IOSR Journal Of Pharmacy And Biological Sciences (IOSR-JPBS)
e-ISSN:2278-3008, p-ISSN:2319-7676. Volume 14, Issue 1 Ser. IV (Jan – Feb 2019), PP 47-60
www.Iosrjournals.Org

In vitro and in vivo biological evaluations of Combretum dolichopetalum leave extract and fractions in the management of some tropical diseases.

[1]Gugu Thaddeus Harrison, [1]Eze Christopher Osita, [2]Kenechukwu Frankline Chima, [3]Nnadi Charles Okeke, [2]Ofokansi Keneth Chibuzor, [2]Attama Anthony Amaechi and [4]Esimone Charles Okechukwu

[1]*Department of Pharmaceutical Microbiology and biotechnology, University of Nigeria, Nsukka*
[2]*Department of Pharmaceutics, University of Nigeria, Nsukka*
[3]*Department of Pharmaceutical and Medicinal Chemistry, University of Nigeria, Nsukka*
[4]*Department of Pharmaceutical Microbiology and biotech., Nnamdi Azikiwe University, Awka*
Corresponding Author: Gugu Thaddeus Harrison

Abstract: *The emergence of drug resistance and infectious diseases is worrisome in most tropical rain forest zone and among rural dwellers as a result of drug abuse and poor health care facilities necessitating the use of herbal medicines as alternatives in the control and treatment of infectious diseases. The aim of this study was to evaluate the active principles of Combretum dolichopetalum against some pathogens of tropical diseases. The active principle of the plant was extracted by cold maceration using methanol respectively and fractioned using column chromatography. Acute toxicity, antibacterial, antifungal, antidiarrheal, anti-trypanosomiasal, anti-leishmanial, anti-plasmodial and cytotoxicity studies were carried out on both the crude extract and fractions following standard methods. The LD50 was found to be >5000 mg/kg and the extracts had significant activity against bacteria and fungi. The anti-diarrhea had a significant water absorption on animal feaces within 4 hours with maximum percentage inhibition of 88.3% for the crude extract at 200 mg/kg and up to 100% for some fractions, while the in vitro and in vivo anti-trypanosomal, anti-plasmodial and anti-leishmanial study revealed that, there was significant reduction of parasite count ($p>0.05$) within 8 days of post infection with both the crude extract and fractions. The cytotoxicity studies showed that both crude extract and ethyl acetate, n-butanol and n-hexane fractions ware safe. The study showed effective activity of Combretum dolichopetalum against the tested organisms which are responsible for most of the tropical diseases and can be used as an alternative in the management of such ailments.*

Date of Submission: 01-02-2019 Date of acceptance:18-02-2019

I. Introduction

Parasitic diseases contribute significantly to the burden of infectious diseases worldwide today and this has become major concern [1]. The emergence of gastrointestinal disorder due to an increase in intestinal and luminal protozoa, as well as enterobacteriaceae has become worrisome and challenging in developing countries especially in the tropical rain forest zone like Nigeria due to poor hygiene [2] bad drinking water [3-4] and other agricultural and environmental hazards to human among the rural dwellers [5]. Not minding the existence of the orthodox drugs like antibiotics, anti-protozoas, anti-motility drugs and other antimicrobial agents, the menace is still on the increase due to evolutionary emergence of drug resistant strains of the causative organisms [6]. On this note, interventions of using extracts as herbal products from medicinal plant that have activity on such organisms are considered as alternative. There is thus, the need to evaluate the ant-bacterial and anti-fungal, anti-protozoal and ant-diarrheal properties of *Combretum dolichopetalum* and it fractions against clinical isolates of both Gram positive and negative bacteria, *Entamoeba histolitica*, *and Trypanosoma brucei* isolated from stool and urine towards identifying more novel medicinal plants for the management of diseases caused by these organisms. The term 'Tropical Diseases' is regarded as neglected tropical diseases (NTDs) in some cases. This can be describe as communicable and non-communicable diseases or disorders emerged due to nutritional deficiencies or environmental conditions (such as heat, humidity, and altitude) [7] that are encountered in geographical areas that lie between, and alongside, the Tropic of Cancer and Tropic of Capricorn belts [8]. In tropical countries, apart from non communicable diseases, a severe burden of disease is caused by an array of different types of micro-organisms, parasites, land and sea animals, and arthropods [9]. These diseases include Chagas disease and vector-borne viral encephalitis, trypanosomiasis, giardiasis, and viral hemorrhagic fevers

