Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
www.phytojournal.com
JPP 2022; 11(4): 01-09
Received: 03-04-2022
Accepted: 08-05-2022

Dada-Adegbola HO
[a] Department of Medical Microbiology and Parasitology, Faculty of Basic Medical Sciences, College of Medicine/University College Hospital, University of Ibadan, Nigeria
[b] Department of Pharmacognosy, Faculty of Pharmacy, University of Ibadan, Nigeria

Moody JO
Department of Pharmacognosy, Faculty of Pharmacy, University of Ibadan, Nigeria

Corresponding Author:
Dada-Adegbola HO
[a] Department of Medical Microbiology and Parasitology, Faculty of Basic Medical Sciences, College of Medicine/University College Hospital, University of Ibadan, Nigeria
[b] Department of Pharmacognosy, Faculty of Pharmacy, University of Ibadan, Nigeria

In vitro activities of the methanolic extracts and fractions of *Bridelia ferruginea* Benth on clinical isolates of multidrug resistant gram-negative bacteria

Dada-Adegbola HO and Moody JO

DOI: https://doi.org/10.22271/phyto.2022.v11.i4a.14437

Abstract
Introduction: Antimicrobial resistance is of major public health concern. There is paucity of data on antimicrobial activity of the fractions and sub-fractions of methanol extract of the stem bark of *Bridelia ferruginea* Benth, Phyllanthaceae on multi-drug-resistant Gram negative bacteria. We explored their susceptibility to fractions of this plant extracts.
Method: Methanolic extract and N-hexane, Dichloro-methane (DCM) and Ethyl acetate (EtoAc) fractions of the dried stem bark of *Bridelia ferruginea*, were tested against twenty multi-drug-resistant GNB clinical isolates using Agar diffusion and Broth-dilution methods.
Results: The zone of inhibition of extracts ranged between 0-22mm, comparable to 0-28mm observed for Imipenem and Ciprofloxacin. The EtoAc fraction exhibited the highest antimicrobial activities against *Klebsiella pneumoniae, Acinetobacter baumanni* and others.
Conclusion: Most of the antimicrobial activities of *Bridelia ferruginea* Benth reside in the ethyl-acetate fraction and further study to isolate the active compound(s) is advocated and desired for development of new drugs against MDR.

Keywords: *Bridelia ferruginea*, multidrug resistant, gram-negative bacteria, clinical isolates, *Acinetobacter baumanni*

Introduction

Drug resistance continues to present an ever-increasing threat to global health, because all major pathogens of both human and animals are involved and all classes of antimicrobial agents are affected [1, 2]. The rate of emergence of antibiotic resistant bacteria seems not matched by the rate of development of new antibiotics to combat them. While the development of new antimicrobials had slowed down, the prevalence of resistance has grown at an astronomical rate.

Microorganisms have deployed what could be termed “advanced technologies” to match human technological development, because as soon as a new antimicrobial is introduced, resistance sets in to make it a short-lived relief. According to a recent assessment by the World health organization (WHO), antibiotics resistance has become a global concern [3] (WHO, 2016). The resistance mechanism is a well-organized activity in microorganisms; therefore, humans definitely must find a decisive means to combat resistance. To further justify the importance of antimicrobial resistance the Infectious Diseases Society of America (IDSA) referred to antibiotic resistance as an epidemic that calls for action [4]. Finding only 22 (5.1%) of all isolates from January to December 1999 from our previous study (Unpublished data), sensitive to penicillin, is an indication that this drug should no longer be used as first line antibiotics as was the practice in some years past. The consequences of the various antimicrobial resistance is that patients with infections caused by drug-resistant bacteria are more likely to stay longer in the hospital and generally are at increased risk of worse clinical outcomes (increase morbidity) and even death (mortality). These categories of patients are found to consume more health-care resources in the form of more investigations, prolonged in-patient care, more expensive antibiotics than patients infected with the same bacteria that are not resistant [5].

If no urgent steps are taken to curtail the rate of resistance either through infection control practices and discovery of new and effective antimicrobial, the world may be heading towards the ‘era of pre-antibiotic’ when common infections were killing many people.

The global increase in resistance to antibiotics has forced researchers to search for other antimicrobials, and plants have been seen as a good source of antimicrobials [6]. Numerous studies on plants have identified compounds that are effective antibiotics [7]. Traditional healing systems around the world that utilize herbal remedies are an important source for the discovery of new antibiotics [8]. Some traditional remedies with effective antimicrobial activity even against resistant strains of bacteria have already been produced [9].
This study, in an effort to expand the spectrum of antibacterial agents from natural resources, selected *Bridelia ferruginea* belonging to Phyllanthaceae family. Bridelia is wide spread in many regions of the world such as Africa, Southern Asia, Australia, and India, was initially placed in the family known as Euphorbiaceae, was first described in 1806, while the genus Bridelia was named in the honor of Samuel Elisẽe Bridel-Brideri by the German botanist Carl Ludwig Willdenow [10, 11]. *Bridelia ferruginea* Benth is one of the many herb that have been in use for decades in my village for the treatment of febrile and debilitated individuals who have developed furred tongue as a result of oral thrush. It is believed to hasten recovery from severe illness. *Bridelia* plants has been employed in various healing activities as a result of the main properties ascribed to them, which include aphrodisiac, astringent, diuretic, febrifuge, laxative and purgative [12]. The medicinal property is believed to reside in the astringent property, which in turn was due to the presence of tannins [12]. Traditionally, the stem bark is prepared as decoctions, soups, chewed raw or extracted with lime and sometimes in combination with other herbs for patient management.
Several literatures actually report chemical investigations, phytochemical analysis, isolation of compounds and pharmacological activities of about 16 species of *Bridelia*, out of the over 60 currently known. It is interesting to note that so far, there are very few published data concerning the antimicrobial activities of the various plant parts and the isolated compounds from these *Bridelia* plants. The few available studies on *Bridelia ferruginea* both within and outside Nigeria has been on the leaves, fruits and roots in management of diseases other than infections [13, 14, 15]. The stem bark is pounded into powder and used to treat hemorrhoids, oral thrush [16, 17]. The water and ethanolic stem bark extracts of *Bridelia ferruginea* Benth showed *in vitro* antimicrobial activities [17, 18].
However, there is paucity of data on antimicrobial studies of the extracts and fractions of stem bark extracts of *Bridelia ferruginea* Benth and very few studies if any, to our knowledge, on the activity of the stem bark extract and fractions on multidrug-resistant microorganisms from clinical specimens.
This study therefore was aimed at investigating the *in vitro* activities of methanolic extracts ahead the isolation of specific compounds and determination of mode of action of *Bridelia ferruginea* Benth against multidrug resistant Gram-negative bacilli, isolated from various clinical specimens.

