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In Vitro Antimalarial, Antimicrobial and Trypanocidal Potentials of the Leaf Extract and Fractions of *Bridelia ferruginea* (Benth.)

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Abstract

Bridelia ferruginea Benth. (Euphorbiaceae) is a plant used for various medicinal purposes including the treatment of protozoan infections in many African countries. The present study was aimed to evaluate the *in vitro* antimalarial, antimicrobial and antitrypanasomal activity of the methanol crude extract and fractions of the medicinal plant, *B. ferruginea*. Crude extract from dried leaves of *B. ferruginea* was prepared by cold maceration and fractionated using a reverse phase column under reduced pressure with water – methanol mixtures. The extracts and fractions were tested to determine their biological activities. *In vitro* antimalarial activity was determined by the plasmodial lactate dehydrogenase culture sensitivity assay while antitrypanosomal activity was tested *in vitro* against a culture of *Trypanosoma brucei promastigotes*. The crude extract of *B. ferruginea* exhibited significant antitrypanasomal activity with an IC_{50} of 8.48 µg/mL which was further enhanced in some of the fractions like the MeOH fraction (BFD) with an IC_{50} of 1.04 µg/Ml which indicates very good activity. Sub – fraction BFF (obtained from 1:3 H_2O:MeOH fraction – BFC) exhibited the best antimalarial activity among all the fractions and sub – fractions with an IC_{50} value of 19.73 µg/mL against *P. falciparum* D6 and 20.49 µg/mL against *P. falciparum* W2. There was no observable antibacterial or antifungal activity for all crude extracts, fractions and sub – fractions. The results from this study have validated the leaves of *B. ferruginea* as potential sources of antimalarial and antitrypanosomal agents which can serve as a lead in drug development.

Keywords: Antimalarial, Antimicrobial, Antitrypanosomal. *Bridelia ferruginea*, *in vitro*

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1. Introduction

Plants have a long history of use in the treatment of several diseases and play an important role in the traditional medicine practice (1). These plants, in particular, have over the ages played a leading medical role in most cultures of the world. Most of these medicinal plants were used against common sicknesses such as coughs, colds, parasitic infections, and inflammation and which was probably based on trial and error. This ancient knowledge of the medicinal properties of plants was orally transferred from one generation to another which conserved it from extermination (2, 3). African traditional medicine has a very wide and varied scope which is due to the rich biological and cultural diversity on the continent (4).

Today, plants still occupy an outstanding and paramount place in modern pharmacy although current biomedicine employs synthetic drugs as therapeutic agents to a noteworthy degree in place of natural products. Plants have over time

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being used as sources of pharmaceutical drugs in the form of isolated plant compounds, as sources of precursors to drugs, or as sources of compounds that have served as representations for synthetic or semi – synthetic drugs (5).Many pharmacological and economically significant medicinal plant species have had multiple uses globally; some of such medicinally useful plants include *Bridelia ferruginea*.

B. ferruginea belongs to the family Euphorbiaceae which is usually found in Savannah regions (6). It is a small non-laticiferous scaly tree or shrub that grows to about 4 meters high when conditions are favourable. It is usually a gnarled shrub which sometimes reaches the size of a tree in suitable conditions (7). *B. ferruginea* has diverse medicinal uses and has been in use in ethnomedicine for treatment of various ailments in many regions of Africa. A decoction of the leaves is usually used to treat diabetes. It is also used as a purgative and a vermifuge (8). The bark extract has been used for the coagulation of milk and also lime juice for the formulation of a traditional gargle "ogun efu" (9). Its potential for water treatment has been reported (10). In Togo, the roots of the plant are used as chewing sticks and the root bark is used for intestinal and bladder disorders as well as skin diseases (11). Also, extracts from the roots and leaves is used to cure piles, diarrhea, female sterility, rheumatic pains (12)

Based on the existing traditional uses of the plant, this present work sought to establish and report the antitrypanosomal, antimalarial, antibacterial and antifungal activities of methanol crude extract and various fractions of *B. ferruginea*.