[10]. Bites from several animal species including snakes, scorpions, and jellyfish cause much morbidity and mortality from envenomation and secondary infections. Skin diseases in returning travellers from the tropics are frequent. The rising success rates of solid organ and hematopoietic stem cell transplantations, with associated immunosuppression, have started to face the impact of neglected tropical diseases transmitted via infected donor tissue [11]. More post-transplantation respiratory viral, bacterial, protozoal, and fungal infections are being recognized. In addition to these, the host of leishmaniasis, onchocerciasis, filariasis, Chagas' disease, African trypanosomiasis, rickettsioses, enteric fever, helminthiases, viral hemorrhagic fevers, and diarrheal diseases that affect billions of people each year remains truly neglected [12-13]. They have extremely high public health impact, and cause significant morbidity and mortality in adults and children. These diseases share population targets, ecological niches, and wide geographical distribution. Respiratory tract infections are common globally and are caused by a variety of bacterial, viral, and fungal pathogens, some of which have restricted geographical distributions [14].

C. dolichopetalum is a tropical medicinal plant (shrub) that grows well in southeastern Nigeria. It is a wild plant whose root extract contains phytochemicals like tannins and saponins and other metabolites with proven anti-ulcerative effect [15]. This plant belongs to the family Combretaceae with the local (Ibo) name 'Olitarangu'. The root of the plant contains high water content which might be responsible for the activities.

Fig. 1.0 The *Combretum dolichopetalum*

The current challenges posed by drug resistance to some of the tropical parasites has resurrected the use of some medicinal plant in the management of some local and tropical diseases. *C. dolichopetalum* extracts and its secondary metabolites have been explored recently as alternative in the management of metabolic imbalance like diabetes and other diseases [16]. This is because of its medicinal contents and values. This research output could not confront the global challenges of the malaria parasite, trypanosomiasis and other environmental and climatic parasites that cause regional threat. This knowledge gap and the use of this plant in local setting for this disease managements prompted further research to on this plant.

This study was aimed at authenticating the local claim on the use of *Combretum dolichopetalum* in the management and treatment of some tropical diseases (TDs). Also to evaluate the anti-microbial and anti-diarrheal activities of *Combretum dolichopetalum* leaves and to investigate, *in vitro* and *in vivo*, the anti-protozoa properties of both crude extract and fractions.

II. Materials

2.1.1 Plant and organisms

Combretum dolichopetalum, Protozoa: Trypamastigotes of *Trypanosoma brucei rhodesiense*, strain STIB900; amastigotes of *Trypanosoma cruzi*, Strain Tulahuen C4 and *Leishmania donovani* axer, Strain MHOM-ET- 67/L82; IEF of *Plasmodium falciparum*, strain NF54, Fungi: *Candida albicans* and *Aspergillus niger;* Bacteria: *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumonia* and *Enterococcus feacalis.*

2.1.2 Animals and animal sample specimens

White albino rats (180-210 g), mice (50-80 g), horse serum, *fetal bovine serum* (FBS) stool, urine samples and L6 cells line of rat skeletal myoblasts

2.1.3 Drug, chemicals and reagents

Melarsoprol (Aventis), benznidazole, diminal® (Standard drug Trypanosomiasis which contains 445 mg diminazene aceturate + 555 mg phenazone/g. Eagle Chemical Company LTD, Ikeja, Nigeria), miltefosine (Sigma), podophyllotoxin (Sigma)), loperamide, metronidazole (Pauco Pharmacy), chloroquine (Juhel Nig ltd) Trypticase-yeast extract-maltose (TYM) medium (pH 6.0), L-glutamine, nutrient broth glucose serum (NBGS) medium, *slime mould* (SM) medium, chlorophenolred-β-D-galactopyranoside (CPRG)/Nonidet, Roswell Park Memorial Institute (RPMI) 1640 medium, Sabouruad dextrose agar (SDA) (Oxoid), nutrient agar (Oxoid), Mueller-Hinton agar (Oxoid), manitol salt agar (Oxoid), Salmonella shigella Agar (SSA) (Oxoid), Maconkey agar (Oxoid), cetrimide agar (Oxoid), Millipore water, methanol HPLC grade (BDH), ethanol (BDH), ethylacetate (BDH), methanol (BDH), n-hexane (BDH), chloroform (BDH), distilled water, normal saline, silica gel, rice powder, etc.