Materials and Methods

Plant collection, authentication and preparation

The 'matured' stem bark of *Bridelia ferruginea* Benth was collected from a primary forest in Obbo-Ile, Ekiti Local Government Area of Kwara state, Nigeria in April 2015. This was air dried under shade, for about four weeks so as to allow it to dry completely. Maturation of stem-bark was determined by the extent of fissuring and redness of the fleshy part. The outer-fissured part of the stem bark-surface was removed so as to expose the red fleshy portion before maceration. The dried stem bark was grounded with mortar and pestle and later milled into a coarse powder with a pepper-milling machine. Authentication of the plant material was done at Department of Pharmacognosy Herbarium, University of Ibadan where a voucher specimen (DPHUI 1708) was deposited.

Methanol extraction

The powdered plant material (3 kg) of the ripe stem bark of *Bridelia ferruginea* (BF) was macerated in 5 L of distilled methanol for 72 hours with constant stirring for 10-15 minutes daily. The extract was filtered with a muslin cloth and the procedure was repeated three times on the marc to ensure exhaustive extraction.

Concentration of extracts of *Bridelia ferruginea* Benth

The methanol extracts were pooled and dried *in vacuo* at 40°C using rotary evaporator RE52-2, Search Tech Instruments. The dried methanol extract was then kept in airtight container for further processing.

Phytochemical analysis of *Bridelia ferruginea* Benth bark

The screening procedures were carried out according to the standard procedure [19, 20, 21, 22].
and the dried bark and the methanol extract were screened for the presence of flavonoids, anthraquinones, saponins, tannins, glycosides, alkaloids, terpenoids and steroids.

Collections and Preparation of Bacterial isolates

Gram negative bacteria (GNB) from various infections affecting different body (anatomic) sites and various age groups were collected from the Department of Medical Microbiology and Parasitology of University College Hospital, Ibadan. The GNB isolates were identified to species level using Microbact (OXOID Microbact™ Identification Kits Microbact™ GNB 24E, Oxoid Ltd Wade Road, Basingstoke, Hants, RG24 8PW, UK) and other biochemical reactions following standard procedure [23, 24]. These bacterial isolates were initially subjected to standard antibiotic susceptibility testing and those found to be multidrug resistant (MDR) or extended drug resistant (XDR) were selected for the study. The GNB isolates selected include 23 GNB (21 clinical isolates and 2 control organisms) such as *Stenotrophomonas maltophilia, Acinetobacter baumanni, Morganella morganii, Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Escherichia coli,* also included were control *Acinetobacter baumanni* NCTC 7363 and *Escherichia coli* ATCC 35218.
Overnight culture broth of the organisms was prepared by inoculating a single colony of the isolate (to ensure pure growth) into a peptone water followed by incubation aerobically at 37°C for 18 – 24 hours. The fresh broth was diluted with sterile normal saline to make 0.05 MacFarland standard, which was used for the bioassays (Agar diffusion or Broth dilution).

Extract preparation for antimicrobial assays

Two hundred milligram (200 mg) each of the crude methanol and aqueous extracts weighed and constituted separately into aqueous solution by the dissolution in 5 millilitres (5 mL) of distilled water respectively. From this stock solution (200 mg in 5000 μL of water), aliquot of 500 µL was removed (20 mg) to prepare the different serial concentration using double dilution in five tubes containing 250 µL of distilled water as follows:

Twenty milligrams (20 mg) from the stock solution (0.5 mL of stock) was added into the first tube (tube 1) thus in the first tube we have 20 mg in 500 microliters. Two hundred and fifty microliters (250 µL) which is 10 mg, was removed from this 0.5 mL and added to the second tube and the process was repeated for the third, fourth and fifth tubes thus given double dilution of 20 mg/mL, 10 mg/mL, 5 mg/mL and 2.5 mg/mL and 1.25mg/mL in tubes 1, 2, 3, 4, and 5 respectively. Each of the dilution was used to determine the minimum inhibitory concentration (MIC) of the extract and also the minimum bactericidal concentration (MBC) in the agar diffusion method. Broth dilution method, using the 96 well micro titre plate, was also used for MIC and MBC determination. Eight (8) serial dilutions from 20 mg/mL were prepared thus giving a dilution of 20, 10, 5, 2.5, 1.250, 0.625, 0.312, and 0.156mg/mL respectively in wells A - H.

One hundred microliter (100 µL) of the extract dilution series was introduced into each well either in the agar or the 96 well micro titre plate, therefore for 10 tested organisms; the volume of extracts solution for each dilution series was 1000 µL (20 mg/mL x 10, 10 mg/mL x 10; 5 mg/mL x 10; 2.5 mg/mL and 1.25 mg/mL x 10, 0.156 mg/mL x10). They were prepared in separate tubes to allow for accuracy in pipetting and were done for each of the extracts (aqueous, and methanol).

Antimicrobial assays using agar well diffusion method

In each Mueller Hinton (susceptibility) agar plate, five or six wells were made with agar borer, according to purpose. The six-welled agar plates were used for comparing the activities of the methanol, aqueous, ethyl acetate, N-hexane and dichloromethane fractions and old aqueous extracts. The five-welled agar plate on the other hand was used for the comparative analysis of the activities of the serial dilutions of the most active fraction, which happens to be the ethyl acetate fraction. The susceptibility of isolates were also determined for the column fractions to select the best fraction with antimicrobial activities and these fractions were selected for further analysis including MIC and MBC determination.