2. Materials and methods

2.1. Plant Samples Collection and Identification

B. ferruginea leaves were collected at the end of the raining season from Eruwa, Oyo state, Nigeria in November 2016. The plant sample was identified and authenticated at the Forestry Research Institute of Nigeria (FRIN), Ibadan, Oyo state, Nigeria with voucher reference number FHI 110711. A voucher specimen of the sample was deposited at FRIN for further reference in the herbarium section.

2.2. Preparation of Plant Samples

The leaves of the plant were air – dried at room temperature under the shade for 7 days, after which they were milled into powder with the aid of an electric hammer mill model TRAPP TRF 80 Hammer mill foliage and finally stored in a moisture free environment until required for further use.

2.3. Extraction of Plant Samples

The powdered leaves were extracted with aqueous methanol as follows. Exactly 7 L of 10% aqueous methanol was added to 1.5 kg of the powdered leaves in a stoppered glass container and tightly covered. The mixture was left for 3 days at room temperature with periodic daily stirring and the extract finally filtered through cotton wool and Whatman 125 mm filter paper No. 1. The methanol extract obtained was concentrated using rotary evaporator model Stuart RE 300B W13 at reduced pressure. The extract was evaporated to dryness with the methanol distilled off at 40 oC to obtain the crude methanolic extract of the sample. The dry extract was later kept in tightly stoppered bottles in a refrigerator until required for further analysis.

2.4. Fractionation of Crude Extracts

The crude methanolic extract of *B. ferruginea* (140 g) was loaded on a reverse phase column under reduced pressure and eluted with 1 litre each of H_2O (100 %), H_2O-MeOH (1:1), H_2O – MeOH (1:4) and MeOH (100 %) in that order to yield 4 fractions (BFA – BFD) respectively. The fractions were concentrated first over a water bath at 40 °C (using vacuum pump) to distill off the methanol and then in a freeze dryer to remove the water. The 1:4 H_2O – MeOH fraction (BFC), 10 g was suspended in water and successively extracted with dichloromethane and ethyl acetate in a separating funnel to afford the corresponding dichloromethane (BFE), ethyl acetate (BFF) and aqueous (BFG) soluble sub – fractions respectively. All the sub – fractions were concentrated on a water bath at 40 °C.

2.5. Assay For In Vitro Antitrypanosomal Activity

Antitrypanosomal activity of the samples was tested *in vitro* against a culture of *Trypano-*

soma brucei promastigotes. They were grown in RPMI 1640 medium supplemented with 10% fetal calf serum (Gibco Chem. Co.) at 26 °C. A 3-day-old culture was diluted to 5 X 105 promastigotes / mL. Drug dilutions were prepared directly in cell suspension in 96-well plates. Plates were incubated at 26 °C for 48 h and growth of trypanaosomal promastigotes was determined by Alamar blue assay as described (13). Standard fluorescence was measured on a Fluostar Galaxy plate reader (BMG Lab Technologies) at excitation wavelength of 544 nm and emission wavelength of 590 nm. Pentamidine, Amphotericin B and Alpha-difluoromethylornithine were used as the standard antitrypanosomal agent. IC_{50} and IC_{90} values were calculated from dose – response curves created by plotting percent growth versus drug concentration. (14)

2.6. Assay For In Vitro Antimalarial Activity

The assay is based on the determination of plasmodial lactate dehydrogenase (pLDH) activity. The pLDH culture sensitivity assay is repeatable, easily interpreted, fast and economical to perform, suggesting field applicability. For the assay, a suspension of red blood cells infected with D6 and W2 strains of *P. falciparum* (200 µL, with 2% parasitemia and 2% hematocrit in RPMI 1640 medium supplemented with 10% human serum and 60 µg/mL Amikacin) was added to the wells of a 96 – well plate containing 10 µL of test samples diluted in medium at various concentrations. The plate was placed in a modular incubation chamber (Billups-Rothenberg, CA) flushed with a gas mixture of 90% N2, 5% O_2 and 5% CO_2, and incubated at 37 °C, for 72 h. Parasitic LDH activity was determined by using MalstatTM reagent (Flow Inc., Portland, OR) (15). Briefly, 20 µL of the incubation mixture was mixed with 100 µL of the MalstatTM reagent and incubated at room temperature for 30 min. 20 µL of a 1:1 mixture of NBT/PES (Sigma, St. Louis, MO) was added and the plate is further incubated in the dark for 1 h. The reaction was stopped by the addition of 100 µL of a 5% acetic acid solution. The plate was read at 650 nm using the EL-340 Biokinetics Reader (Bio-Tek Instruments, Vermont). IC_{50} values were calculated from the dose – response curves. Artemisinin and chloroquine were incorporated in each assay as the drug controls. DMSO (0.25%) was employed as vehicle control (14).