2.2 Methods

2.2.1 Collection and identification of plant material

The *Combretum dolichopetalum* was collected and identified by a taxonomist Mr. A. O. Ozioko from Diogbe town in southeastern Nigeria in the month of October 2014.

2.2.2 Extraction and fractionation

The plant was dried under the room temperature, pulverized and extracted using Soxhlet extraction method and thereafter, the resultant crude extract was fractionated by column chromatographic method using different solvents: methanol, ethyl-acetate, n-butanol, N-hexane respectively.

2.2.3 Phytochemical evaluation of *Combretum dolichopetolum* crude extract

The plant extract was subjected to phytochemical analysis to determine the presence or the absence of secondary metabolites including saponnins, tannins, flavonoids, steroids,terpinoids and cardiac glycosides by adopting standard method [17-19].

2.2.4 Isolation of the test organisms

The test organisms used were isolated from clinical sample of stool and urine of infected patients using appropriate and selective media/medium prepared following standard methods [20]. The isolates were stored in an appropriate storage medium in the refrigerator for further works. Also, all the protozoa used for both *in vitro* and *in vivo* experiments were isolated with their appropriate medium and methods

2.2.5 Evaluation of antibacterial and antifungal property

2.2.5.1 Preparation of media and plant extract.

Roswell Park Memorial Institute (RPMI) 1640 medium, Sabouruad dextrose agar (SDA), nutrient agar, Mueller-Hinton agar, manitol salt agar, Salmonella shigella Agar (SSA), Maconkey agar, cetrimide agar used were prepared following manufacturer's specification by dispersing the required quantity of the powder in distilled water, homogenized and then sterilized in the autoclave at 121^{o}C for 15 min. Graded concentration of the plant extract: 400, 200, and 100 mg/ml, were prepared with dimethylsulphoxide (DMSO) using two fold serial dilution.

2.2.5.2 Evaluation of sensitivity

The agar well diffusion method was adopted [21]. Overnight broth cultures of the *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumonia, Enterococcus feacalis*, *Candida albicans* and *Aspergillus niger* were used to obtain McFarland 0.5 ~ approximately 1.5×10^{8}CFU/ml of bacteria and in each case 0.1 ml of the test organism was inoculated into 20 ml sterile molten Mueller-Hinton agar

medium maintained at 45 °C in a sterile petri-dish and the mixtures were rotated both clockwise and anticlockwise to ensure distribution of the organisms in the medium and allowed to solidified on cooling. Seven holes of 8 mm respectively were bored on each of the labeled seeded agar plate with a sterile cork borer after solidification. With the aid of sterile syringe, the wells were filled with 0.1 ml of different dilutions of the extract and allowed for pre-diffusion for 25 to 30 mins and then incubated at 37 ^{o}C for 24 h (bacteria) and 25 ^{o}C for 48 h (fungi) respectively. The inhibition zone diameter of each concentration against the test organisms were determine and recorded for further analysis

2.2.5.3 Determination of minimum inhibitory concentration (MIC)

The MIC was determine using agar dilution method [21]. A 1 ml volume of the graded concentrations of the extract solution was diluted with 19 ml molten Mueller-Hinton agar and the resultant concentrations of 400, 200, 100, 50, 25, and 12.5, 6.25, and 3.125 mg/ml of extracts and agar were obtained in the sterile Petri-dish and allowed to solidify. The test organisms were streaked on the agar surface and incubated at 37^{o}C for 24 h, thereafter, the plates were observed and the result recorded.