Mueller Hinton agar was prepared in 25 mm Petri dish, and in the agar plate, wells were made at almost equidistance to each other at the periphery and one well was placed centrally, the agar borer was sterilized over Bunsen-burner flame, to ensure no microbial contamination. Meanwhile, space was deliberately left for two commercial antibiotic discs and each well was labeled. Each bacterial isolate was prepared in peptone water and diluted to 0.05 MacFarland standard. Sterile cotton swab was dipped in the organism broth and excess was gently strained out and the swab was used to evenly inoculate the bored plate by swabbing. The media surface was allowed to dry for about 30 minutes, after which 100 µL of the different pre-determined concentrations of the extract were carefully introduced into the wells. Triplicate samples were prepared for each organism to validate zone of inhibition by extracts and commercial antibiotic discs, depending on which organism is inoculated, were introduced for comparison. The plates were incubated at 37°C for 18 - 24 hours or overnight. After the incubation, the diameter of the clear zone around the antibiotic disc and the wells were measured with a divider, which was then placed against a ruler. The three different readings of the zones of inhibition on the three plates were recorded and used for final analysis and determination of the mean and standard deviation.

Broth dilution method using 96 well micro titre plate

The minimum inhibitory concentration was done using two-fold serial dilutions from the lowest concentration used in the antimicrobial assay to 1/16th of that particular concentration in a 96 well micro titre plate. Each well containing varying concentrations were further sub cultured on Mueller Hinton agar and the point at which no visible growth was seen was taken as the minimum inhibitory concentration [25]. Mueller-Hinton broth was added to each microwell and 10mg/100 µl of the extract was then constituted and serially diluted. A pre-determined concentration of microbes (0.5 MacFarland standard) was prepared in Bijou bottles. Approximately 10 µl (a loopful) of organism broth was introduced into each well containing the serial dilution of extract.

A well with only the organism broth was included as positive control and another well with only the extract or fraction that is being tested as negative controls. Included in the experiment were control organisms. The wells with no obvious multiplication (clear, non-turbid) were noted, the first well with no growth (the highest dilution at which the sample just prevents growth of the microorganism) was taken as the minimum inhibitory concentration (MIC) of the extract.

Determination minimum inhibitory and bactericidal concentration (MIC and MBC) using broth dilution method

For the determination of the minimum inhibitory and minimum bactericidal concentration, the 96 round-bottom microtitre plates were used. Each column (A - H) was used for serial dilution of the extracts and the rows (1 - 12) were for different isolates. Each well contains 100 µL of the serial dilution of the extract as 10, 5, 2.5, 1.25, 0.625, 0.312, 0.156, 0.078 mg dispensed into wells A – H while 12 organisms were tested on a plate against ethyl acetate fraction, another plate for N-hexane and another for dichloromethane (DCM) fraction. The wells were inoculated with a loopful (≈ 10 µL) of overnight organism broth and wells were observed for turbidity which is a sign of visible growth and the well without visible growth was taken as the concentration at which multiplication of the organism was inhibited and interpreted as minimum inhibitory concentration (MIC). The concentrations of the extract in such wells were recorded. However, because this extract is coloured red, observing for turbidity was difficult i.e. not very convincing, but possible as this will appear as an unusual thickening of the broth. To ascertain that there was actually an inhibition of growth, each well was sub-cultured unto a solid non-inhibitory agar (Nutrient agar and Blood agar) and incubated at 37°C for 18 - 24 hours or overnight.

Each agar plate was divided into four, and in each quadrant, the broth from eight wells (A-H) were inoculated which represents double dilutions from 10, 5, 2.5, 1.25, 0.625, 0.312, 0.156, 0.078 mg/mL of the extract. The concentration in the first well where no turbidity occurred was interpreted as MIC while the subculture was done on solid agar to determine which well will yield growth. The first well with the least concentration where there was no growth of organism was reported as MBC.

Results

Susceptibility of multi-resistant Gram-negative bacteria (GNB) clinical isolates and control GNB to Bridelia extracts and fractions.

Twenty-three different strains of Gram-negative bacteria (GNB), consisting of 21 clinical isolates from various human specimens and 2 control organisms were tested against the different extracts and fractions of *Bridelia ferruginea* Benth. The zone of inhibition ranged between 0 – 22 (± 2.56), 0 – 16

(± 7.01) mm for EtOAc, DCM and hexane fractions respectively, 0 – 18 (± 7.42) mm and 0 – 20 (± 8.14) mm for methanol and aqueous extracts respectively. The lower dilutions of ethyl acetate fraction also inhibited several of the tested organisms. Two control organisms *Escherichia coli* ATCC 35218 and *Acinetobacter baumanni* NCTC 7363 were included to compare the susceptibility with that of clinical isolates. Two standard antibiotic discs of a quinolone (Ciprofloxacin 5 µg), and a carbapenem (Meropenem or Imipenem 10µg) were included for comparison of the activity of the extracts and standard antibiotics that are currently employed in the treatment of infections caused by these organisms. The zones of inhibition of these standard antibiotics were 0 – 36 (± 11.35) mm and 0 – 30 (± 10.02) mm for Ciprofloxacin and Imipenem respectively. Nineteen GNB isolates and the two control GNB which implies 91.30% of tested GNB were inhibited by ethyl acetate fraction of Bridelia while 86.96% and 47.83% of all GNB tested were inhibited by imipenem and ciprofloxacin respectively (Table 1 and Figure 1)

Table 1: Susceptibility of multidrug-resistant (MDR) Gram negative bacteria clinical isolates to Bridelia extracts and fractions.

