Nigerian Research Journal of Chemical Sciences **(ISSN: 2682-6054)**
Volume 8, Issue 2, 2020

Chemical Constituents and Biological Evaluation of Hexane Fraction from *Bridelia ferruginea* (Benth.) Leaves Crude Extract

.*[1,2]Afolayan Michael O., [2]Asekun Olayinka T., [2]Familoni Oluwole B.

[1]Chemistry Advanced Research Centre, Sheda Science and Technology Complex (SHESTCO), Garki – Abuja, Nigeria.

[2]Department of Chemistry, University of Lagos, Lagos, Nigeria.

* Corresponding Author: afolayanmic@yahoo.com

ABSTRACT

The present investigation was carried out to determine the possible bioactive components from the hexane fraction of crude methanolic extract of *Bridelia ferruginea* leaves using chromatographic techniques (Thin layer and Column chromatography). The oil obtained from the hexane fraction after separation and purification was orange in colour and was methylated using dry methanol before subjecting it to Gas Chromatography – Mass Spectroscopy (GC – MS) analysis for identification of its different components. Twelve (12) compounds were identified from the analysis of the GC – MS spectra while two compounds were unidentified. Squalene having a percentage composition of 53.66% was the most abundant constituent. Other notable compounds in the oil were α – amyrin (7.55%), Stigmastan-3,5-diene (6.92%), 8-formyl-6,7-bis[2-(methoxycarbonyl)ethyl]-1,3,5-trimethyl-2,4-divinylporphyrin (4.15%), Vitamin E (3.91 %), (+) gamma tocopherol (2.03%), 4,8,12,16– tetramethylheptadecan–4–olide (1.86%), dl-alpha tocopherol (1.72%) and 2-Pentadecanone, 6,10,14-trimethyl (1.19%). Biological evaluation of the hexane fraction was also carried out. Antimalarial assay of the oil revealed an IC_{50} of 20.44 X 10^3ng/mL against *Plasmodium falciparum* D6 and 22.40 X 10^3ng/mL against *Plasmodium falciparum* W2. It showed 73% inhibition against *Leishmania donovani* and 98% inhibition against *Trypanasoma brucei* pathogen. The chemical constituents and biological activities of the hexane fraction of *Bridelia ferruginea* leaves may be a lead to the discovery of novel drugs for the treatment of some diseases and ailments.

Keywords: Bioactive, biological, *Bridelia ferruginea*, chromatography, hexane, leaves.

INTRODUCTION

Plants have remained an integral part of human existence as man have had to rely on plants for food, fuel, shelter, clothing and very importantly medicine [1]. Plants form the basis of a sophisticated traditional medicine from antiquity to date and humans have long used naturally occuring substances for medical purposes in the treatment of diseases and maintenance of healthy living [2]. Most medicinal plants over the ages have been used against common ailments such as coughs, colds, parasitic infections, inflammation and even against more serious diseases like cholera, diarrhea, diabetes and cancer. These plants have been found effective and employed in traditional medicine from antiquity till date.

The phytochemicals that are responsible for therapeutic effect of plants are varied from one plant to the other hence the medicinal use of one plant will very likely differ from another. Also, the compositions of different parts of the same plant are almost always different and so medicinal uses of the parts of a plant are also multifarious. Likewise, phytochemical constituents of a plant may change over seasons and weather hence giving the same plant different uses as seasons change. This leaves traditional healers with a large repository of medicinal plants to choose from in the treatment of illnesses and diseases. Medicinal plants are abundantly found and frequently used for various purposes across Asia (China, Japan, Thailand, India, Pakistan), and Africa (Ghana, Congo, Mali, Cameroon, South Africa and Nigeria). Traditional medicine also exists in southern America and Australia, but these are not as developed as in Asia and Africa [3].