2.2.6 *In vivo* evaluation of the plant extracts

2.2.6.1 Acute toxicity test

The LD_{50} was carried out using modified Dietrich Lorke's method [22]. A total of 15 mice were used and it was carried out in two phases. A total of nine (9) mice, 3 groups of 3 mice each per group were used. They were grouped into; Group one received 10 mg/kg, group two received 100 mg/kg while group three received 1000 mg/kg. The animals were constantly monitored 2 h interval for the period of 24 - 48 h for behavioral changes and mortality. From the result of the first phase, the doses for the second phase was decided. In thesecond phase, the remaining 8 mice were grouped into four (4) groups of two animals each per group. Group1 received 2000 mg/kg, group two received 3000 mg/kg, group three received 4000 mg/kg while group four received 5000 mg/kg respectively. The animals were observed as in the first phase and the rate of mortality was recorded.

2.2.6.2 Evaluation of anti-diarrhea property

Adopting castor oil-induced diarrhea method of preventive therapy as described [23]. Twenty five (25) albino mice of both sex (wt. 80-100 g) were divided into five groups of five animals each. They were fasted for 24 h prior to the test, while allowed free access to water. Group 1 was treated with 0.2 ml/kg of normal saline, which served as negative control; Groups 2, 3 and 4 received different doses of the extract (200, 400 and 800 mg/kg) respectively. Group 5 received standard drug (Loperamide 2 mg/kg). All doses were administered orally. The animals were then housed singly in cages lined with transparent paper. One hour after pre-treatment with the extract, the animals were challenged with 0.5 ml of castor oil orally. Thereafter, they were observed for 4 h for the presence of diarrhea defined watery (wet) and unformed stool.

2.2.6.3 Evaluation of *in vivo* anti-trypanosome property

The *in vivo* activity of the leaf extracts were evaluated using rat model [24]. Thirty (30) white albino rats used were divided into six (6) groups of five rats each. The rats in four groups were intraperitoneally infected with *T. brucei* per 100 g body weight (b.w) and the level of parasitaemia was monitored and estimated daily using Herbert method [25] for the 7 days period of the experiment. The following treatment protocol was followed:

- Group one (1) received 100 mg/kg
- Group two (2) received 200 mg/kg
- Group three (3) received 400 mg/kg of the extract orally
- Group four (4) received 7 mg/kg of Diminal® was also given intraperitoneally
- Group five (5) sixth group was left untreated (infected control), whereas group six (6) was maintained as uninfected untreated (normal) control.

The treatments were commenced a day after the parasites were first detected in the bloodstream within 24-72 h and completed for seven days post infection. The pre-infection and terminal packed cell volumes of all the rats were determined by the microscopic and microheamatocritic method.

2.2.7 Evaluation of *in vitro* antiprotozoan activity and cytotoxicity

2.2.7.1 *In vitro* anti-*Plasmodium falciparum* activity

In vitro activity against erythrocytic stages of *P. falciparum* was determined by a modified [3H]-hypoxanthine incorporation assay [26], using the chloroquine- and pyrimethamine-resistant K1 strain and the standard drug chloroquine. The parasite cultures were incubated in RPMI 1640 medium with 5% AlbuMAX (Lipid-rich bovine serum albumin for cell culture) and exposed to serial drug dilutions in micro-titer plates.

After 48 h of incubation at 37 °C, in a reduced oxygen atmosphere, 0.5 μCi 3-Hhypoxanthine was added to each well. The cultures were incubated for a further 24 h before they were harvested onto glass-fiber filters and washed with distilled water. The radioactivity was counted using a Betaplate TM liquid scintillation counter. The results were recorded as counts per minute (CPM) per well at each drug concentration and expressed as percentage of the untreated controls. IC50 values were calculated from the sigmoidal inhibition curves using Microsoft Excel. Chloroquine was the reference drug used.

2.2.7.2 *In vitro* anti-*Trypanosoma brucei rhodesiense* activity

Trypanosoma brucei rhodesiense, STIB 900 strain, and the standard drug, melarsoprol, were used for the assay. This stock was isolated from a human patient in Tanzania and after several mouse passages cloned and adapted to axenic culture conditions. Minimum Essential Medium (50 μL) supplemented with 25 mM HEPES, 1g/L additional glucose, 1% MEM non-essential amino acids (100x), 0.2 mM 2- mercaptoethanol, 1 mM Na-pyruvate and 15% heat inactivated horse serum was added to each well of a 96-well micro-titer plate [24]. Serial drug dilutions of seven 3-fold dilution steps covering a range from 0.123 to100 μg/mL were prepared. Then, the bloodstream forms of *T. b. rhodesiense* STIB 900 in 50 μL was added to each well and the plate was incubated at 37 °C under a 5% CO_2 atmosphere for 72 h, and thereafter, 10 μL Alamar Blue (resazurin, 12.5 mg in 100 mL double-distilled water) was then added to each well and incubation continued for a further 2–4 h. The plates were then read in a Spectramax Gemini XS microplate fluorometer using an excitation wavelength of 536 nm and an emission wavelength of 588 nm. Data were analysed using the micro plate reader software Softmax Pro Molecular Devices.