	Zone of Inhibition, produced by different concentrations of Ethyl acetate fractions/others/standard antibiotics, in millimetres (mm) (SD)									
	Ethyl acetate fraction (mg)				**DCM**	**N-hexane**	**MeOH**	**Aqueous extract**	**Standard antibiotics**	
GNB	10	5	2.5	1.25	10 mg	10 mg	10 mg	10 mg	CIP 5µg	IMP 10µg
St.malt	12 ±1.3	10 ±0.3	0±0.0	0 ±0.0	14 ±0.0	10 ±0.2	10 ±0.6	13 ±1.0	0 ±0.0	0 ±0.0
*M.m*bp 1	16 ±1.6	12±1.1	10±1.1	0±0.0	10±0.0	8±0.5	10±0.6	12±1.7	24±1.0	28±1.1
*A.Iwofi*i	18±1.0	14±2.0	10±0.6	8±0.5	12±1.1	10±0.5	10±0.0	12±1.7	0±0.0	20±2.0
St.Malt	16±1.6	10±0.6	0±0.0	0±0.0	12±1.1	0±0.0	12±1.1	12±1.6	0±0.0	25±2.5
*M.*subsp. M	16±0.5	10±1.1	0±0.0	0±0.0	12±0.5	10±0.6	16±1.6	14±2.0	20±0.0	28±3.0
*M.morg*bg1	14±0.6	13±1.0	12±0.0	0±0.0	0±0.0	10±0.0	18±1.0	20±0.6	0±0.0	28±3.0
P. stuarti	16±2.0	12±0.1	0±0.0	0±0.0	12±1.7	8±0.6	16±1.6	12±1.7	20±0.6	26±4.6
S. malto	14±2.0	11±1.7	10±1.1	10±0.0	0±0.0	0±0.0	16±1.6	14±1.0	0±0.0	0±0.0
*M.m*biogp 1	12±1.1	10±0.5	0±0.0	0±0.0	0±0.0	0±0.0	10±1.1	10±0.0	0±0.0	24±1.0
S.malto	12±1.7	10±0.0	8±0.6	0±0.0	0±0.0	0±0.0	8±0.6	10±0.6	24±2.0	24±1.0
A. baum	0±0.0	0±0.0	0±0.0	0±0.0	14±1.0	0±0.0	0±0.0	0±0.0	0±0.0	16±1.6
P. aerug	20±0.6	10±0.6	0±0.0	0±0.0	10±0.6	0±0.0	0±0.0	14±0.0	14±0.0	24±2.5
S.malto	0±0.0	0±0.0	0±0.0	0±0.0	0±0.0	0±0.0	0±0.0	0±0.0	0±0.0	16±2.0
E. coli ATCC 35218	14±0.6	12±0.0	12±1.1	12±1.1	10±0.0	10±0.0	10±0.0	10±0.0	36±1.0	24±2.0
A.bauma NCTC7363	22±1.5	20±0.5	18±1.0	18±1.0	15±1.6	10±1.1	16±1.6	15±1.6	24±1.0	28±3.0
P. aerug	22±2.0	20±0.6	18±0.6	16±1.6	14±2.0	12±0.0	16±2.0	20±1.5	0±0.0	22±1.5
P. aerug	18±0.6	16±0.6	14±1.6	14±1.6	12±1.7	12±1.7	12±1.7	18±1.0	0±0.0	0±0.0
K. pneu	20±0.6	16±0.6	14±0.0	14±1.6	12±0.0	14±0.5	10±0.0	12±1.7	26±2.5	28±0.0
E. coli	18±0.0	18±1.0	16±1.7	16±0.6	16±0.6	16±0.0	0±0.0	10±1.7	24±3.0	30±0.0
P. mirab	22±2.0	18±1.0	16±0.0	16±0.6	16±0.6	14±0.6	14±1.7	16±0.6	14±0.0	24±0.0
S. malto	16±0.6	12±1.7	12±1.7	12±1.7	0±0.0	0±0.0	10±1.1	16±0.6	0±0.0	22±1.5
P. stuart	18±0.6	12±1.7	12±1.7	10±0.6	0±0.0	0±0.0	10±0.6	10±0.6	0±0.0	24±2.0
K. Oxytoca	19±1.5	15±1.6	12±1.7	12±0.6	8±0.6	10±0.0	14±1.6	12±1.1	15±1.6	22±0.0

Key: *M.morggb1= Morganella morganii biogroup 1; P. stuarti= Proteus stuartii; S. malto= Serratia maltophilia: St.malto= Stenotrophomonas maltophilia; A. baum= Acinetobacter baumanni; P. aerug= Pseudomonas aeruginosa; E. coli= Escherichia coli; K. pneu= Klebsiella pneumonia; P. mirab = Proteus mirabilis; P. stuart= Proteus stuarti; K. oxytoca= Klebsiella oxytoca; M. subsp.m= Morganella morgani subspecies morgani. Enterobacter gergoviae.* CIP = Ciprofloxacin, IMP= Imipenem

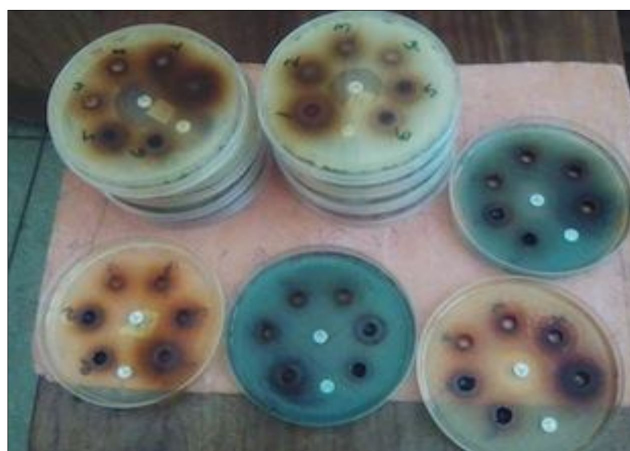


Fig 1: Susceptibility of MDR GNB to Bridelia extracts and fractions.

Minimum inhibitory concentration of GNB isolates

The minimum inhibitory concentration (MIC) was determined for the Bridelia fractions so as to know the lowest concentration that will inhibit the visible growth of microorganisms. Ethyl acetate fraction gave MIC of 0.312 – 0.625 mg for clinical isolate and 0.032 mg for control organisms, while DCM gave 0.156 – 0.625mg for clinical isolates and 0.031 and 0.625 mg for the control organism. Hexane inhibited at a higher concentration of 0.625 – 2.50 mg (Table 2 and Figure 2).

