International Journal of Scientific Engineering and Applied Science (IJSEAS) – Volume-9, Issue-5, May 2023
ISSN: 2395-3470
www.ijseas.com

Combined Effect of Some Herbal Extracts in The Treatment of Buruli Ulcer in Mbutu Mbaise Imo State, Nigeria

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Abstract

Buruli ulcer caused by *Mycobacterium ulcerans* is a chronic necrotizing skin disease. The combined effect of extracts (*Azadiricta indica, Moringa oleifera and Mucuna pruriens)* on Buruli ulcer was studied in Nigeria. Swab specimens were obtained from 55 presumptive Buruli ulcer patients and examined microscopically for acid-fast bacilli using Ziehl-Neelsen technique and culture. The infection was 54.5% in females. Age group 1-20 years had 40.0%. Demographic characteristics and distribution of buruli ulcer showes farmers had 30.9%, rural dwellers 63.6%. Illiterates had 41.8%, unmarried 41.8%. Antimicrobial activity of the combined *Azadrichta indica* leaf, *Moringa oleifera* root and *Mucuna pruriens* seed extracts against *M. ulcerans* had the highest inhibition zone of 25.6±0.1 mm. Patients were treated with the combination of the extracts. 77.8% of patients had their ulcers healed, 13.3% did not heal due to diabetes. These plant species could be a source of a new class of herbal drug against buruli ulcer.
Keywords: *Combined effect, Herbal extract, Treatment, Buruli ulcer*

1. Introduction

Buruli ulcer is a chronic, indolent, necrotizing disease of the skin and soft tissue. It is the third most common virulent mycobacteria disease of the immunocompetent host, after tuberculosis and leprosy. Buruli ulcer (BU) is caused by Mycobacterium ulcerans which is an environmental pathogen. It is a disfiguring disease of the skin and occasionally the bones. It generally begins as a painless dermal papule or subcutaneous nodule, which over a period of weeks to month's breaks down to form a necrotic ulcer with extensively undermined edges. Lesions heal with scarring that can be a significant source of morbidity (Portaels, Silva & Meyers, 2009).

Mycobacterium ulcerans release a lipid toxin, mycolactone which functions as immune suppressant, necrotising agent and activator of cellular death (Vander-Werf, Groothuis & Knell, 2003). Healing may occur spontaneously but more often the disease is slowly progressive with further ulceration, scarring, and contractures. Secondary infection may occur with other nodules developing and infection may occur in bone. Although seldom fatal, the disease results in considerable morbidity and deformity (Portaels, Silva & Meyers, 2009).

The rising trend in the use of plants to treat disease is a global phenomenon which is seen both in the developed and developing countries. The primary benefit of using herbal drugs is that they are relatively safer and cheaper than the synthetic alternatives (Alyegoro & Okoh, 2009). In addition, herbal mixture is a complex mixture of different phytochemicals, which makes it difficult for pathogens to develop resistance (Daferera, Ziogas & Polission, 2003).

Some plants have been discovered to be rich in secondary metabolites, such as tannins, alkaloids, flavonoids, phenols, terpenoids, steroids and volatile oil. These compounds are responsible for the

therapeutic activities. Also, some plant parts have been used as antimicrobial agents, respectively their extracts either as decoctions, infusions or oral administration (Gajalakshmi, Vijayalakshmi & Davirajeswar, 2013).

Azadirachta indica is a very useful traditional medicinal plant in the sub-continent. The plant is native to Asia, but has now naturalized in West Africa and is widely cultivated in Nigeria as an ornamental as well as medicinal plant. The plant has anthelmintic, antifungal, antidiebetic, antibacterial, antiviral, contraceptive, sedative, and antiulcer effects. The leaves have also been used to treat skin diseases (Ruchi, Amit-Kumar & ShoorVir, 2014).

Moringa oleifera is commonly known as drumstick tree, indigenous to Northwest India. Most of the parts of the plant possess antimicrobial activity. Various parts of this plant such as the leaf, root, seed, bark, fruit, flower and immature pod possess antitumor, antipyretic, antiepileptic, anti-inflammatory, antiulcer, antispasmodic, diuretic, antihypertensive, cholesterol lowering, antioxidant, antidiabetic, hepatoprotective, antibacterial and antifungal activities (Kumar, Mishra, Ghosh & Panda, 2010).

Mucuna pruriens is a tropical legume native to Africa and tropical Asia and widely naturalized and cultivated. It is also known for its medicinal values. Researchers have evaluated its medicinal properties which include, antifungal, antidiebetic, antibacterial, antiviral, contraceptive, sedative, antiulcer, antimalarial, anticancer, and antifertility effects (Rathi, Grover & Vats, 2009).

2. Materials and Methods

2.1 Study Area

The study area was Mbutu Mbaise in Imo State.

2.2 Collection of Plant Material

Azadrichta indica, Moringa oleifera and Mucuna pruriens were collected from Imo State. They were identified at the herbarium unit, Botany Department, Abia State University, Uturu.

Collection of Herbal Remedies

The herbal remedies made from *Azadrichta indica, Moringa oleifera and Mucuna pruriens* were collected from the native doctor at Mbutu Mbaise. The samples were coded in forms in which they existed and their therapeutic claims were collected and documented. Samples were kept in the refrigerator for further use and analysis.

Collection and Preparation of Plant Extract (*Azadrichta Indica, Moringa Oleifera* and *Mucuna Pruriens*)

Neem leaves were collected from Mbaise in Imo state. It was ensured that the plant was healthy and uninfected. Leaves were washed under running tap water to remove any traces of soil particles and other dirt. Then washed with distilled water, air dried and cut in to small pieces and dried for 15 days in shade. Then the leaves were ground and sieved to get fine powder.

Two kilograms of *Moringa oleifera* roots were washed, pulverized, air dried, powdered and processed. The processed roots were packed in a thimble (cloth material) and fixed to a soxhlet reflux extractor apparatus to obtain a crude aqueous extract. The crude aqueous extract obtained was re-suspended in cool distilled water and filtered. The filtrates were partitioned with chloroform, ethyl acetate and n-butanol based on their order of polarity. The *Moringa oleifera* crude aqueous root extracts and the various organic solvent extracts were subjected to qualitative chemical screening for identification of various classes of active chemical constituents such as carbohydrates, tannins, steroids, flavonoids and alkaloids. These

Phytochemical analyses were done according to the standard methods described by Trease and Evans (1997).