2.7. Assay For In Vitro Antimicrobial Activity

All organisms were obtained from the American Type Culture Collection (Manassas, VA) and included the fungi *Candida albicans* ATCC 90028, *Cryptococcus neoformans* ATCC 90113, and *Aspergillus fumigatus* ATCC 90906, the methicillin-resistant *Staphylococcus aureus* ATCC 43300 (MRS), *Escherichia coli* ATCC 35218, *Pseudomonas aeruginosa* ATCC 27853, and *Mycobacterium intracellulare* ATCC 23068. Susceptibility testing was performed using a modified version of the CLSI (formerly NCCLS) method (16-19). Samples were serially diluted in 20% DMSO/saline and transferred in duplicate to 96 – well flat bottom microplates. Microbial inocula were prepared by correcting the OD630 of microbe suspensions in incubation broth to afford final target inocula. Drug controls (Ciprofloxacin & Meropenem [ICN Biomedicals, Ohio] for bacteria and Amphotericin B & Fluconazole [ICN Biomedicals, Ohio] for fungi were included in each assay as the drug controls. All organisms were read at either 630 nm using the EL-340 Biokinetics Reader (Bio-Tek Instruments, Vermont) or 544ex/590em, (*M. intracellulare*, *A. fumigatus*) using the Polarstar Galaxy Plate Reader (BMG LabTechnologies, Germany) prior to and after incubation. Minimum Inhibitory concentrations (MICs) were determined by removing 5µL from each clear well, transferring to agar and incubating. The MIC is defined as the lowest test concentration that kills the organism (allows no growth on agar). Percent growth was plotted versus test concentration to afford the IC_{50}. (20).

2.8. Statistical Analysis

Results were expressed as mean±standard deviation (SD).

3. Result and discussion

3.1. Antitrypanosomal Activity of the Crude Extract and Fractions

Antitrypanosomal activity of the crude extract and fractions were evaluated against *Trypanasoma brucei* promastigotes and the results

Table 1. Primary (Percent Inhibition) Antitrypanosomal Activity of *B. ferruginea* Crude Extracts & Fractions.

Samples	Test Concentration (µg/mL)	Percent Inhibition
BF	20	90 ± 2.38
BFA	20	95 ± 1.03
BFB	20	76 ± 0.33
BFC	20	17 ± 1.08
BFD	20	98 ± 0.83
BFE	20	97 ± 1.02
BFF	20	86 ± 1.23
Pentamidine	10	100
Amphotericin B	2	99
Alpha-difluoromethylornithine	20	99

shown below. The primary assay showing percent inhibition is shown in Table 1 while the secondary and tertiary assays showing IC_{50} and IC_{90} values respectively were recorded and presented in Table 2.

The crude extract and all the fractions of *B. ferruginea* showed excellent primary activity against *T. brucei* with very high percent inhibition except fraction BFC (Fractions BFE and BFF almost comparable to the reference drug) as seen in Table 1. However, activity dropped a little with advancement to the secondary assay. Fractionation of the crude extract also gave significant increase in antitrypanosomal activity as seen in fraction BFD with an IC_{50} value of 1.05 µg/mL (Table 2) which indicates very good activity.

3.2. Antimalarial Activity of the Crude Extract and Fractions

The result of this assay is presented in Table 3.