2.2.7.3 *In vitro* anti- *Trypanosoma cruzi* activity

Rat skeletal myoblasts (L6 cells) were seeded in 96-well microtitre plates at 2000 cells/well in 100 μL RPMI 1640 medium with 10% fetal bovine serum (FBS) and 2 mM L-glutamine. The medium was removed after 24 h and replaced with 100 μL per well containing 5000 trypomastigotes of *T. cruzi* Tulahuen strain C4 containing the β-galactosidase (Lac Z) gene. After 48 h, the medium was removed from the wells and replaced with 100 μL fresh medium with serial dilution of extract and fractions covering a range from 100 to 0.123 μg/mL. After 96 h of incubation, the plates were inspected under an inverted microscope to confirm growth and sterility of the controls. The substrate chlorophenolred-β-D-galactopyranoside (CPRG)/Nonidet (50 μL) was added to all wells. A color reaction which developed within 2–6 h was read photometrically at 540 nm. Data were transferred into the graphic programme Softmax Pro Molecular Devices, which calculated IC50 values. Benzmidazole was the reference drug used.

2.2.7.4 *In vitro Leishmania donovani a*ctivity

Amastigotes of *L. donovani* strain MHOM/ET/67/L82 were grown in axenic culture at 37 °C in *slime mould* (SM) medium at pH 5.4 supplemented with 10% heat-inactivated fetal bovine serum under an atmosphere of 5% CO_2 in air. One hundred microlitre (100μl) of culture medium with 105 amastigotes from axenic culture with solutions of extract, fractions and control drug were seeded in each of the 96 well microtitre plates. Serial dilutions of extract and fractions with control drug covering a range from 100 to 0.123 μg/mL were prepared. After 72 h of incubation the plates were inspected under an inverted microscope to confirm growth and sterility of the controls. Ten microliter Alamar Blue (12.5 mg resazurin dissolved in 100 mL distilled water) was then added to each well and all the plates were incubated for another 2 h. The plates were then read with a Spectramax Gemini XS microplate fluorometer using an excitation wavelength of 536 nm and an emission wavelength of 588 nm. Data were analyzed using the software Softmax Pro Molecular Devices. Decrease of fluorescence (inhibition) was expressed as percentage of the fluorescence of control cultures and plotted against the drug concentrations. From the sigmoidal inhibition curves the IC50 values were calculated. Miltefosine was used as the reference drug.

2.2.8 Cytotoxicity test against L6 Cells

The assays was performed in 96-well microtiter plates, each well containing 100 μL of RPMI 1640 medium supplemented with 1% L-glutamine (200 mM) and 10% fetal bovine serum, and 4 × 104 L6 cells (a primary cell line derived from rat skeletal myoblasts). Serial drug dilutions of seven 3-folddilution steps covering a range from 0.123 to100 μg/mL were prepared. After 72 h of incubation, the plates were inspected under an inverted microscope to confirm growth and sterility of the controls. Ten microliter of Alamar Blue solution was then added to each well and the plates incubated for another 2 h. The plates were then read with a Spectramax Gemini XS microplate fluorometer using an excitation wavelength of 536 nm and an emission wavelength of 588 nm. Data were analysed using the microplate reader software Softmax Pro. Podophyllotoxin was the reference drug used.

III. Results And Discussion

Table 1.0 Phytochemical constituents of the crude extract.

S/no	Parameters	Results
1	Alkaloids	+
2	Saponins	+++
3	Tannins	+++
4	Flavonoids	++
5	Steroids	-
6	Terpenoids	-
7	Glycosides	+
8	Proteins	++

Keys: (+) indicates presence, (-) Indicates absence

- +++ Relatively high abundance of compound; ++ = Moderate abundance of compound
- + Relative low presence of compound.