Table 2: Minimum inhibitory concentration of fractions on selected GNB isolates

Serial No.	Isolate	Ethyl acetate (mg/mL)	DCM (mg/mL)	N-hexane Fraction (mg/mL)
1.	*Stenotrophomonas maltophilia*	0.625	0.156	1.250
2.	*Escherichial coli*	0.312	0.312	0.625
3.	*Morganella morgani biogrp 1*	0.625	0.312	0.625
4.	*Acinetobacter iwofii*	0.625	0.312	1.250
5.	*Proteus stuarti*	0.625	0.625	1.250
6.	*Stenotrophomonas xanthomonas*	0.625	0.625	0.625
7.	*Morganella morgani subsp morgani*	0.625	0.625	0.625
8.	*Acinetobacter baumanni*	0.625	0.625	2.500
9.	*Pseudomonas aeruginosa*	0.312	0.625	2.500
10.	*Serratia maltophilia*	0.312	0.625	1.250
11.	*Escherichia coli* ATCC 35218	0.031	0.031	Not done
12.	*Acinetobacter baumanni* NCTC 7363	0.031	0.625	Not done

Minimum bactericidal concentration of GNB

The minimum amount of extract that will inhibit the physical growth and kill the organism using the broth dilution method gave a concentration of 0.312 – 0.625 mg/mL for both of the ethyl acetate and dichloromethane (DCM) fractions and 0.625 – 1.250 mg/mL for hexane fraction. One isolate of the *Stenotrophomonas maltophilia* and one *Acinetobacter baumanni* were not inhibited by lower concentrations of the extract and fractions (Table 3).

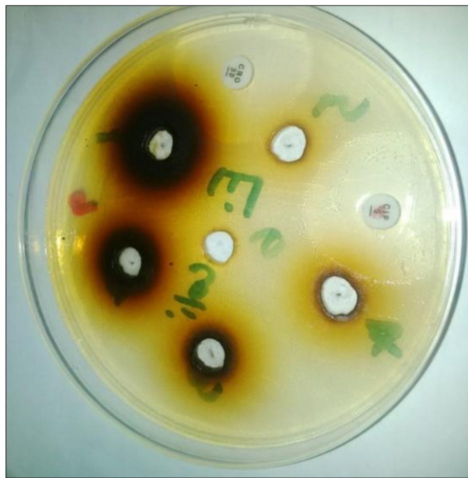
Table 3: Minimum bactericidal concentration (MBC) of *Bridelia ferruginea* solvent-partitioned fractions on Gram negative bacilli (GNB)

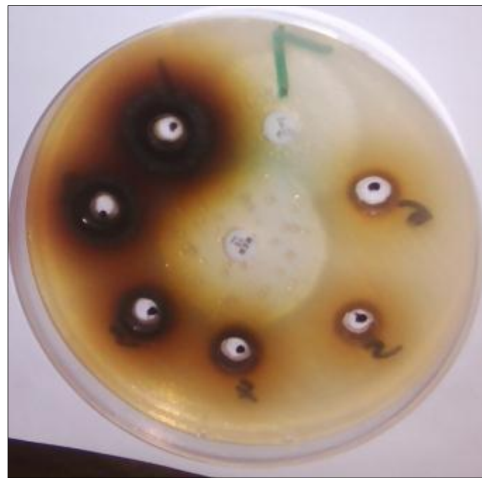
Serial No.	Isolate	Ethyl acetate fraction (mg/mL)	DCM fraction (mg/mL)	N-Hexane fraction (mg/mL)
1.	*Stenotrophomonas maltophilia*	0.312	0.312	0.625
2.	*Morganella morganii biogp1*	0.625	0.625	0.625
3.	*Acinetobacter iwofii*	0.625	0.625	0.625
4.	*Stenotrophomonas maltophilia*	0.625	0.625	1.250
5.	*Morganella morganii subsp. morganii*	0.625	0.625	1.250
6.	*Morganella morganii biogp1*	0.625	0.625	1.250
7.	*Proteus stuarti*	0.625	0.625	1.250
8.	*Stenotrophomonas xanthomonas*	0.312	0.625	1.250
9.	*Morganella morganii biogp1*	0.625	0.625	1.250
10.	*Stenotrophomonas maltophilia*	0.625	0.625	1.250
11.	*Acinetobacter baumanni*	0.625	0.625	1.250
12.	*Pseudomonas aeruginosa*	0.625	0.625	1.250
13.	*Stenotrophomonas maltophilia*	0.312	0.625	1.250
14.	*Acinetobacter baumanni control*	0.625	0.625	1.250
15.	*Escherichia coli control*	0.312	0.625	1.250
16	*Serratia maltophilia*	0.625	0.625	1.250
17.	*Enterobacter gergoviae*	0.312	0.625	1.250
18.	*Proteus mirabilis*	0.312	0.625	1.250
19.	*Pseudomonas aeruginosa*	0.312	0.625	0.625
20.	*Klebsiella oxytoca*	0.312	0.625	0.625

Table 4: Comparison of inhibitory activity of different concentration of ethylacetate fraction with standard antibiotics on selected MDR GNB.

Zone of inhibition, produced by standard antibiotics and different concentrations of Ethyl acetate fractions in millimetres (mm) (SD)									
Serial No.	**Isolate**	**MER 10 µg**	**CIP 5 µg**	**10 mg**	**5 mg**	**2.5 mg**	**1.25 mg**	**0.65 mg**	**0.312 mg**
1.	*S. maltop*	**0±0.0**	**0±0.0**	**18±1.5**	**16±1.0**	0±0.0	0±0.0	0±0.0	0±0.0
2.	*E. coli*	**0±0.0**	**0±0.0**	**13±0.6**	**11±1.7**	**10±2. 0**	0±0.0	0±0.0	0±0.0
3.	*M. morg*	**30±3.0**	0±0.0	**28±1.5**	16±1.6	12±1.7	11±1.7	0±0.0	0±0.0
4.	*A. iwoffii*	32±2.5	0±0.0	20±2.0	18±1.5	16±1.6	15±1.5	0±0.0	0±0.0
5.	*P. stuarti*	**34±2.0**	0±0.0	**36±1.6**	**34±1.5**	22±1.7	11±1.7	0±0.0	0±0.0
6.	*S. xantho*	28±1.5	29±1.5	24±2.0	23±0.6	22±0.6	09±1.1	0±0.0	0±0.0
7.	*M. mor Sm*	**33±2.5**	0±0.0	**34±1.1**	23±1.0	21±1.7	10±0.0	0±0.0	0±0.0
8.	*A. bauma*	**29±2.5**	0±0.0	**32±0.6**	20±1.7	0±0.0	0±0.0	0±0.0	0±0.0
9.	*P. aerugi*	28±2.5	0±0.0	21±1.1	19±1.1	12±1.1	09±1.1	0±0.0	0±0.0
10.	*S. malto*	**29±1.5**	**0±0.0**	**0±0.0**	**0±0.0**	**0±0.0**	**0±0.0**	**0±0.0**	**0±0.0**
11.	*E. coli* ATCC 35218	**24±1.0**	**29±1.0**	**32±2.5**	**28±1.5**	**27±3.0**	**24±1.0**	**22±2.0**	**20±1.0**
12.	*A. bauma* NCTC 7363	**28±3.0**	**26±1.0**	**28±1.5**	**26±0.0**	**26±0.6**	**22±2.0**	**20±1.0**	**18±0.6**