Mucuna seeds were collected and grinded into powdered form. The powdered crude drug was macerated with 80% ethanol. However, methanol was also used as solvent for the extraction of *Mucuna pruriens*. The solvent was then evaporated at a constant temperature of 72^0C until a very concentrated extract was obtained.

2.3 Phytochemical Testing

Aim: To identify the active constituents present in the plants

Procedure: The herbal remedies/preparation was chemically tested for the presence of its active ingredients using standard procedure as described by Trease and Evans (1997).

Preparation of Aqueous Extract

All the chemicals and reagents used in this study were of analytical grade. The powdered leaves (20g) were extracted successively in double distilled water and at 50-60^0C for 18 hours using Soxhlet apparatus. The solvents used were recovered under pressure until dry extracts were obtained.

Tests for Alkaloids

To the extract, dilute hydrochloric acid was added, shaken well and filtered. With the filtrate, the following tests were performed.

Mayer's Reagent Test

To 3 ml of filtrate, few drops of Mayer's reagent were added along sides of tube. Formation of creamy precipitate indicates the presence of alkaloids.

Tests for Carbohydrates

Molisch Test

2 ml of aqueous extract was treated with 2 drops of alcoholic α-naphthol solution in a test tube and then 1 ml of concentrated sulphuric acid was added carefully along the sides of the test tube. Formation of violet ring at the junction indicates the presence of carbohydrates.

Tests for Reducing Sugars

Fehling's Test

To 1 ml of aqueous extract, 1 ml of Fehling's A and 1 ml of Fehling's B solutions were added in a test tube and heated on a water bath for 10 minutes. Formation of red precipitate indicates the presence of reducing sugar.

Tests for Flavonoids

Alkaline Reagent Test

The extract was treated with few drops of sodium hydroxide solution separately in a test tube. Formation of intense yellow colour, which becomes colourless on addition of few drops of dilute acid indicates the presence of flavonoids.

Tests for Glycosides

Borntrager's Test

To 3 ml of test solution, dilute sulphuric acid was added, boiled for 5 minutes and filtered. To the cold filtrate, equal volume of benzene or chloroform was added and it was shaken well. The organic solvent layer was separated and ammonia was added to it. Formation of pink to red colour in ammonical layer indicates the presence of anthraquinone glycosides.

Legal's Test

1 ml of test solution was dissolved in pyridine. 1 ml of sodium nitropruside solution was added and made alkaline using 10% sodium hydroxide solution. Formation of pink to blood red colour indicates the presence of cardiac glycosides.

Tests for Tannin and Phenolic Compounds

A small amount of extract was dissolved in distilled water. To this solution 2 ml of 5% ferric chloride solution was added. Formation of blue, green or violet colour indicates presence of phenolic compounds.

Tests for Protein and Amino Acids

3 ml of the test solution was heated with 3 drops of 5% Ninhydrin solution on a water bath for 10 minutes. Formation of blue colour indicates the presence of amino acids.

Tests for Triterpenoids and Steroids

The extract was treated with chloroform and filtered. The filtrate was added with few drops of concentrated sulphuric acid, shaken and allowed to stand. If the lower layer turns red, sterol is present (Bisanda, Ogola & Tesha, 2003).

Alkaloids

Alkaloids are basic nitrogenous compounds with definite physiological and pharmacological activity. Alkaloid solution produces white yellowish precipitate when a few drops of Mayer's reagents was added (Bisanda, Ogola & Tesha, 2003). Most alkaloids are precipitated from neutral or slightly acidic solution by Mayer's reagent (Trease and Evans, 1997).The alcoholic extract was evaporated to dryness and the residue was heated on a boiling water bath with 2% hydrochloric acid. After cooling, the mixture was filtered and treated with a few drops of Mayer's reagent. The samples were then observed for the presence of turbidity or yellow precipitation.

Anthraquinones

About 0.5g of the extracts was boiled with 10% HCl for few minutes in a water bath. It was filtered and allowed to cool. Equal volume of HCl was added to the filtrate. Few drops of 10% NH3 were added to the mixture and heat. Formation of rose-pink colour indicates the presence of authraquinones.

Reducing Sugar

To 0.5 ml of extract solution, 1 ml of water and 5-8 drops of Fehling's solution was added at hot and observed for brick red precipitate.

Tannins

To 0.5 ml of extract solution 1 ml of water and 1-2 drops of ferric chloride solution was added. Blue colour was observed for Gallic tannins and green black for catecholic tannins (Bisanda, Ogola & Tesha, 2003).

Saponins

About 0.2 g of the extract was shaken with 5ml of distilled water and then heated to boil. Frothing (appearance of creamy miss of small bubbles) shows the presence of saponins.

Phlobatanins

The extract (0.5g) was dissolved in distilled water and filtered. The filtrate was boiled with 2% HCl solution. Red precipitate shows the presence of phlobatanins.

2.4 Collection of the wound specimen (Ulcer)

Fifty five swab specimens were collected from the lesion of Buruli ulcer patients' after a routine thorough site cleaning with chlorohexidine hypochlorite. This reduces the risk of contamination of the culture. The specimens were collected from the base and sides of the ulcer. Specimens were stored in a sterile container without any additive and were kept cool at 4^0C.

2.5 Patients Treatment

The patients suspected to have *Mycobacteriun ulcerans* were placed on treatment with the combination of herbs from *Azadrichta indica, Moringa oleifera* and *Mucuna pruriens* extracts (leaves, roots and seeds) which were produced in powdered and infusion. The powered form of the herbal mixture was applied on the various lesions every morning and night. The infusion was also administered every morning and night. All doses (one glass cup each) received by the patients were given under direct observation by the native doctor.

2.6 Experiments

Fasting Blood Sugar

Aim: To determine if the ulcer is caused by diabetes mellitus or *Mycobacterium ulcerans*.

Procedure: The patient's blood specimens were taken and examined on Accu-chek test strip machine to determine the level of sugar present in their blood.

Isolation of the Organism (*Mycobacterium ulcerans*) From the Swab Specimen

Aim: To isolate and identify the causative organism of Buruli ulcer.

Procedure: The swab was cultured on Lowenstein Jensen (L.J) agar medium for the growth of mycobacterium at 30^0C – 33^0C for 8days. Smear was made on glass slide and Ziehl Neelsen staining technique was used to determine AFB.