Antimalarial assay of the crude extract and fractions were evaluated using two plasmodium strains (*P. falciparum* D6 and *P. falciparum* W2). The results of this assay as presented in Table 3 revealed that the crude extract exhibited weak antimalarial activity. However, fractionation and further partitioning of the fractions gave fractions and sub – fractions with significant antimalarial activity. Fraction BFC from *B. ferruginea* crude extract had an IC_{50} value of 28.19 µg/mL against *P. falciparum* D6 and 25.44 µg/mL against *P. falciparum* W2 while BFD had an IC_{50} value of 20.44 µg/mL against *P. falciparum* D6 and 22.40 µg/mL against *P. falciparum* W2. This was quite better than the IC_{50} values of the crude extract which indicates better activity. Sub – fraction BFF (obtained from BFC) exhibited the best antimalarial activity among *B. ferruginea* fractions and sub – fractions with an IC_{50} value of 19.73 µg/mL against *P. falciparum* D6 and 20.49 µg/mL against *P. falciparum* W2. It can be observed that fractionation and fur-

Table 2. Secondary and Tertiary (IC_{50} and IC_{90}) Antitrypanosomal Activity of *B. ferruginea* Crude Extracts & Fractions.

Samples	Test Concentration (µg/mL)	*T. brucei* (IC_{50})	*T. brucei* (IC_{90})
BF	20-0.8	8.48	19.32
BFA	20-0.8	6.92	17.50
BFB	20-0.8	10.26	>20
BFC	20-0.8	>20	>20
BFD	20-0.8	1.05	1.63
BFE	20-0.8	12.15	16.49
BFF	20-0.8	16.83	>20
Pentamidine	10-0.008	0.002	0.003

Table 3. Antimalarial Activity of *B. ferruginea* Crude Extracts & Fractions.

Code	Test Concentration (µg/mL)	*P. falciparum* D6 IC_{50}	*P. falciparum* W2 IC_{50}
BF	47-5.29	40.84	35.35
BFA	47-5.29	>47	>47
BFB	47-5.29	>47	>47
BFC	47-5.29	28.19	25.44
BFD	47-5.29	20.44	22.40
BFE	47-5.29	45.76	>47
BFF	47-5.29	19.73	20.49
Artemisinin	2.38-0.026	<0.026	<0.026
Chloroquine	2.38-0.026	<0.026	0.15

ther partitioning enhances antimalarial activity for the extract; however, the crude extract and all the fractions were less active than the reference drugs (artemisinin and chloroquine).

3.3 Antimicrobial Activity of the Crude Extracts and Fractions

The primary assay showing percent inhibition is shown in Table 4; while, the secondary assay showing IC_{50} values were recorded and presented in Table 5.

The antibacterial activity of the crude extract and fractions were evaluated against five bacteria strains; these are the Methicillin-resistant *Staphylococcus aureus*, *E. coli*, *P. aeurginosa*, *K. pneumonia* and Vancomycin-resistant Enterococci. The results as presented in Table 4 shows slight primary antibacterial activity for some fractions against *E. coli* while secondary assay as seen in Table 5 shows no observable antibacterial activity for the crude extracts, all fractions and sub – fractions of *B. ferruginea*. The IC_{50} values were above 200 µg/mL.

Antifungal assay was also carried out on the crude extracts and fractions and this was evaluated against three fungi strains; these are *C. albicans*, *A. fumigatus* and *C. neformans*. The results is presented in Table 6 and 7 and shows no observable antifungal activity for all crude extracts, fractions and sub – fractions of *B. ferruginea*.

The crude extract of *B. ferruginea* exhibited excellent antitrypanasomal activity with 90% inhibition and an IC_{50} of 8.48 µg/mL which was further enhanced in some of the fractions like the 1:4 H_2O:MeOH fraction (BFD) which had an IC_{50} of 1.04 µg/mL, this may be a case of purification

Table 4. Primary (Percent Inhibition) Antibacterial Activity of *B. ferruginea* Crude Extracts & Fractions.