MER= Meropenem, CIP = Ciprofloxacin, S. maltop= *Stenotrophomonas maltophilia*, A. bauma = *Acinetobacter baumannii*, P. aerugi = *Pseudomonas aeruginosa*, E. coli = Escherichia coli; A. iwofii = *Acinetobacter iwofii*; M. mor Sm= *Morganella morganii subsp. Morganii; P. stuarti = Proteus stuarti, M. Morg = Morganella morganii;* S. xantho = *Stenotrophomonas xanthomonas.*





E. coli and EtOAc *S. xanthomonas* and EtOAc

Fig 2: Microtitre plate for the MBC Determination

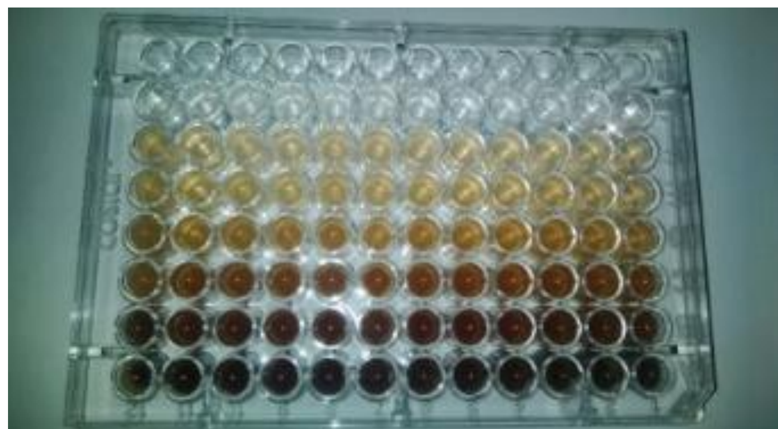


Fig 3: Microtiter Plate for MIC Determination

Acinetobacter baumanni *Escherichia coli*

Fig 4: Comparison of susceptibility of column fractions on selected organisms

Discussion

Stenotrophomonas maltophilia has recently emerged as a threatening nosocomial pathogen and difficult to treat, as it is intrinsically resistant to a wide range of commonly used drugs. Infection by this organism is predominant mainly in immune compromised individuals with different types of organs affection [26]. It is a globally emerging GNB that is widely spread in environment and hospital equipment.

Pseudomonas aeruginosa, a Gram-negative bacterium that is ubiquitous and responsible for several opportunistic nosocomial infections and has been found to be responsible for a wide range of infections that may present high rates of antimicrobial resistance.

The genus *Klebsiella* belongs to the tribe Klebsiellae, a member of the family Enterobacteriaceae, in recent years, have become important pathogens in nosocomial infections, where endotracheal intubation, impaired host defenses, and antimicrobial use have been associated with oropharyngeal carriage [27]. Klebsiellae have also been incriminated in HA infections, with common sites being the urinary tract, lower respiratory tract, biliary tract, and surgical wound sites. The presence of invasive devices, contamination of respiratory support equipment, use of urinary catheters, and use of antibiotics are factors that increase the likelihood of nosocomial infection with *Klebsiella* species [28, 29, 30].

Acinetobacter is a group of non-fermentative, aerobic, opportunistic, Gram-negative cocci bacillary rod widely distributed in soil and water. They pose very minimal risk to healthy individuals and normally inhabits skin, mucous membranes, and soil [31] but are capable of causing disease in people with weakened immune systems and therefore are isolated from outbreaks in intensive care units (ICUs) and healthcare settings where *Acinetobacter baumannii* is said to show resistance rate of 60 - 80% to Carbapenems and responsible for up to 53.3% mortality in critical care unit [32, 33, 34].

The bark of *Bridelia ferruginea* is rich in saponins, tannins and flavonoids while alkaloids, steroids and anthraquinones were moderately present. Terpenoids were only minimally present. However, cardiac glycosides were not detected by the bio-chemical tests employed. The presence of tannins and flavonoids may be responsible for the antimicrobial activities exhibited by the extracts and fractions tested, while the presence of alkaloids may be responsible for other anti-cancer and antioxidant activities reported in the literature. Flavonoids are hydroxylated phenolic substances that occur as a C6-C3 unit linked to an aromatic ring. They have been reported to be synthesized for defense by plants in response to microbial infections [35], and have been found *in vitro* to be effective antimicrobial substances against a wide array of microorganisms. The antimicrobial activity is probably linked with their ability to complex with extracellular and soluble proteins and to complex with bacterial cell walls, similar to what is obtainable for quinones and it was found that even the more lipophilic flavonoids is capable of disrupting microbial membranes [35]. Therefore, it is not surprising that the extract is exhibiting a broad-spectrum antimicrobial activity.

The extracts and fractions of Bridelia were found to inhibit a higher percentage of the tested isolates than the standard antibiotics, thereby comparing favourably with them. Both extracts and all the fractions of *Bridelia ferruginea* exhibited good activities against the Gram negative bacteria with more of all GNB tested being inhibited by extracts more than by the standard drugs: imipenem/meropenem and ciprofloxacin, which are typical example of the current drugs employed in the management of infections caused by these organisms. Bridelia extracts and fractions exhibited a higher percentage of GNB inhibition of 91.30% versus 86.96% for carbapenems and 47.83% for quinolone (ciprofloxacin), while the ethyl acetate fraction even showed a better inhibitory activity than other fractions and standard antibiotics (Table 4).