Biochemical Test

Procedure: This was done according to the methods described by Cheesbrough (2006). The tests carried out were arylsulphatase test, nitrate reduction test and tween 80 hydrolysis tests, etc.

Arylsulphatase Test

The test organism was inoculated in 2ml of sterile Middlebrook OADC broth containing phenolpthalien disulphate tri-potassium. It was incubated at 25 – 37^oC for 18 days. Test for free phenolphthalein was done by adding 2 drops of 40 g/l sodium hydroxide.

Nitrate Reduction Test

About 0.5ml of sterile nitrate broth was inoculated with a heavy growth of the mycobacterium ulcerans. It was incubated at 35 – 37^{o}C for 4 hours. 1 drop of sulphanilic acid reagent and alpha-naphthylamine reagent were added and mixed and observed for the presence of red colour.

Tween 80 Hydrolysis Test

Exactly 4ml of sterile tween 80 phosphate buffered substrate was inoculated with a loopful of growth of the test organism. It was incubated at 35 – 37^{o}C for 18 days. It was examined 5, 10 and 18 days for a change in colour of the substrate from amber to pink-red.

Coagulase Test

A drop of physiological saline was placed on each end of the two separate slides. A colony of the test organism was emulsified in each of the drops to make two thick suspensions. A drop of plasma was added to one of the suspensions and was mixed. Clumping of the organisms was observed within 10 seconds.

Oxidase Test

A piece of filter paper was placed in a clean Petri dish and 2 drops of freshly prepared oxidase reagent was added. A glass rod was used to remove a colony of the test organism, and was smeared on a filter paper. The development of a blue –purple colour was observed within a few seconds.

Indole Test

A sterile straight wire was used to inoculate 5ml of sterile MIU medium with a smooth colony of the test organism. An indole paper strip was placed in the neck of the MIU tube above the medium; the tube was closed with a stopper and was incubated at 35^{o} C overnight. An indole production was observed by looking for a reddening of the lower part of the strip.

Catalase Test

About 2-3 ml of hydrogen peroxide solution was poured into a test tube. A sterile wooden stick was used to remove a good growth of the test organism and was immersed in a hydrogen peroxide solution. An immediate bubbling was observed as catalase positive.

2.7 Susceptibility Patterns of *M. ulcerans* on the Herbal Preparation

Aim: To determine the antibacterial activities of the herbal preparation on the organism (*Mycobacterium ulcerans*).

Procedure: The antibacterial activity of the herbal remedies was determined by agar well-diffusion method according to Ahmed, Mehmood & Mohammed, (1998). 0.5 McFarland standard (approx 10^{8} cfu/ml) was prepared using the test organism and 0.1 of the microbial species was mixed with the agar medium and poured in pre-sterilized Petri plates. A cock borer (6mm diameter was used to punch wells in solidified medium and was filled with extracts of 100mg/ml final concentration of herbal remedies. The plates were incubated at 37^{o}C for 72 hours in an incubator and the diameter of the zones of inhibition was measured in millimetre, each sample was assayed and the mean value was observed. The antibacterial activity was interpreted from the size of the diameter of zone of inhibition measured to the nearest millimetre (mm) as observed from the clear zones surrounding the wells.

2.8 Statistical Analysis

Data obtained from this study were analysed by descriptive statistics and presented as mean ± standard deviation of mean (SEM) of three determinations, using the statistical software package SPSS for windows version 25.0 (SPSS Inc. Chicago IL). Differences between means were separated using the ANOVA and multiple comparison tests. Values of $p<0.05$ were taken as significant.

2.9 Ethical and Administrative Considerations

An ethical approval to carry out the study was obtained from Abia State University ethical committee. Advocacy visits was paid to the Native Doctor to obtain approval, to enable the researcher conduct the study. Informed consent was sorted from the patients who were receiving treatments. Honesty was maintained during reporting an analysis of the data. Confidentiality and respect for human dignity was observed during the study.

3. RESULTS

Table 1: Quantitative Phytochemical Screening of *Azadrichta indica* Leaf Extracts

Phytochemical Constituents	Cold water extract (µg/ml)	Ethanolic extract (µg/ml)
Alkaloids	1.38±0.01	2.26+0.04
Flavonoids	0.43±0.01	0.08±0.02
Saponins	0.72±0.01	0.19±0.02
Steroids	0.20±0.00	1.38±0.01
Terpenoids	0.16±0.05	3.12±0.02
Tannins	0.23±0.01	4.26±0.06
Cardiac glycosides	0.01±0.01	3.83±0.02

Concentration of extracts in 100µg/ml

Table 2: Quantitative Phytochemical Screening of *Moringa oleifera* Root Extracts

Phytochemical Constituents	Cold water extract (µg/ml)	Ethanolic extract (µg/ml)
Alkaloids	3.07±0.00	2.26+0.04
Flavonoids	3.56±0.03	3.83±0.02
Saponins	1.46±0.03	1.72±0.05
Steroids	3.21±0.00	3.12±0.02
Terpenoids	4.84±0.05	4.26±0.06
Tannins	9.36±0.04	9.19±0.02
Antraquinone	11.68±0.04	10.86±0.06
Carotenoids	1.16±0.05	0.0±0.02
Cardiac glycoside	0.36±0.03	0.19±0.02

Concentration of extracts in 100µg/ml

Table 3: Quantitative Phytochemical Screening of *M*ucuna *pruriens* Seed Extracts

Phytochemical Constituents	Cold water extract (µg/ml)	Hot water extract (µg/ml)	Ethanol extract (µg/ml)
Flavonoids	0.42+00.2	0.35±0.02	0.31±0.02
Alkaloids	1.07±0.01	0.88±0.01	0.37±0.02
Tannins	0.28±0.00	0.09+0.00	0.08+0.00
Saponins	0.47±0.02	0.39±0.02	0.37±0.02
Phenoids	2.82±0.01	0.33±0.01	0.32+0.02
Cardiacglycosides	0.43±0.02	0.24±0.00	0.22+0.01

Concentration of extracts in 100µg/ml

The qualitative phytochemical constituents of *Azadrichta indica* leaf extracts revealed that the ethanolic extract was a better solvent for extracting the qualitative constituents. The ethanolic extracts of the leaf had highest level of tannins (4.26±0.06 µg/ml) than the other secondary metabolites which cardiac glycosides had the lowest of 0.01±0.01µg/ml (Table 1). The quantitative phytochemical constituents of *Moringa oleifera* root extracts showed that antraquinone was highest in cold water (11.68±0.04 µg/ml), while carotenoids was absent in ethanolic extract (Table 2). Phenols was highest in the cold water extract of *Mucuna pruriens* seed (2.82±0.01µg/ml) and the lowest was tannins (0.08±0.00 µg/ml) (Table 3).