Samples	Test Concentration (µg/mL)	MRSA*	*E. coli*	*P. aeurginosa*	*K. pneumoniae*	VRE**
BF	200	0	13±5.36	17±0.14	0	6±1.25
BFA	200	0	12±2.14	0	11±0.71	0
BFB	200	0	26±4.84	0	1±0.06	0
BFC	200	0	37±1.98	21±0.42	0	7±2.03
BFD	200	0	16±2.21	2±0.14	1±0.96	1±0.28
BFE	200	0	30±0.91	6±0.28	0	0
BFF	200	0	32±0.18	18±0.57	7±1.50	10±0.86
Ciprofloxacin	100	0	100	100	5	15
Vancomycin	100	99	60	27	26	18
Methicillin	100	99	11	69	5	21
Cefotaxime	100	98	100	84	3	22
Meropenem	100	75	99	79	98	22

*MRSA=Methicillin-resistant *Staphylococcus aureus*

**VRE=Vancomycin-resistant Enterococci

Table 5. Secondary (IC_{50}) Antibacterial Activity of *B. ferruginea* Crude Extracts & Fractions.

Samples	Test Conc. (µg/mL)	MRSA*	*E. coli*	*P. aeurginosa*	*K. pneumoniae*	VRE**
BF	200-0.8	>200	>200	>200	>200	>200
BFA	200-0.8	>200	>200	>200	>200	>200
BFB	200-0.8	>200	>200	>200	>200	>200
BFC	200-0.8	>200	>200	>200	>200	>200
BFD	200-0.8	>200	>200	>200	>200	>200
BFE	200-0.8	>200	>200	>200	>200	>200
BFF	200-0.8	>200	>200	>200	>200	>200
Ciprofloxacin	10-0.4	>10	<0.01	0.03	>10	>10
Meropenem	100-4	1.43	7.28	2.87	35.02	>100

*MRSA=Methicillin-resistant *Staphylococcus aureus.*

**VRE=Vancomycin-resistant Enterococci

enhancing activity. This is in line with the report by Ekanem at al., on the Trypanocidal potential of methanolic extract of *B. ferruginea* benth bark in *Rattus novergicus*. Antimalarial assay of the crude extract showed weak antimalarial activity but fractionation significantly increased its activity. This might be attributed to the presence of flavonoids and terpenoids in these fractions as these phytochemicals have been variously implicated in antiplasmodial activities of many plants (21). Aqueous stem bark extract of *B. ferruginea* was reported to have considerable antimalarial activity in an earlier report (21), this might imply that the stem bark contained specific phytochemicals that aided its antimalarial activity which may be absent in the leaves. Both crude extract and all fractions showed no observable antimicrobial activity and this is in agreement with earlier reports (22).

Table 6. Primary (Percent Inhibition) Antifungal Activity of *B. ferruginea* Crude Extracts & Fractions.

Sample	Test Concentration (µg/mL)	*C. albicans*	*A. fumigatus*	*C. neformans*
BF	200	0	4±0.86	0
BFA	200	0	1±0.00	0
BFB	200	2±0.57	6±0.45	0
BFC	200	4±0.15	0	9±2.40
BFD	200	0	0	0
BFE	200	0	0	0
BFF	200	0	12±0.01	8±0.64
Fluconazole	100	74	3	75
Amphotericin B	100	97	96	97

Table 7. Secondary (IC_{50}) Antifungal Activity of *B. ferruginea* Crude Extract & Fractions.

Samples	Test Concentration (µg/mL)	*C. albicans*	*A. fumigatus*	*C. neformans*
BF	200 – 0.8	>200	>200	>200
BFA	200 – 0.8	>200	>200	>200
BFB	200 – 0.8	>200	>200	>200
BFC	200 – 0.8	>200	>200	>200
BFD	200 – 0.8	>200	>200	>200
BFE	200 – 0.8	>200	>200	>200
BFF	200 – 0.8	>200	>200	>200
Fluconazole	100 – 4	0.726	>100	2.85
Amphotericin B	100 – 4	<0.1	1.14	0.14

4. Conclusion

In vitro antimalarial, antimicrobial, and trypanocidal activity of methanolic extract and fractions of *B. ferruginea* leaves have been assessed with a view to justifying the various claims of its use in traditional medicine and also establishing the possibility of developing novel natural compounds from the plant extract for effective and safe antimalarial and trypanocidal agents for combating malaria and trypanosomiasis which are protozoan diseases that has continued to be of immense economic and health importance in many tropical countries of the world, especially in Africa.

Conflict of Interest

None declared.

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