Bridelia ferruginea belongs to the family *Euphorbiaceae* and is commonly found in the Savannah regions. It is a small non-laticiferous scaly tree or shrub that grows to about 4 meters in height. It is usually a gnarled shrub and can sometimes reach the size of a tree when conditions are favourable. The plant often bears spines and may be slash crimson in colour. The leaves of *Bridelia ferruginea* may be small to medium sized, simple, alternate, spiral or distichous, broadly elliptic and pubescent. They are also pinnately veined with entire margin and an acuminate or acute apex [4]. *B. ferruginea* has been employed in the treatment of various ailments across Africa in traditional ethno medicine [5]. The bark, roots, fruits and leaves are used mainly as decoctions and have been employed in ethno medicine for treatment of many ailments including malaria fever [6]. A decoction of *B. ferruginea* leaves has been reportedly used to treat diabetes and also used as a purgative and a vermifuge [7]. Extracts from the roots

and leaves are used to cure piles, diarrhea, female sterility, rheumatic pains [8], dysentery and to treat elephantiasis of the scrotum [9]. Roots of the plant are used as chewing sticks and the root bark is used for intestinal and bladder disorder remedies as well as skin diseases [10].

Several pure compounds with varying biological activities have also been isolated from different parts of *Bridelia ferruginea* which include quercetin 3-methyl ether, quercetin 3,7,3′,4′-tetramethyl ether, myricetin 3′,4′,5′-trimethyl ether, Quercetin 3-neohesperidoside (rutin), quercetin, myricetin, gallocatechin, epigallocatechin [11].

The present study aims to explore the chemical compositions and medicinal potentials of the oil isolated from hexane fraction of crude methanolic extract of *Bridelia ferruginea* leaves grown in Nigeria.

MATERIALS AND METHODS

Solvents and chemicals

Analytical grade solvents were used for all purposes and distilled before use to remove any trace impurities. Methanol, hexane, dichloromethane, ethyl acetate and acetone (all 95% purity) were purchased from Sigma-Aldrich and Fisher Scientific. Deionized water was also used for all purposes. All thin layer chromatography analyses were performed at room temperature using pre-coated silica gel 60 $_{F254}$ (Merck, Germany). Column chromatography was performed using silica gel (60 – 120 mesh)

Plant samples collection and identification

Bridelia ferruginea leaves were collected at the end of the rainy season from Eruwa, Oyo state, Nigeria in November 2016. It was identified and authenticated by a botanist, Mr. A. Adeyemo, of the Forestry Research Institute of Nigeria (FRIN), Ibadan, Oyo State, Nigeria with Voucher reference number FHI 110711. A voucher specimen was deposited at FRIN for future reference in the herbarium section.

Preparation of plant samples

The plant samples were air – dried at room temperature away from sunlight after collection for 21 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.

Extraction of plant samples

The powdered plant material was extracted with aqueous methanol as follows: About 7 L of 10% aqueous methanol was added to 1.5 kg of the powdered plant material in stoppered glass container and tightly covered. The mixture was left for 7 days at room temperature with periodic daily stirring and then 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. The dry extract was later kept in tightly stoppered bottle in a refrigerator until required for further analysis.

Fractionation of crude extract

The crude methanolic extract (140 g) was loaded on a reverse phase column under reduced pressure and eluted with 1 litre each of H_2O, 50% H_2O – MeOH, 25% H_2O – MeOH and MeOH in that order to yield 4 fractions respectively. The column was finally washed with acetone to remove the chlorophyll and other undissolved components. The fractions were concentrated first over a water bath at 40 ºC (using vacuum pump) to distill off the methanol and then in a freeze drier to remove the water.

Normal phase column chromatography of the methanol fraction was carried out on silica gel with acetone – hexane gradient to yield 30 fractions. Fraction 2 (obtained from 100% hexane) was oily, showed a single spot on TLC and was subjected to GCMS to determine its components.

Gas chromatography/mass spectrometry (GC/MS)

The GC/ MS system consisted of an Agilent 7890A gas chromatograph and an Agilent 5975C mass selective detector. The injections were made with an Agilent 7693 autosampler. The system was controlled by ChemStation software (version E.02). A 30 m × 0.25 mm fused silica capillary GC column coated with a 0.25 μm film of 5% phenyl methylpolysiloxane (HP-5MS) from J&W Scientific was used for the oil analysis. The inlet temperature was set at 250 $^{\circ}$C, and the injector was operated in a split mode with a split ratio of 25:1. The oven temperature was kept at 50 $^{\circ}$C for 5 min, programmed to 200 $^{\circ}$C at a rate of 2 $^{\circ}$C /min, then programmed to 280 $^{\circ}$C at a rate of 8 $^{\circ}$C / min, and kept constant at 280 $^{\circ}$C for 30 min. The mass spectrometer was operated in a scan mode over a mass range of 50 − 650 atomic mass units (amu) with the electron impact (EI) voltage at 70 eV.