Table 4: Characterisation and Identification of Isolated *Mycobacterium ulcerans*

Parameter	*M. ulcerans*
Pigmentation in the dark	+
Pigmentation in the light	+
Growth at 33^{o}c	+
Catalase	–
Tween 80 hydrolysis (10 days)	–
Urease activity	–
Niacin Production	–
Nitrate reduction	–
Acid phosphates activity	–
Morphology	R

Key +: Positive
- : Negative
R: Rough

Table 5: Age and Gender Distribution of Presumptive Buruli Ulcer among the Study Population

Age group (years)	Males (%)	Females (%)	Total
1 – 20	10 (18.2)	12(21.9)	22 (40.0)
21 – 40	2 (3.6)	3(5.5)	5 (9.1)
41 – 60	5(9.1)	6(10.9)	11(20.0)
61 and above	8(14.5)	9(16.3)	17 (30.9)
Total	**25(45.5)**	**30(54.5)**	**55 (100)**

A total of 55 presumptive Buruli ulcer patients including 25 males and 30 females were enrolled into the study. The infection was more prevalent in females (54.5%), than in males (45.5%). The age group of 1-20 years had the highest prevalence rate of 40.0% to Buruli ulcer, 60 years and above had 39.9% and 21-40 years had the least prevalence rate of 9.1% (Table 5).

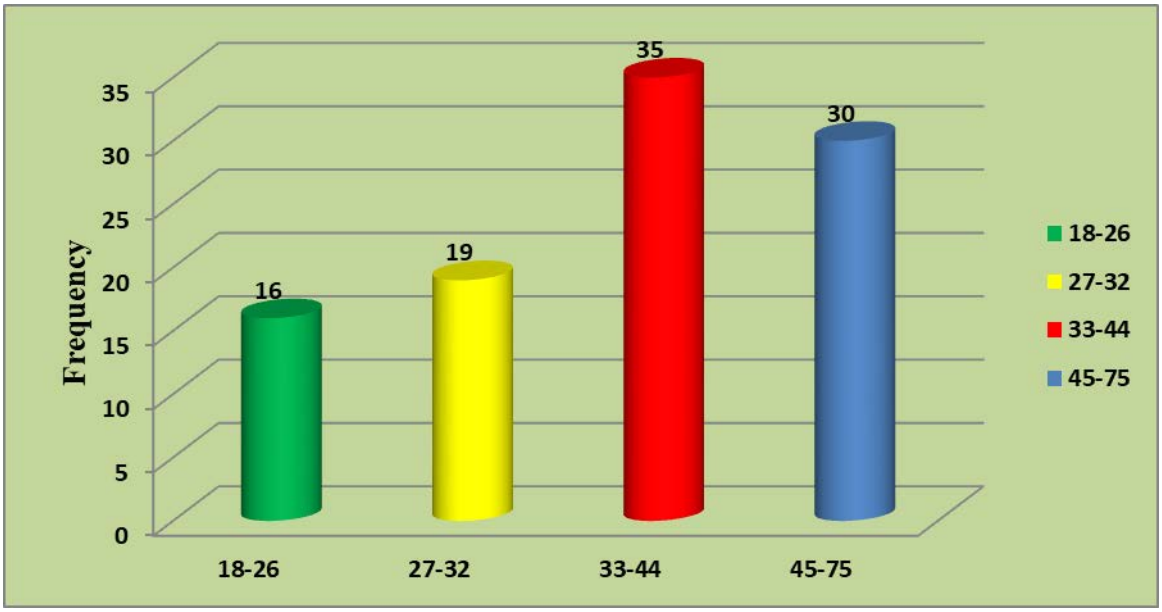


Figure 1: Age range of the participants

Table 6: Demographic Characteristics and Distribution of Buruli Ulcer among the Study Population

Variables	Total No examined	Buruli ulcer +	Buruli ulcer -
Occupation			
Farmers	20	17(30.9)	3 (5.5)
Fishers	14	11(20.0)	3(5.5)
Students	12	9 (16.3)	3(5.5)
Civil Servant	9	8(14.5)	1(1.8)
Location/Residence			
Rural Dwellers	40	35(63.6)	5 (9.1)
Urban	15	10(18.2)	5(9.1)
Level of Education			
No Formal Education	24	23(41.8)	1(1.8)
Primary	15	10(18.2)	5(9.1)
Secondary	11	9 (16.3)	2(3.6)
Tertiary	5	3(5.5)	2(3.6)
Marital Status			
Single	28	23(41.8)	5(9.1)
Married	19	16(29.1)	3(5.5)
Divorced /Separated	8	6(10.9)	2(3.6)