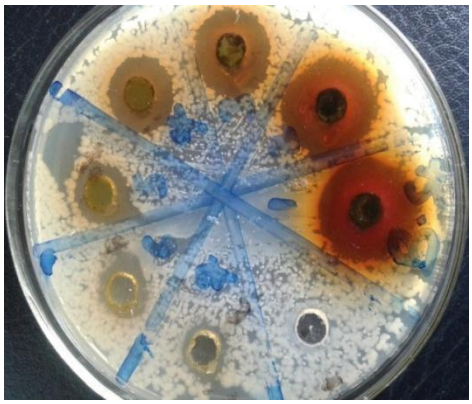
In the Table 1.0 above, the results of the qualitative phytochemical analyses showed that the extract contained secondary metabolites like tannin, alkaloids, flavonoids, saponins, glycoside with the relative abundance of tannin and saponin, possibly responsible for its biological activities like anti-bacteria, anti-fungi activity, anti-protozoa etc. Similar constituents were also detected on the leaf from a previous study [27] with addition of steroid and terpinoids. These types of phytoconstituents have been known to exhibit antibacterial activity [28]. Thus one or more of these phytochemicals could possibly be contributing to the observed antifungi activity of the extract [29]. In addition, some species of Combretaceae family have been reported to contain different chemical constituents such as ellagic acid, gallic acid, ellagitannins and gallotannins and have been known for a long time to show pharmacological effects like antioxidant and antidiabetic activities. [28]

3.2 Sensitivity pattern of *C. dolichopetalum* against bacteria and fungi

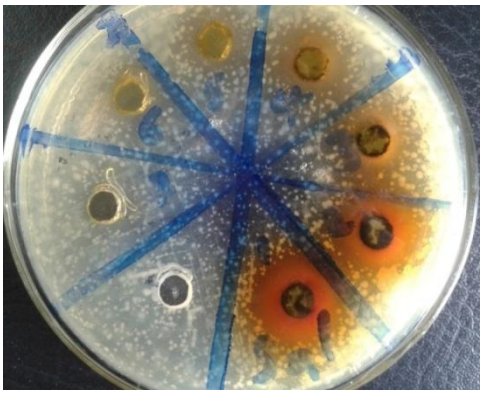
Table 2 .0 Sensitivity pattern of *C. dolichopetalum* crude extract and fractions.

Extract and fractions	Conc. (mg/ml)	Test organisms							
		S. aereus	*E. coli*	*E. feacalis*	*B. subtilis*	*P. aerug.*	*K. pneum.*	*S. typhi*	*C. alb.*
		Inhibition zone diameter (mm)							
	400	16	18	17	15	12	13	15	10
Crude extract	200	14	16	15	13	10	11	13	8
	100	12	14	13	11	8	9	11	6
Ethyl acetate	100	6	8	6	14	12	8	6	4
	50	4	6	4	12	10	6	4	2
N-butanol	100	10	12	10	12	10	10	8	0
	50	8	10	8	10	8	8	6	0
N-hexane	100	8	6	6	7	10	8	6	2
	50	6	4	4	5	8	6	4	0

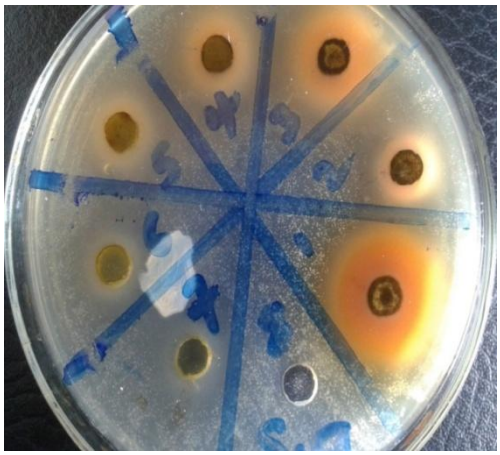
Test organisms: *Staphylococcus aereus, Escherichia coli, Enterococcus feacalis, Bacillus subtilis, Pseudomonas aeruginosa, Klebsiella pneumonia, Salmonella typhi, Candida albicans.*