Biological assays

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 reproducible, easily interpreted, rapid and inexpensive to perform, suggesting field applicability. For the assay, a suspension of red blood cells infected with D6 or 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% N_2, 5% O_2 and 5% CO_2, and incubated at 37 $^{\circ}$C, for 72 h. Parasitic LDH activity was determined by using Malstat^{TM} reagent (Flow Inc., Portland, OR) according to the procedure of Makler and Hinrich [12]. Briefly, 20 µL of the incubation mixture was mixed with 100 µL of the Malstat^{TM} reagent and incubated at room temperature for 30 min. 20 µL of a 1:1 mixture of NBT/PES (Sigma, St. Louis, MO) was then added and the plate is further incubated in the dark for 1 h. The reaction was then 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 computed from the dose – response curves. Artemisinin and chloroquine were included in each assay as the drug controls. DMSO (0.25%) was used as vehicle control [12].

Assay for *in vitro* antileishmanial activity

Antileishmanial activity of the compounds was tested *in vitro* against a culture of *Leishmania donovani* and *Trypanasoma brucei* promastigotes. They were grown in RPMI 1640 medium supplemented with 10% fetal calf serum (Gibco Chem. Co.) at 26 $^{\circ}$C. A 3-day-old culture was diluted to 5 X 10^5 promastigotes/mL. Drug dilutions were prepared directly in cell suspension in 96-well plates. Plates were incubated at 26 $^{\circ}$C for 48 h and growth of leishmania promastigotes was determined by Alamar blue assay as described [12]. 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 and Amphoterecin B were used as the standard antileishmanial agents. IC_{50} and IC_{90} values were computed from dose – response curves generated by plotting percent growth versus drug concentration [12].

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 bacterium *Escherichia coli* ATCC 35218, *Pseudomonas aeruginosa* ATCC 27853, and *Klebseila pneumoniae* ATCC 23068. Susceptibility testing was performed using a modified version of the CLSI (formerly NCCLS) method [13]. 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 [ICN Biomedicals, Ohio] for bacteria and Amphotericin B [(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 bactericidal concentrations (MBCs) were determined by removing 5μL from each clear well, transferring to agar and incubating. The MBC is defined as the lowest test concentration that kills the organism (allows no growth on agar). Per cent growth was plotted versus test concentration to afford the IC_{50} [14, 15].

RESULTS AND DISCUSSION

Extraction and fractionation of *Bridelia ferruginea* leaves

Powdered *B. ferruginea* leaves (1.5 kg), was extracted with 7 L of 10% aqueous methanol to give 158.1 g dried crude extract representing 10.54% yield. The crude extract was dark green and gummy in nature. Exactly 140 g of the crude extract was fractionated with acetone - hexane mixture in different proportions to give different fractions. The 100% hexane fraction gotten was 45.7 g representing 32.64 % yield.

After several chromatographic purifications, the oily mass which was orange yellow in colour obtained from the hexane fraction after separation was subjected to Gas Chromatography – Mass Spectroscopy analysis for identification of its different components.

GC–MS spectra of the oil are shown in Figure 1 while the interpretation and analysis of the spectra are shown in Table 1:

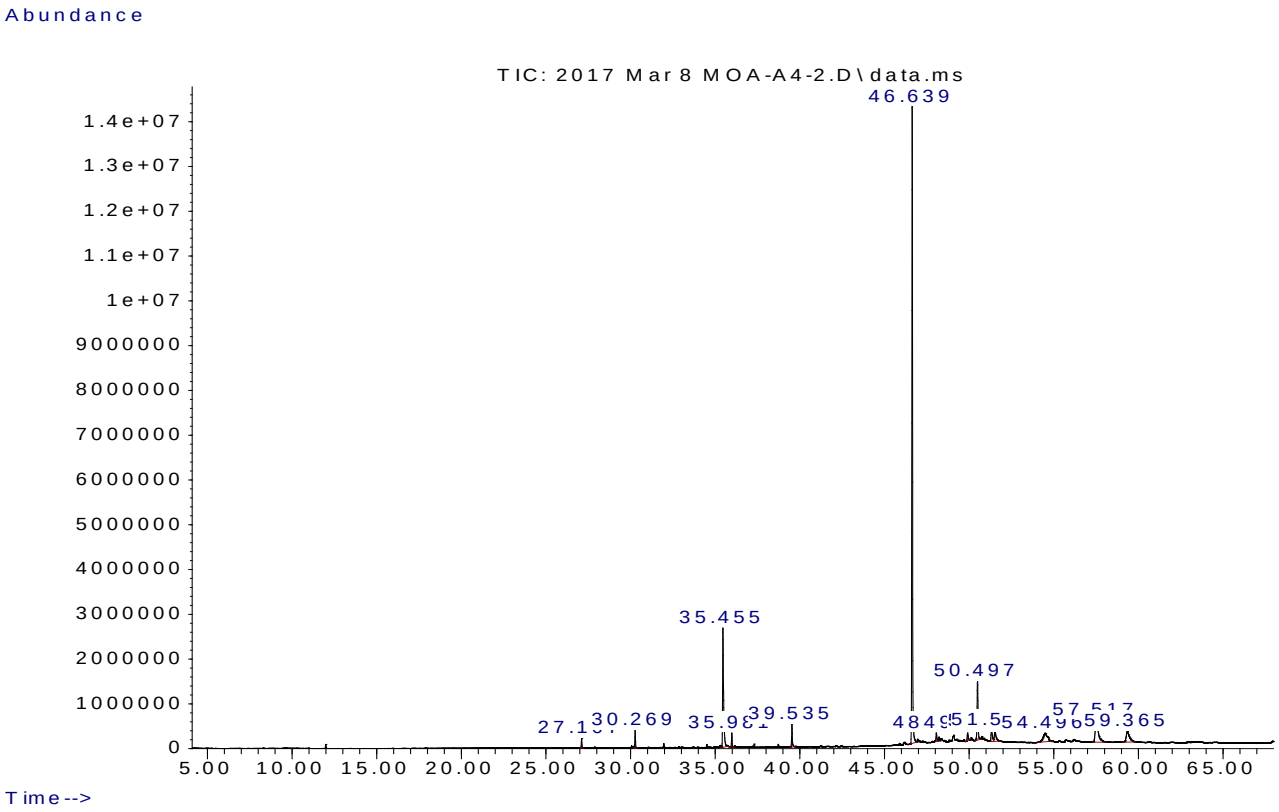

Figure 1: Gas Chromatogram of purified hexane fraction of *Bridelia ferruginea* crude methanolic extract

Table 1: GC – MS analysis of purified hexane fraction of *Bridelia ferruginea* crude methanolic extract

Compound number (#)	RT (min)	Area (Ab*s)	Peak Width 50% (min)	% Composition	Hit Name	Mol. Weight (amu)
1	27.107	5326601	0.037	0.65	2-Propenoic acid, n-pentadecyl ester	282.256
2	30.271	9724160	0.04	1.19	2-Pentadecanone, 6,10,14-trimethyl	268.277
3	35.457	1.13E+08	0.061	13.84	2-Hexadecen-1-ol, 3,7,11,15-tetramethyl-, [R-[R*,R*-(E)]]-	296.308
4	35.98	8143197	0.041	0.99	1,2,3,4,5-Pentamethyl-Cyclopentane	156.126
5	39.537	15212425	0.045	1.86	4,8,12,16-Tetramethylheptadecan-4-olide	324.303
6	46.638	4.38E+08	0.045	53.66	Squalene	410.391
7	48.064	6197477	0.048	0.76	Solanesol	630.574
8	49.917	6316083	0.054	0.77	9,10-Methanoanthracen-11-ol, 9,10-dihydro-9,10,11-trimethyl-	250.136
9	50.501	56483338	0.069	6.92	Stigmastan-3,5-diene	396.376
10	51.329	14039025	0.075	1.72	dl-alpha-Tocopherol	430.381
11	51.526	16548865	0.089	2.03	(+)-.gamma.-Tocopherol	430.381
12	54.493	33910511	0.226	4.15	8-formyl-6,7-bis[2-(methoxycarbonyl)ethyl]-1,3,5-trimethyl-2,4-divinylporphyrin	604.269
13	57.52	61610705	0.154	7.55	Alpha-amyrin	426.386
14	59.367	31900670	0.164	3.91	Vitamin E	430.381

Fourteen (14) chemical constituents were detected from the purified hexane fraction of *Bridelia ferruginea* methanolic crude extract through the use of a hyphenated Gas chromatographic – Mass Spectroscopy system. It can be observed from the spectra that compound 6 with retention time of 46.639 min had the dominant peak and is shown to be the major component with a percentage composition of 53.66%. This compound from the analysis in Table 1 as gotten from the GC – MS raw data is squalene. It has a quality of 99% and molecular weight of 410.391 amu.