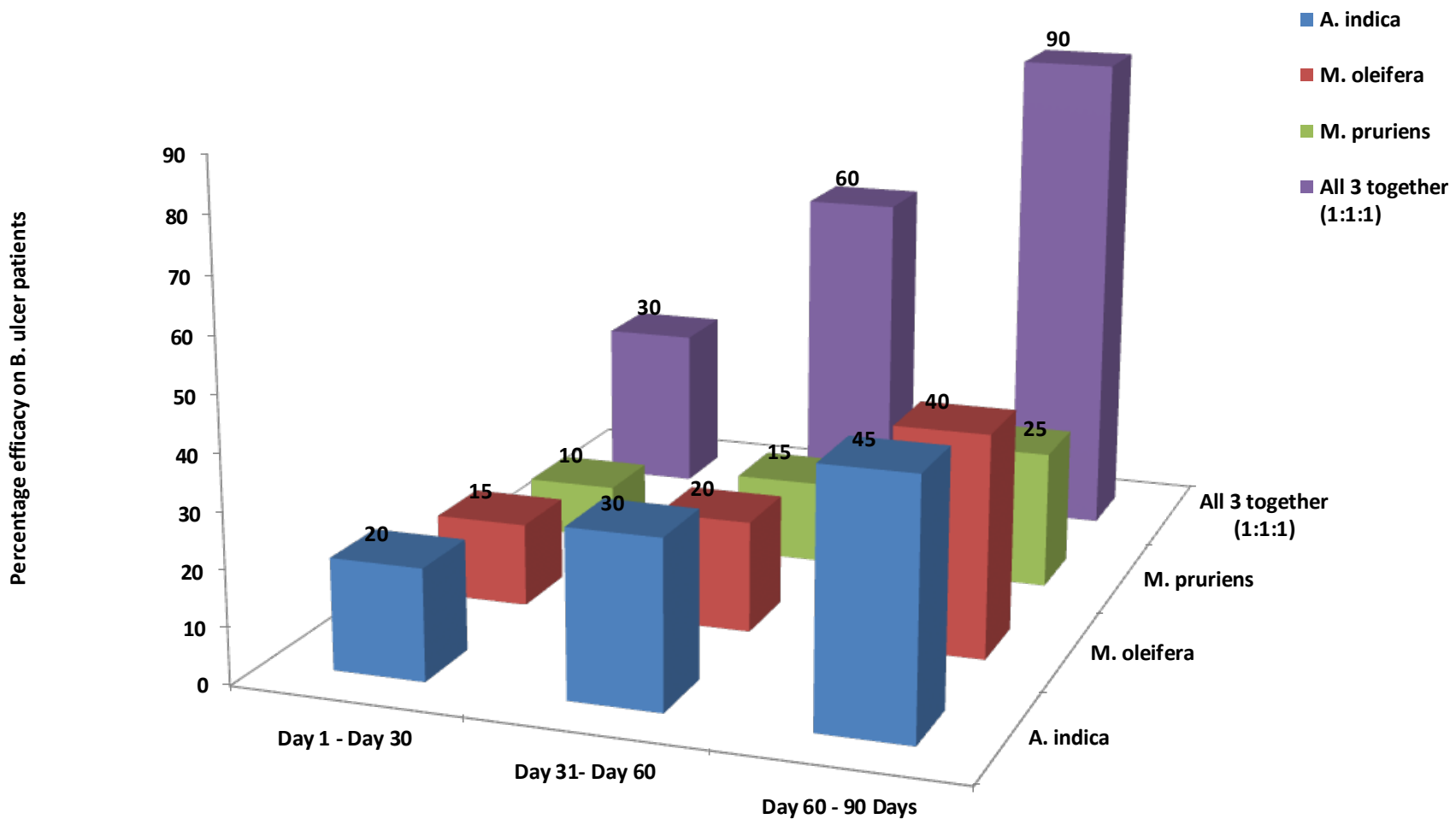


Fig.2: Efficacy of Herbal Remedies on Buruli Ulcer Patients

Table 7: Antimicrobial Activity of *Azadrichta indica* Leaf Extracts against Bacterial Isolates from Buruli Ulcer (mm)

Organisms	Cold water Extract	Hot water Extract	Ethanolic extract	Control (Cefuroxime)	Control (Itraconazole)
M. ulcerans	9.0±0.2	11.0±0.2	13.9±0.1	15.8±0.3	ND
S. aureus	10.0±0.1	13.0±0.4	15.0±0.6	19.4±0.7	ND
Streptococcus pyogenes	8.9±0.1	13.0±0.3	14.9±0.6	16.6±0.9	ND
Escherichia coli	10.2±0.3	11.0±0.1	12.7±0.4	21.9±0.3	ND
Pseudomonas aeruginosa	10.5±0.3	12.0±1.0	13.9±0.2	21.0±0.1	ND
Proteus vulgaris	11.2±0.1	14.3±0.4	16.8±0.8	20.8±0.5	ND
Klebsiella aerogenes	10.6±0.7	16.1±0.2	19.5±0.2	19.6±0.2	ND
Candida sp.	6.5±0.1*	10.1±0.7	14.3±1.0	ND	20.1±0.2
Fusarium sp.	7.0±0.4*	12.8±0.6	15.6±0.5	ND	21.1±1.0
Trichosporon sp.	7.8±0.5*	15.0±0.1	18.0±0.4	ND	24.1±0.1
Aspergillus sp.	7.4±0.2*	13.7±0.4	15.9±0.7	ND	22.0±0.5

Interpretative standard: Clinical and Laboratory Standards Institute (CLSI) (2006).
Values are mean of three replicates and expressed as mean±SD; Cork borer diameter: 6mm. P>0.05; not significantly different from control except values with asterisks (*).
Key: ND = Not determined; *M. ulcerans* =*Mycobacterium ulcerans*, *S. aureus* = *Staphylococcus aureus*

Table 8: Antimicrobial Activity of *Moringa oleifera* Root Extracts against Bacterial Isolates from Buruli Ulcer (mm)

Organisms	Cold water Extract	Hot water Extract	Ethanolic extract	Control (Cefuroxime)	Control (Itraconazole)
M. ulcerans	8.0±1.0	10.0±0.6	11.0±0.5	15.8±0.3	ND
S. aureus	9.2±0.5	11.0±0.1	13.1±0.1	19.4±0.7	ND
Streptococcus pyogenes	8.2±0.2	10.2±0.1	11.3±0.1	16.6±0.9	ND
Escherichia coli	10.0±0.1	10.9±0.6	12.0±0.4	21.9±0.3	ND
Pseudomonas aeruginosa	9.0±0.2	11.1±1.1	12.1±0.2	21.0±0.1	ND
Proteus vulgaris	10.0±0.1	12.0±0.9	13.4±0.6	20.8±0.5	ND
Klebsiella aerogenes	9.6±0.4	13.0±0.4	15.0±0.2	19.6±0.2	ND
Candida sp.	6.0±0.3*	9.7±0.2	13.2±0.9	ND	20.1±0.2
Fusarium sp.	6.9±0.5*	10.5±0.1	13.9±0.4	ND	
Trichosporon sp.	6.8±0.6*	11.1±0.3	15.1±0.7	ND	24.1±0.1
Aspergillus sp.	7.0±0.9	11.9±0.7	16.2±0.8	ND	22.0±0.5

Interpretative standard: Clinical and Laboratory Standards Institute (CLSI) (2006).
Values are mean of three replicates and expressed as mean±SD; Cork borer diameter: 6mm. P>0.05; not significantly different from control except values with asterisks (*).
Key: ND = Not determined; *M. ulcerans* =*Mycobacterium ulcerans*, *S. aureus* = *Staphylococcus aureus*