A clinical isolate of *Stenotrophomonas maltophila* was resistant to all the standard drugs but was inhibited by methanol and aqueous extracts and ethyl acetate fraction of *Bridelia ferruginea*. Another clinical MDR isolate, *Pseudomonas aeruginosa* was inhibited by Bridelia, even at a lower concentration of 1.25mg of the EtOAc fraction (Table 4). One of the strains of *Acinetobacter baumanni* was resistant to all tested standard drugs and extracts except the DCM fraction that inhibited with a zone of 14 mm diameter. Infections with these MDR are very devastating with poor clinical outcome in the absence of antibiotics to treat such cases. In all, extracts and fractions of *Bridelia ferruginea* (BF) exhibited good antimicrobial activity against MDR Gram negative bacteria that can be explored for solution to combating infections by MDR. The earlier study by Burkil in 2004 reported only activity against GPC and no activity on GNB [12]. It has been reported that GNB were more susceptible to Bridelia extracts [18] although other previous studies on its antimicrobial activities focused on common isolates, some of which are environmental or even control organisms, our current study was targeted mainly on clinical isolates with multi-drug resistant features and emerging organisms such as *Acinetobacter baumanni* and *Stenotrophomonas maltophilia*. Irobi and colleagues [18] also reported EtOAc fraction as being the most active of the three when tested against ten selected MDR GNB and the activity was compared with standard drugs. The findings from the current study as contained in Table 4 showed that 10 mg/mL of EtOAc fraction has comparative activity and even more superior to the ciprofloxacin 5 µg and meropenem 10 µg. Even, the isolates of *Stenotrophomonas maltophilia* and *Escherichia coli* that were not inhibited by these standard drugs, was appreciably inhibited 5 mg/mL of the extract of *Bridelia ferruginea*. Some other antibiotics that were compared with BF but not showed in the results include amikacin, ceftazidime, with the extracts exhibiting good if not better activity than most of these available antibiotics. There is a good comparative activity against *Proteus stuarti, Acinetobacter baumanni* and several other GNB as shown in Table 4. Most previous studies did not work on fractions of BF. All the fractions in this study had a good minimum inhibitory concentration (MIC) of 0.625-1.25mm, however, ethyl acetate (EtOAc) and dichloromethane (DCM) fractions are most active while EtOAc had the overall best activity against both clinical isolates and control organisms. Although all the three fractions demonstrated good bactericidal activities, the EtOAc and DCM fractions exhibited ability to kill GNB at a lower concentration than the hexane fraction. The minimum bactericidal concentration (MBC) was almost similar to the MIC, which is an indication of good activity. The inhibitory and bactericidal activities were concentration dependent to some extent, although a higher concentration does not seem to offer additional benefit for the inhibitory activity in the initial antimicrobial screening tests, however a more detailed study will be required to confirm this observation.

Following bioactivity of the fractions obtained from solvent-solvent partitioning, the ethyl acetate fraction gave the best antimicrobial activity. It is also noteworthy that the Bridelia

extracts effectively inhibited MDR isolates that were resistant to meropenem and ciprofloxacin (Table 4 and figure 4). The MIC and MBC of 0.312-2.5 mg/mL obtained for the clinical isolates and control organisms and the fact that isolates which failed to respond to standard drugs were inhibited effectively by the Bridelia extracts, fractions and sub-fractions indicates a powerful antimicrobial activity.

In conclusion, the observed antimicrobial activity of the extracts, partitioned fractions and column sub-fractions against multi-drug-resistant clinical isolates of bacteria is comparable and even superior to the standard drugs. The extract of the stem-bark of *Bridelia ferruginea* Benth appear to contain bioactive constituents required to fight against multi-drug resistant organisms, which are responsible for disability and death among those affected.

The results of this study provide some justifications for the use of BF in the treatment of infections in Nigerian ethnomedicine and also warrants further research into the isolation, characterization and utilization of the bioactive templates in drug development for the management of infections and especially MDR and related disease conditions.