Squalene is a linear triterpene which is usually synthesized in plants, animals and microbes. It is a precursor for the synthesis of various hormones, vitamins and sterols in plants and animals [16]. Squalene has been reported to have various medicinal values which enhances its use for example in weight and cholesterol control leading to a cardioprotective effect by decreasing the production of cholesterol. Other bioactive uses of squalene include antioxidant, emollient and moisturizer, detoxification and anticancer. Squalene is also used as a drug administrative agent and as a natural emollient in skin protection [17]. This major component of the oil may be a pointer to its medicinal value and other prospective uses.

Other notable components of the purified hexane fraction which may equally contribute to the bioactivity of the oil include alpha – amyrin with a percentage composition of 7.55%. Alpha – amyrin is a pentacyclic triterpene which is commonly found in medicinal compounds. It has been reported to have antibacterial, antifungal and anti-inflammatory activities [18]. It also has analgesic effects, antihepatoxic, antihyperglycaemic and antioxidant activities [19].
Stigmastan-3,5-diene which is apparently the most important of the stigmastandienes has a percentage composition of 6.91% in the oil isolated. Stigmastandienes are dehydration compounds of sitosterol and they belong to the sterenes family [20].

Vitamin E is also present in the oil with a percentage composition of 3.91%. Vitamin E is a vitamin synthesized only in plants and hence an essential nutrient in the diet of animals and humans. It plays a lot of important roles in the human body because of its antioxidant activity [21]. Vitamin E consists of two families, tocopherols and tocotrienols. Some other forms of vitamin E present in the isolated oil are dl-alpha-Tocopherol (1.72 %) and (+)-.gamma.-Tocopherol (2.03 %). This implies that the oil extracted from hexane fraction of *Bridelia ferruginea* is very rich in Vitamin E which makes it a potential source of Vitamin E that could be of immense use in pharmaceuticals, nutraceuticals and cosmetics if well exploited.

Other components of the oil that could contribute to its medicinal value and identified from the chromatogram are 2-pentadecanone, 6,10,14-trimethyl (1.19 %), 2-propenoic acid, n-pentadecyl ester (0.65 %), 1,2,3,4,5-pentamethyl-cyclopentane (0.99 %), 4,8,12,16-tetramethylheptadecan-4-olide (1.86 %), solanesol (0.76 %) and 8-formyl-6,7-bis[2-(methoxycarbonyl)ethyl]-1,3,5-trimethyl-2,4-divinylporphyrin (4.55 %).

Squalene and vitamin E (tocopherol) are important components of the oil with notable medicinal values and being reported for the first time to the best of our knowledge from oil isolated from *Bridelia ferruginea* leaves.

Biological assay results

Results of the various biological assays carried out on the hexane fraction of *Bridelia ferruginea* leaves are shown in Tables 2 – 4:

Table 2: Antimalarial assay results (IC_{50}) of hexane fraction of *Bridelia ferruginea* leaves

Sample	*P. falciparum* D6	*P. falciparum* W2	Test Concentration (ng/mL)
Hexane fraction	20436.8	22400.8	47600 – 5288.9
Artemisinin	< 26.4	< 26.4	238 – 26.4
Chloroquine	< 26.4	150.6	238 – 26.4

Table 3: Antimicrobial assay results (percentage inhibition) of hexane fraction of *Bridelia ferruginea* leaves

	Sample	*C. albicans*	*A. fumigatus*	*C. neformans*	Test Conc.
Antifungal	Hexane fraction	0	0	0	200 µg/mL
	Fluconazole	74	3	75	100 µg/mL
	Amphotericin B	97	96	97	100 µg/mL
	Sample	*E. coli*	*P. aeurginosa*	*K. pneumoniae*	Test Conc.
Antibacterial	Hexane fraction	16	2	1	200 µg/mL
	Ciprofloxacin	100	100	5	100 µg/mL
	Vancomycin	60	27	26	100 µg/mL

Table 4: Antileishmanial assay results (percentage inhibition) of hexane fraction of *Bridelia ferruginea* leaves