Table 9: Antimicrobial Activity of *Mucuna pruriens* Seed Extracts against Bacterial Isolates from Buruli Ulcer (mm)

Organisms	Cold water Extract	Hot water Extract	Ethanolic extract	Control (Cefuroxime)	Control (Itraconazole)
M. ulcerans	7.1±0.4	7.9±0.1	10.2±0.6	15.8±0.3	ND
S. aureus	7.8±0.1	9.1±0.2	11.0±0.4	19.4±0.7	ND
Streptococcus pyogenes	8.0±0.1	9.9±0.3	10.3±0.8	16.6±0.9	ND
Escherichia coli	10.1±0.9	10.9±0.6	11.8±0.1	21.9±0.3	ND
Pseudomonas aeruginosa	8.1±0.1	9.9±1.0	12.0±0.1	21.0±0.1	ND
Proteus vulgaris	9.1±0.2	10.9±0.1	13.1±0.8	20.8±0.5	ND
Klebsiella aerogenes	9.0±0.5	10.5±0.1	11.9±0.5	19.6±0.2	ND
Candida sp.	6.0±0.8	8.0±0.1	8.7±0.1	ND	20.1±0.2
Fusarium sp.	6.0±0.9	9.1±0.3	10.9±0.5	ND	21.1±1.0
Trichosporon sp.	6.0±0.5	9.9±0.2	11.7±0.8	ND	24.1±0.1
Aspergillus sp.	6.0±0.1	9.5±0.6	10.9±0.2	ND	22.0±0.5

Interpretative standard: Clinical and Laboratory Standards Institute (CLSI) (2006).
Values are mean of three replicates and expressed as mean±SD; Cork borer diameter: 6mm. P>0.05; not significantly different from control except values with asterisks (*).
Key: ND = Not determined; *M. ulcerans =Mycobacterium ulcerans, S. aureus = Staphylococcus aureus*

Table 10: Antimicrobial Activity of Combined *Azadrichta indica* leaf, *Moringa oleifera* Root and *Mucuna pruriens* Seed (1:1:1) Extracts against Bacterial Isolates from Buruli Ulcer (mm).

Organisms	Cold water Extract	Hot water Extract	Ethanolic extract	Control (Cefuroxime)	Control (Cefuroxime + erythromycin+ Ofloxacine)	Control (Itraconazole)	Control (Itraconazole +Griesofulvin + Ketoconazole)
M. ulcerans	14.2±0.2	22.4±0.6	25.6±0.1	15.8±0.3	26.1±1.0	ND	ND
S. aureus	11.8±1.0	18.1±0.3	22.2±0.5	19.4±0.7	20.5±0.2	ND	ND
Streptococcus pyogenes	12.0±0.3	19.7±0.4	21.8±1.3	16.6±0.9	20.0±0.1	ND	ND
Escherichia coli	13.2±0.1	24.8±1.0	26.3±0.4	21.9±0.3	24.2±0.9	ND	ND
Pseudomonas aeruginosa	14.3±0.5	23.0±0.7	26.7±0.3	21.0±0.1	23.6±0.8	ND	ND
Proteus vulgaris	15.3±0.1	26.1±0.5	30.5±1.0	20.8±0.5	24.6±0.3	ND	ND
Klebsiella aerogenes	17.1±1.2	20.4±0.1	23.8±1.1	19.6±0.2	25.7±0.4	ND	ND
Candida sp.	15.9±0.1	19.3±0.9	20.4±0.4	ND	ND	21.1±1.0	30.5±0.9
Fusarium sp.	11.8±0.6	20.0±0.7	22.1±0.6	ND	ND	24.1±0.1	27.6±0.5
Trichosporon sp.	11.9±0.5	20.8±0.1	22.9±0.4	ND	ND	22.0±0.5	28.8±0.7
Aspergillus sp.	12.6±0.1	21.6±0.6	22.8±0.5	ND	ND	20.1±0.2	29.0±0.3

Interpretative standard: Clinical and Laboratory Standards Institute (CLSI) (2006).
Values are mean of three replicates and expressed as mean±SD; Cork borer diameter: 6mm. P>0.05; not significantly different from control except values with asterisks (*).
Key: ND = Not determined; *M. ulcerans* =*Mycobacterium ulcerans*, *S. aureus* = *Staphylococcus aureu*

The efficacy of herbal remedies on Buruli ulcer patients showed that the plants extracts had increasing percentage efficacy on B. ulcer patients. The combined plants in a ratio of 1:1:1 had the highest efficacy (Fig. 1).

The antimicrobial activity of *Azadrichta indica* leaf extracts was assessed by the measurement of zones of inhibition of various extracts against *Mycobacterium ulcerans.* The plant leaves showed broad spectrum antibacterial activity. The ethanolic extract produced the highest zone of inhibition of 15.8±0.3 mm against *Mycobacterium ulcerans*, followed by an inhibition zone of 11.0±0.2 mm by hot water extract and the least inhibition zone of 9.0±0.2 mm by cold water extract. Other microbial isolates had an inhibition zone range of 6.5±0.1*mm to 19.5±0.2 mm. The results revealed that there was no significant difference at $p>0.05$ between the inhibition zones of the extracts and that of the positive controls except the values with asterisks (Table 7).

The extracts of Moringa *oleifera* root produced an antimicrobial activity of 11.0±0.5 mm, 10.0±0.6 mm and 8.0±1.0 mm against *M. ulcerans*. Ethanolic extract produced the highest inhibition zone of 16.2±0.8 mm against *Aspergillus* sp and while the cold water extract had the least inhibition zone of 6.0±0.3* mm against *Candida* species (Table 8).