References

1. Levy SB, Marshall B. Antibacterial resistance worldwide: causes, challenges, and responses. Nature Medicine. (Abstract, Science Direct online), 2004;10:S122-S129.
2. Olayinka AT, Olayinka BO, Onile BA. Antibiotic susceptibility and plasmid pattern of *Pseudomonas aeruginosa* from the surgical unit of a University teaching hospital in north central Nigeria. International Journal of Medicine and Medical Sciences. 2009;1(3):079-083.
3. World Health Organization. Antimicrobial resistance Fact Sheet 2016. www.who.int/mediacentre/factsheets.
4. Spellberg B, Guidos R, Gilbert D, Bradley J, Boucher HW, Scheld WM, *et al*. The epidemic of antibiotic-resistant infections: a call to action for the medical community from the Infectious Diseases Society of America. Clin Infect Dis. 2008;46(2):155-64. Doi: 10.1086/524891.
5. Capone A, Giannella M, Fortini D, Giordano A, Meledandri M, Ballardini M, *et al.* High rate of colistin resistance among patients with carbapenem-resistant *Klebsiella pneumonia* infection accounts for an excess of mortality. Clin Microbiol Infect. 2013;19:E23-30. http://dx.doi.org/10.1111/1469-0691.12070.
6. Mahesh B, Satish S. Antimicrobial activity of some important medicinal plants against plant and human pathogen, World Journal of Agricultural Science. 2008;4:839-843.
7. Basile A, Sorbo S, Giordano S, Ricciardi L, Ferrara S, Montesano D, *et al*. Antibacterial and allelopathic activity of extract from Castanea sativa leaves. Fitoterapia. 2000;71:110-116.
8. Okpekon T, Yolou S, Gleye C, Roblot F, Loiseau P, Bories C, *et al*. Antiparasitic activities of medicinal plants used in Ivory Coast. J Ethnopharmacol. 2004;90:91-97.
9. Kone WM, Kamanzi AK, Terreaux C, Hostettmann K, Traore D, Dosso M. Traditional medicine in North Cote-d'Ivoire screening of 50 medicinal plants for antibacterial activity. J Ethnopharmacol. 2004;93:43-49.
10. Willdenow, Carl Ludwig von. Species Plantarum. Editio quarta 1806;4(2):978-979. in Latin. https://www.biodiversitylibrary.org/page/566705#page/347/mode/1up
11. Govaerts R, Dransfield J, Zona SF, Hodel DR, Henderson A. World Checklist of Phyllanthaceae Facilitated by the Royal Botanic Gardens, Kew 2011. Published on the Internet; http://apps.kew.org/wcsp/ Retrieved 2015-06-14.
12. Burkil HM. The Useful Plants of West Tropical Africa. Royal Botanic Gardens; Kew, 2004. http://www.aluka.org/
13. Oliver-Bever B. Medicinal Plants in Tropical West Africa. Cambridge University Press, 1986.
14. Onunkwo GC, Akah PA, Udeala OK. Studies on *Bridelia ferruginea* leaves: Stability and hypoglycemic actions of the leaf extract tablets. Phytother. Res. 1996;10:418-420.
15. Oloyede OI, Babalola SO. *In vitro* Antioxidant activity of ethanol extract of *Bridelia ferruginea*. J. Acad. Res. Int. 2012;2(3):2223-2244.
16. Orafidiya LO, Lamikanra A, Adediji JA. Coagulation of milk as an index of astringency of the bark extract of *Bridelia ferruginea* Benth and lime juice for the formulation of a traditional gargle 'Ogun Efu'. Phythother. Res. 1990;4(5):189-194.
17. Dada-Adegbola HO, Oluwatoba OA, Adebiyi OE, Odikagbue AN. *In vitro* evidence of anti-infective activity of crude aqueous extract obtained by boiling ripe stem-bark of *Bridelia ferruginea* Benth. Journal of Pharmacognosy and Phytotherapy. 2010;2(4):43-48. https://www.academicjournals.org/jpp
18. Irobi ON, Moo-Young M, Anderson WA, Daramola SO. Antimicrobial activity of bark extracts of *Bridelia ferruginea* (Euphorbiaceae). Journal of Ethnopharmacology. 1994;43:185-190.
19. Evans WC. Trease and Evans Pharmacology 14th Edition, WB sanders company Ltd, 2000, 224-293.
20. Ayoola GA, Coker HAB, Adesegun SA, Adepoju-Bello AA, Obaweya K, Ezennia EC, *et al*. Phytochemical Screening and Antioxidant Activities of Some Selected Medicinal Plants Used for Malaria Therapy in Southwestern Nigeria. Tropical Journal of Pharmaceutical Research. 2008;7(3):1019-1024. (http://www.tjpr.org)
21. Adebayo EA, Ishola OR. Phytochemical and antimicrobial screening of the crude extracts from the root, stem bark and leaves of *Bridelia ferruginea*. Afr. J. Biotech. 2009;8(4):650-653.
22. Adefuye AO, Ndip RN. Phytochemical analysis and antibacterial evaluation of the ethyl acetate extract of the stem bark of *Bridelia micrantha*. Pharmacognosy Magazine. 2013;9(33):45-50. Doi:10.4103/0973-1296.108139.
23. Cheesbrough M. Medical laboratory manual for Tropical countries. Volume II. Microbiology. Doddington. Tropical Health Technology, 1987, 99-108.
24. Bauer AW, Kirby WM, Sherris JC, Turck M. Antibiotics Susceptibility testing by a standardized single disc method. AMJ Clin Pathol. 1966;45:493-496.
25. Andrews JM. Determination of minimum inhibitory concentrations. J Antimicrob Chemother. 2001;48(suppl_1):5-16. https://doi.org/10.1093/jac/48.suppl_1.5
26. Brooke JS. *Stenotrophomonas maltophilia*: an Emerging Global Opportunistic Pathogen. Clin Microbiol Rev. 2012;25(1):2-41. https://doi.org/10.1128/CMR.00019-11.

27. Dao TT, Liebenthal D, Tran TK, Ngoc Thi Vu B, Nbog Thi Nguyen D, Thi Tran HK, *et al*. *Klebsiella pneumoniae* Oropharyngeal Carriage in Rural and Urban Vietnam and the Effect of Alcohol Consumption. PLoS ONE. 1999;9(3):09. https://doi.org/10.1371/journal.pone.0091999.
28. Yang Qi-wen, XU Ying-chun, XIE Xiu-li, WANG Hui, HU Yun-jian *et al*. Antimicrobial Resistance Surveilance on Hospital- and Community-acquired Pathogens in 10 Teaching Hospitals in China. Chinese Journal of Nosocomiology 2009. (Abstract)
29. Yang Huai-de, Zhang Jij-jun, LI Xue-mei. *et al.* Analysis on drug resistance of *Klebsiella pneumonia* in clinical infection. International Journal of Laboratory Medicine, 2010, 11. (Abstract)
30. CDC Healthcare associated Infections (HAI): Diseases and organisms. https://www.cdc.gov/HAI/organisms/acinetobacter.html. Nov 24, 2010.
31. Fournier PE, Richet H, Weinstein RA. The Epidemiology and Control of *Acinetobacter baumannii* in Health Care Facilities. Clin. Infect. Dis. 2006;42:692-699.
32. Wang L, Shi XH, Xiao D. Drug resistance of *Acinetobacter baumannii* infection in SICU. Xinjiang Medicine. 2013:43:141-142. (Google scholar/CrossRef))
33. Inchai J, Pothirat C, Limsukon A, Khositsakulchai W, Liwsrisakun C. Prognostic factors associated with mortality of drug-resistant *Acinetobacter baumannii* ventilator-associated pneumonia. Journal of Intensive Care. 2015;3:9. DOI 10.1186/s40560-015-0077-4.
34. Dada-Adegbola HO, Brown BJ, Sanusi A, Nwaokenye J, Obebe OO. Antibiotic Susceptibility Pattern of *Acinetobacter* Species Isolated in Clinical Specimens from the University College Hospital, Ibadan, Nigeria. African Journal of Health Sciences. 2020;33(5):18-32.
35. Brantner A, Grein E. Antibacterial activity of plant extracts used externally in traditional medicine. J Ethnopharmacol. 1994;44(1):35-40. Doi:10.1016/0378-8741(94)90096-5