Sample	*L. donovani*	*L. donovani* AMAST	*T. brucei*	Test Concentration
Hexane fraction	73	94	98	20 µg/mL
Pentamidine	100	93	100	10 µg/mL
Amphotericin B	95	97	99	2 µg/mL

The purified hexane fraction of *Bridelia ferruginea* leaves exhibited moderate anti – malarial activity with IC_{50} of 20436.8 ng / mL against *P. falciparum* D6 strain and 22400.8 ng / mL against *P. falciparum* W2 strain as shown in Table 2 above. The antimalarial activity exhibited by the hexane fraction is remarkable and quite better than that exhibited by the crude extract (40838.6 ng / mL for *P. falciparum* D6 strain and 35353.8 ng / mL for *P. falciparum* W2 strain) but not as much as the antimalarial activity exhibited by the polar fraction against both strains of *P. Falciparum* tested. This may be attributed to the expected presence of some phytochemicals like alkaloids and flavonoids which may be present in the polar fractions and absent in the non – polar fractions [15].

The oil had very poor antimicrobial activity against all bacteria and fungi tested (Table 3). It only had 16% inhibition against *E. coli* and no detectable activity at all against all the fungi. This shows that it cannot be possibly employed as an antimicrobial agent and may be explained by the phytochemicals present in it.

Antileishmanial assay of the hexane fraction revealed quite impressive results (Table 4). The oil exhibited excellent activity against *Trypanasoma brucei* with 98% inhibition. This is quite high, the highest among all the fractions gotten and competes favourably with the standard drugs used as drug controls in the test. *Trypanasoma brucei* is the causative organism for African sleeping sickness and shows that the hexane fraction of *Bridelia ferruginea* is a potential drug candidate that can be employed in the treatment of sleeping sickness. It equally exhibited very good activity against two strains of Leishmania parasite – *L. Donovani* (73%) and *L. donovani* AMAST (94 %).

CONCLUSION

The chemical constituents of hexane fraction of *Bridelia ferruginea* have been analyzed using Gas Chromatography – Mass Spectrometry hyphenated system to reveal fourteen components that could be responsible for its medicinal value. Prominent among the components detected in the fraction are squalene, vitamin E (alpha tocopherol) and alpha amyrin. Before now, there was no published report of isolation of these compounds from *Bridelia ferruginea*. Biological evaluation of the fraction showed excellent antitrypanasomal activity against *Trypanasoma brucei* which is the causative organism for African sleeping sickness. This shows that the hexane fraction from crude methanolic extract of *Bridelia ferruginea* leaves is a potential candidate for the treatment of sleeping sickness.

ACKNOWLEDGEMENT

The authors would like to acknowledge the technical assistance of Drs. Mei Wang and Radhakrishnan Srivedavyasasri of the National Centre for Natural Products Research, University of Mississippi, USA for the GC- MS analysis.

REFERENCES

[1] Newman, D.J., Cragg, G.M. & Snader, K.M. (2000). The influence of natural products upon drug discovery. *Natural Product Reports*, 17, 215-234.

[2] Gurib-Fakim, A. (2006). Medicinal plants: traditions of yesterday and drugs of tomorrow. *Molecular Aspects of Medicine*, 27, 1-93.

[3] Mukhtar, M., Arshad, M., Ahmad, M., Pometrantz, R.J., Wigdahl, B. &Parveen, Z.(2008). Antiviral potentials of medicinal plants. *Virus Research*, 131, 111-120.

[4] Ekanem, J. T., Kolawole, O. M., & Abbah, O. C. (2008). Trypanocidal potential of methanolic extract of *Bridelia ferruginea* Benth. bark in Rattus novergicus. *African Journal of Biochemistry Research*, 2(2), 045-050.

[5] Okwu, D. E., & Ukanwa, N. (2010). Isolation and characterization of flavonoids chalcones and anthocynidines from *Bridelia ferruginea* benth. *Der Chemica Sinica*, 1(2), 21-28.

[6] Mbah, C. C., Akuodor, G. C., Anyalewechi, N. A., Iwuanyanwu, T. C., & Osunkwo, U. A. (2012). In vivo antiplasmodial activities of aqueous extract of *Bridelia ferruginea* stem bark against *Plasmodium berghei* in mice. *Pharmaceutical biology*, 50(2), 188-194.