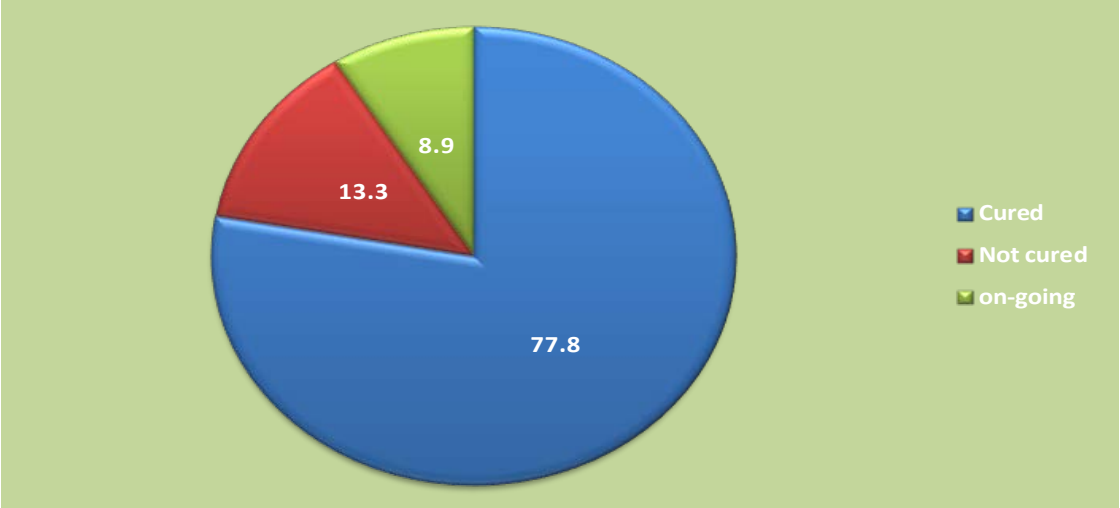
The extracts of *Mucuna pruriens* seed produced lower antimicrobial activity of 10.2±0.6 mm, 7.9±0.1 mm and 7.1±0.4 mm against *M. ulcerans*. Ethanolic extract produced the highest inhibition zone of 12.0±0.1 mm against *Pseudomonas aeruginosa* and while the cold water extract had the least inhibition zone of 6.0±0.1mm against *Aspergillus* species (Table 9).

Table 10 shows the Antimicrobial activity of the combined *Azadrichta indica* leaf, *Moringa oleifera* root and *Mucuna pruriens* seed extracts against *M. ulcerans* and bacterial isolates from Buruli ulcer. The combined ethanolic extract had the highest inhibition zone of 25.6±0.1 mm against *M. ulcerance.*

Table 11: Outcome of Buruli ulcer treatment

Treatment category	No	Percentage (%)
Cured	35	77.8
Not cured	6	13.3
On-going	4	8.9
Total	**45**	**100**

Out of the confirmed Buruli ulcer patients, 35 (77.8%) of the patients had their ulcer completely healed after 9 months of treatments, 6 (13.3%) of the patients were not cured after 9 months, while 4 (8.9%) were still on-going treatment after 9 months (Table 11).



4. Discussion

The phytochemical constituents of *Azadrichta indica* leaf extracts revealed the ethanolic extract as a better solvent for extracting the qualitative constituents. According to Anyanwu and Dawet (2005) these constituents found in plants are known to have anti-protozoal and antibacterial activities. Flavonoids especially, are of a potential benefit to human health (Makeri, Maikai & Nok, 2007). *Azadrichta indica* extracts from the seeds, leaves and bark has been reported to have strong biological activities against insect pests, but with very low toxicity to mammals and the environment (Makeri, Maikai & Nok, 2007). Therefore, the wide use of the neem plant is attributable to the presence of these bioactive compounds, which may explain its many traditional uses against various ailments.

Moringa oleifera is highly esteemed by people in the tropics and sub-tropics in many ways, it is used nutritionally by people and medicinally by local herbalist. Some of these traditional uses reflect the nutritional content of the various parts of the plant. Laboratory investigations have confirmed the efficacy of this plant as found in leaves, flowers, pods, roots, root bark and stem bark, gum, seeds and seed oil. The root of *Moringa oleifera* plants have been known to be used in the treatment of dental caries, common cold, fever, Diarrhoea, flatulence and Edema (Fuglie,2001). There is an increasing awareness that many components of traditional medicine are beneficial while others are harmful, hence WHO encourages and supports countries to identify and provide safe and effective remedies for use in the public and private health services (Sofowora, 1993). The present study showed that the roots of *Moringa oleifera* have pharmacologically important chemical compounds such as carbohydrates, saponins, cardiac glycosides, terpenes, steroids, flavonoids and alkaloids. *Moringa oleifera* root extracts had antraquinone as the highest in aqueous (11.68±0.04 µg/ml).

The chemical constituents present in *Moringa oleifera* root extracts have many known therapeutic values. Though carbohydrates have no known therapeutic effect, it was earlier concluded by Fuglie (2001) that the presence of carbohydrates may be responsible for resistance shown by bacteria. The cardiac glycosides detected in the present study shows the potential of the plant root extract as a therapeutic agent. Glycosides are known to exert pronounced physiological effects on the heart, even though they may be poisonous to animals and man at certain doses. Despite its toxicity, cardiac glycosides are the drug of choice for the treatment of congestive heart failure.

Furthermore, some glycosides are known to have laxative, diuretic and antiseptic properti6es (Fahey, 2005). It is therefore necessary to study this plant for these possible therapeutic values. Saponins found in *Moringa oleifera* aqueous root extract in this study are glycocidic in nature and have the physical characteristics of producing soapy foam. The medicinal value of saponins is its expectorant actions which are very useful in the management of inflammation of the upper respiratory tract. Saponins present

in many plants parts are known to be cardio tonic in nature. Saponins could also be hydrolysed, which may be steroids or triterpenes. Steroids are known to have stimulatory effect on bone marrow resulting in increased erythropoiesis. The presence of steroids in *Moringa oleifera* aqueous root extract is an indication that it may have erythropoitic properties. Further study may be needed to ascertain this.

The presence of alkaloids in *Moringa oleifera* aqueous root extract in this study points towards the potential of the extract to have an analgesic, antiinflammatory and adaptogenic effects, which help the host (man and animal) to develop resistance against disease and endurance against stress (Anwar & Rashid, 2007). Flavonoids detected in *Moringa oleifera* roots could substantiate the use of the products by the traditional medicine healer who use it to treat various disease conditions like oedema, toothache, fever, common cold, diarrhoea and dental caries. These could be possible as the root extracts contains some antibacterial activities. The flavonoids are acting on bacteria by inhibiting its protein synthesis (Kumar, Mishra, Ghosh, & Panda, 2010).

Phenols were highest in the cold water extract of *Mucuna pruriens* seed (2.82±0.01µg/ml). Phenolic compounds prevent oxidative damage in living systems. Saponins was present in all the plants extract. This compound has shown to have immense significance as antihypercholesterol, hypotensive and cardiac depressant properties (Ekeleme, Nwachukwu, Ogodo, Nnadi, Onuabuchi & Osuocha, 2013).

