture was added to 5 ml of 60% H2SO4 and allowed to stand for 5 minutes. sreading was taken at absorbance of 565 nm.

Glycosides

This was carried out using Buljet's reagent. One gram (1 g) 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_2HPO4) solution before the addition of freshly prepared Buljet's reagent. The absorbance was taken at 550 nm. Flavonoids

Five millitres of the extract was mixed with 5 ml of dilute hydrochloric acid (HC1) and boiled for 30 minutes. The boiled extract was allowed to cool and then filtered. One millitre (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.

Phenolics

Ten millitres (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.

Tannins

Ten millitres (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 0.1 NHCL. The absorbance was read at 120 nm.

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_2SO4 was continuously added to the mixture and allowed to cool. The absorbance was taken at 420 nm.

Saponins

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

Determination of Extractive Value

The concentrations of the extracts were determined by evaporating 1.0 ml of the extracts in evaporating dish of known weight in as oven to dryness and weighed. The dish containing the residue was allowed to coot and men 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 (Nwobu *et al.,* 2016).

Maintenance of Test Organisms

The isolated test organisms were used for the antibacterial sensitivity testing. Prior to the test, the organisms were sub-cultured on nutrient agar plate at 37°C for 24 h. Then the 24 h cultures were transferred into nutrient broth and incubated anaerobically using gas pak at 37°C for 24 h (Cheesbrough *et al*., 2015).

Standardization of Inoculum

The inocula were prepared from the stock cultures, which were maintained on nutrient agar slant at 4^0C and sub-cultured 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 (Cheesbrough *et al*., 2015).

Test Organisms

Staphylococcus aureus, Streptococcus pneumoniae, Streptococcus klebsiella and *Haemophilus pneumoniae* were bacterials 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 Cheesbrough *et al.,* (2015) was carried out. Pure cultures of the isolates were maintained in appropriate media for future use.

Preparation of Test Sample

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 method of NCCLS (2013). Here, 2.5 g of the extract was dissolved in each of the extracting solvents.

Antibacterial Assay

Medicinal plants using agar well diffusion method.

This was carried out by using agar well diffusion techniques. In this method, each of the labelled plates was uniformly inoculated with the organisms using pour plate techniques. A sterile cork borer of 6 mm diameter was used to make wells on the medium. O.1m of the various extract concentrations were dropped into each labelled well. After that, the plates were incubated an aerobically at 37^0c for 24 h. Antibacterial activity was determined by measuring the diameter of zones of inhibition (mm) produced after 48 h of incubation. 0.05% Cephalosporin was used as control.

Determination of minimum inhibitory concentration (MIC)

Here, various concentrations of the extracts were obtained using double- fold serial dilution. Each dilution was assayed against the test bacterial using tube dilution techniques. One millilitre of test organism was added into each dilution incubated anaerobically at 37 for 24 h. The MIC was defined as the lowest concentration able to inhibit any visible bacterial growth. This was determined and recorded (Shahidi-Bunjar, 2014).

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 pure plate and incubated anaerobically at 37^0c for 24 h. The lowest concentration of the extracts that killed the test bacterial was taken as the MBC (Shahidi-bunjar, 2014).

RESULT

The phytochemical studies of the constituents of *Asplenium bulbiferum* leaf extracts revealed the presence of alkaloids, saponin, flavoids, phenolics, tannins and steroids (table). The test organisms used for this study were isolated from sputum sample of infected patients. The test organisms include *S. aureus, S. pneumoniae, H. influenzae* and *K. pneumoniae*. Extract showed pronounced activities in against the test organisms. The ethanolic extract showed more activity than aqeous extract. Has the concentrations of the extract increases, the antibacterial activity increase has indicated by increased in diameter zones of inhibition. The leaf extracts inhibited *S. aureus* the most followed by *S. pneumoniae, H. influenzae* and *K. pneumonia* least. The inhibition produced by the leaf extracts differed significantly ($p \leq 0.05$) from that of the control antibiotics (Cephalosporin). The results showed that aqueous extracts of *Asplenium bulbiferum* accelerate the progression of lung infection healing activity. As the concentrations of the extract increase, the rate of lung healing increase.

Table 1: characteristics and identities of the tested organisms

Parameter	*S.pneumoniae*	*S. aureus*	*K. pneumonia*	*H. influenza*
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	-

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.

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

Key: CEP = Cephalosporin

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

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

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

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

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

DISCUSSION

There are many factors which are responsible for the lung infections or damage such as hydrocarbons, chemical, drugs and microbes. Lung infections, human health is dependent on the individual health of various internal systems. Within the respiratory system, our lungs are vital to life supportive respiratory or inhalation and exhalation of air. Lung infections affect the organs principal function of transporting atmospheric oxygen into the blood stream and realising carbondioxide back into the atmosphere. Evidence of multiple drugs resistance by microbes (superburg) and the increasing complexity of lung infection pointed to the continuing search for new antimicrobials from other sources including plant extracts, these plants then emerged as compounds with potentially significant theatric application against human pathogen (Kather *et al.,*2013).Most of the investigations shown that medicinal plant used for treating many diseases that are caused by many pathogens, due to their chemical components(Hamilton- miller,2015,Toda *et al;* 2018).

Apslenium bulbiferum phytochemical screening revealed presence of flavonoids, saponins, Alkaloids, Phenolics, tannins, Resins, Steroids and glycocides in both ethanol and aqueous extracts (Table2) Farooq *et al*. (2017) 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, Waller and Nowacki (2018) reported that phytochemicals 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 than the aqueous leaf extract. This could be due to the fact that ethanol is an organic and polar solvent and dissolve more of the phytochemicals which are mainly organic in nature. The extracts inhibited *S. aureus* most followed by *S. Pneumoniae, H. Influenzae* and *K. Pneumoniae* was the least. This means that extracts could be easily used to manage enteric infections or any infection associated with *S. aureus or* S. *Pneumoniae.*

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 (Chung *et al.,* 2018; Abu- Shanab *et al.,* 2015); Gulmez, *et al.,* 2016). The aqueous extract displayed broad spectrum of activity, i.e. G+ and G^- bacteria were inhibited with *A. bulbiferum* extracts (Abu-Shanab *et al.,* 2015).

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*.

CONCLUSION

This study has shown that the phytochemicals and antibacterial 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 in treating pneumonia infection and infections caused by pathogenic microorganisms (superburgs).

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