[7] Cimanga, K., De Bruyne, T., Apers, S., Pieters, L., Totté, J., Kambu, K., & Labadie, R.

(1999). Complement-inhibiting constituents of *Bridelia ferruginea* stem bark. *Planta medica*, 65(03), 213-217.

[8] Pettit, G. R., Searcy, J. D., Tan, R., Cragg, G. M., Melody, N., Knight, J. C., & Chapuis, J. C. (2016). Antineoplastic Agents. 585. Isolation of *Bridelia ferruginea* Anticancer Podophyllotoxins and Synthesis of 4-Aza-podophyllotoxin Structural Modifications. *Journal of natural products*, 79(3), 507-518.

[9] Mcneely JA. (1990). *Conserving the World's Biological diversity*. Transaction Books. New Brunsick P. 208.

[10] Bruyne, T. D., Cimanga, K., Pieters, L., Claeys, M., Dommisse, R., & Vlietinck, A. (1997). Gallocatechin–(4′→ 0→ 7)-epigallocatechin, a new biflavonoid isolated from *Bridelia Ferruginea*. *Natural Product Letters*, 11(1), 47-52.

[11] Akinpelu, D. A., & Olorunmola, F. O. (2000). Antimicrobial activity of *Bridelia ferruginea* fruit. *Fitoterapia*, 71(1), 75-76.

[12] Bharate, S. B., Khan, I. S., Yunus, N.A.M., Chauthe, S. K., Jacob, M. R., Tekwani, B.l., Khan, I. A. & Singh, I. P. (2007) Antiprotozoal and antimicrobial activities of O-alkylated and formylated acylphloroglucinols. *Bioorganic & Medicinal Chemistry*, 15, 87–96.

[13] NCCLS, (2002) Reference method for broth dilution antifungal susceptibility testing of yeast; Approved Standard M27-A2.*National Committee on Clinical Laboratory Standards*, *22* (15), pp. 1-51.

[14] Mohamed M. R., Susan P. M. & Ross, S. A. (2007). Two New Sulfated Sterols from the Marine Sponge *Lenden feldia dendyi*. *Natural Product Communications*, 2(1), 901 – 904.

[15] Ngueyem, T.A., Brusotti, G., Caccialanzaa, G. & Vita Finzi, P. (2009). The genus Bridelia: A phytochemical and ethnopharmacological review. *Journal of Ethnopharmacology*, 124, 339–349.

[16] Ghimire, G. P., Nguyen, H. T., Koirala, N., & Sohng, J. K. (2016). Advances in biochemistry and microbial production of Squalene and its derivatives, *Journal of Microbiology and Biotechnology*, 26(3), 441–451.

[17] Azalia Lozano-Grande, Shela Gorinstein, Eduardo Espitia-Rangel, Gloria D´avila-Ortiz, & Alma Leticia Mart´ınez-Ayala. (2018). Plant Sources, Extraction Methods, and Uses of Squalene. *International Journal of Agronomy*, 1 – 13. Doi.org/10.1155/2018/1829160

[18] Carretero, M. E., López-Pérez, J. L., Abad, M. J., Bermejo, P., Tillet, S., Israel, A. & Noguera, P, B. (2008). Preliminary study of the anti-inflammatory activity of hexane Extract and fraction from *Bursera simaruba* (Linneo) Sarg. (Burseraceae) leaves, *Journal of Ethnopharmacology*, 116, 11-15. ISSN: 0378-8741

[19] Nnamonu, L. A., Tor-Anyiin, T. A., Ugbenyo,N. O. and AnyamJ. V. (2016). Isolation and Characterization of α –Amyrin from Stem Bark of *Ficus exasperata* (Vahl), *Biotechnology Journal International* 16(4), 1-7

[20] Amelio, R. R. & Flavio, V. (1998). Separation of Stigmasta-3,5-diene, Squalene Isomers, and Wax Esters from Olive Oils by Single High-Performance Liquid Chromatography Run. *Journal of the American Oil Chemists' Society*, 75, 527–530. DOI: 10.1007/s11746-998-0259-5

[21] Rizvi, S., Raza, S.T., Ahmed, F., Ahmad, A., Abbas, S. & Mahdi, F. (2014). The Role of Vitamin E in Human Health and Some Diseases. *Sultan Qaboos University Medical Journal*, 14(2), 157-165