The presence of alkaloids has been implicated in its detoxifying and antihypertensive properties as a result of its stimulatory effects (Ekeleme, Nwachukwu, Ogodo, Nnadi, Onuabuchi & Osuocha, 2013). The ethanolic extracts of the leaf had highest level of tannins (4.26±0.06 µg/ml) than the other secondary metabolites. The presence of tannins suggests the ability of these plants to play a role as anti-microbial agents. Tannins contribute various medicinal properties such as antimicrobial, anti-inflammatory and astringent activity. This perhaps justifies the already locally established function of the plant in the treatment and management of diseases.

Cardiac glycoside was seen in little quantity. This has been used for centuries as stimulants in cases of cardiac failure (Chumark, Khamawat, Sanvarinda, Phornchiraslip & Morales, 2008). Flavonoids are potent antioxidants with reported antimutageneic and anticarcinogenic effects. The age group of 1-20 years had the highest prevalence rate of 40.0% to Buruli ulcer, 60 years and above had 39.9% and 21-40 years had the least prevalence rate of 9.1%. Our study agrees with previous studies that found that the highest rate of the disease is observed among children <15 years of age in disease-endemic communities (Portaels, Silva & Meyers, 2009).

In addition, 54.5% of cases occurred in females, than in males (45.5%). Previous studies did not observe any statistical differences in the occurrence of cases among the sexes. This difference may be attributable to a higher proportion of females participating in sensitisation and project activities in the communities, and therefore those with lesions had a higher likelihood to be screened and assessed, as census data suggest no female sex dominance in the population (Portaels, Silva & Meyers, 2009).

In this study, farmers had the highest prevalence rate of 30.9%. Rural dwellers had the highest prevalence rate of 63.6%, persons with no formal education had the highest prevalence rate of 41.8% while the unmarried persons were most prevalent (41.8%). This is in accordance with Chukwuekezie, (2007) who examined Buruli ulcer in Nigeria (results of a pilot case study in three rural districts). The efficacy of herbal remedies on Buruli ulcer patients showed that the plants extracts had increasing percentage efficacy on B. ulcer patients. The combined plants in a ratio of 1:1:1 had the highest efficacy. The growth of microbial resistance to antibiotics is a threat to the world population with an increasing recurrence of infectious diseases due to the emergence of multidrug resistant microorganisms that hinder chemotherapy (Ekeleme, Nwachukwu, Ogodo, Nnadi, Onuabuchi & Osuocha, 2013).

The antimicrobial activity of *Azadrichta indica* leaf extracts against *Mycobacterium ulcerans* showed broad spectrum antibacterial activity. The ethanolic extract had the highest zone of inhibition of 15.8±0.3 mm against *Mycobacterium ulcerans*, followed by an inhibition zone of 11.0±0.2 mm by hot water extract and the least inhibition zone of 9.0±0.2 mm by cold water extract. Other microbial isolates had an

inhibition zone range of 6.5±0.1*mm to 19.5±0.2 mm. There was no significant difference at p>0.05 between the inhibition zones of the extracts and that of the positive controls. The extracts of *Moringa oleifera* root produced an antimicrobial activity of 11.0±0.5 mm, 10.0±0.6 mm and 8.0±1.0 mm against *M. ulcerans*. Ethanolic extract produced the highest inhibition zone of 16.2±0.8 mm against *Aspergillus* sp and while the cold water extract had the least inhibition zone of 6.0±0.3* mm against *Candida* species.

The extracts of *Mucuna pruriens* seed produced lower antimicrobial activity of 10.2±0.6 mm, 7.9±0.1 mm and 7.1±0.4 mm against *M. ulcerans*. Ethanolic extract produced the highest inhibition zone of 12.0±0.1 mm against *Pseudomonas aeruginosa* and while the cold water extract had the least inhibition zone of 6.0±0.1mm against *Aspergillus* species.

Antimicrobial activity of the combined *Azadrichta indica* leaf, *Moringa oleifera* root and *Mucuna pruriens* seed extracts against *M. ulcerans* and bacterial isolates from Buruli ulcer had the highest inhibition zone of 25.6±0.1 mm against *M. ulcerans.* Out of the confirmed Buruli ulcer patients, 35 (77.8%) of the patients had their ulcer completely healed after 9 months of treatments, 6 (13.3%) of the patients were not cured after 9 months, while 4 (8.9%) were still receiving treatment after 9 months. This confirmed the claims that a number of Buruli Ulcer patients in the rural areas are being successfully treated with herbs since the herbal preparations demonstrated anti-*M. ulcerans* activity. Herbs are traditionally used for the treatment of sores, wounds and ulcers. Herbal therapy would be worth considering and, actively pursued as a BU treatment option, since a single herb is never a single compound but a group of compounds, which potentiate each other or create synergy (Portaels, Silva & Meyers, 2009).

The use of herb or herbal cocktail would simulate combination therapy, which may prevent, or delay the development of antimicrobial resistance. Observations in BU patients demonstrate that *M. ulcerans* disseminates into regional lymph nodes, visceral organs and bone marrows, with resultant osteomyelitis (Portaels, Silva & Meyers, 2009).

Conclusion

Herbal therapy should be investigated further since the Buruli ulcer herbal preparations provided by traditional herbal practitioner inhibited all the Mycobacterium ulcerans isolates investigated, hence, suggesting that herbal therapy could eventually be considered as one of the Buruli ulcer treatment option. This could also offer scientists who are interested in isolating bioactive compounds from plants a wide range of choices for Buruli ulcer drug research and development.

Recommendations

- Early detection of cases at the community level, and information, education and communication
- Training of health workers and village health workers
- Laboratory confirmation of cases
- Early management to prevent disability/rehabilitation

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Biography

Dr. Cosmas Nnadozie Ezejindu is a researcher, author, adviser, speaker and lecturer. His extensive research is widely recognized in NGOs, academic circle and the globe. His researches have received numerous awards and have been cited by many scholars. He obtained his first degree in University of Calabar in the Department of geology. He earned his Postgraduate Diploma in Public Health microbiology, Masters in Public Health microbiology and Ph.D. in Public Health. Currently lecturing at Davd Umehi Federal University of Health Science Uburu, Ebonyi State Nigeria and Abia State University Uturu, Abia State.