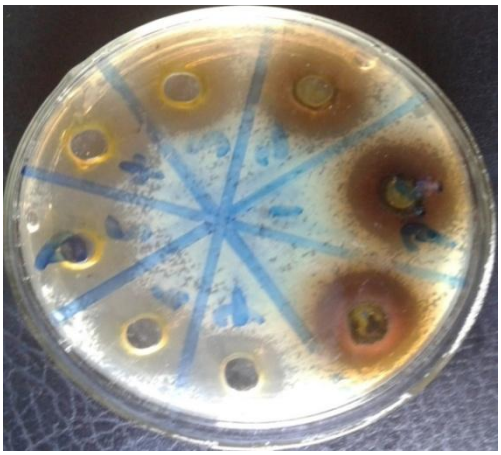
i *Bacillus subtilis* (crude extract)



ii *Salmonella typhi* (crude extract)



iii *Staph. aureus* (crude extract)



Iv *Pseudomonas aeruginosa* (E/Acetate fraction)

Fig. 2.0 Sensitivity pattern against some test bacterial

In the Figure 2.0 above, the antibacterial activity was evaluated, the extracts used were obtained with different solvents (methanol, ethyl-acetate, n-hexane, and n-butanol). Activity was observed against the following bacterial species: *Pseudomonas aeruginosa, Staphylococcus aureus, E.coli, Salmonella typhi, Enterococcus feacalis, Klebsiella pneumonia*, and *Bacillus subtilis* [30]. In addition, antifungal activity against *Candida albicans* was noted [31]. The sensitivity pattern of both crude and fractions showed good activity against both Gram positive and negative bacteria as well as fungi used in the studies. The concentrations of the crude extract used include 400,200, 100 mg/ml and so on with maximum inhibition zone diameter (IZD) of 18mm and 17 mm against *E. coli* and *E. feacalis* and this effect has been recorded more against *E. coli*. [32]. Though root extract seems to be more active than the leave and this could be as a result of accumulation of more active metabolite by toot part [33] and phytoconstituents contained in the leave part of the plant as reported in some recent studies. The activity was poor against two fungi used generally, though, the crude extract good activity against *C. albicans* with up 400mg/ml showing 12 mm IZD and also, ethyl acetate fraction had significant activity of 4 mm at 100mg/lm. This activity against fungi confirmed a report on the Metabolites from *Combretum dolichopetalum* and its associated endophytic fungus Nigrospora oryzae [33].

3.2.1 The minimum inhibitory concentration of both crude extract and fractions of *C. dolichopetalum* against both bacteria and fungi tested

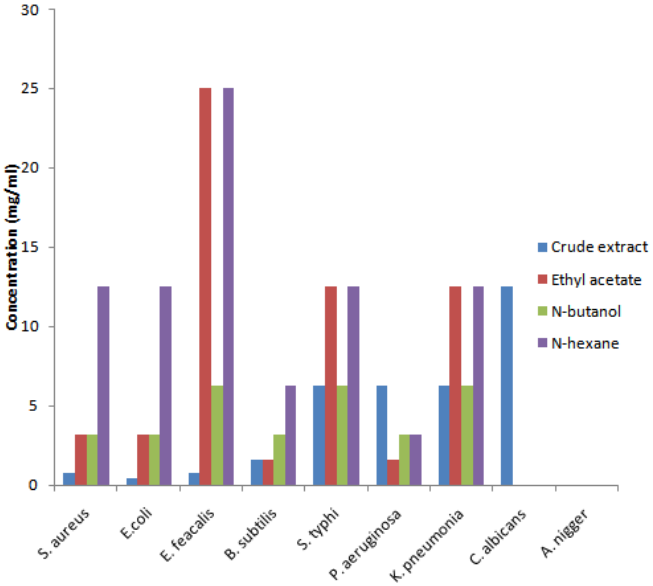


Fig. 3.0 The minimum inhibitory concentration (mg/ml) of both crude extract and fractions of *C. dolichopetalum* against both bacteria and fungi tested

Figure 3.0 showed the minimum inhibitory concentration of both crude and fractions of *C. dolichopetalum* showed that both crude and fraction had good activity against tested organisms. The effect of crude extract were more against Gram positive organisms especially with *S. aureus, E. feacalis* and *B. subtilis* with 0.781, 0.7891 and 1.5625 mg/ml respectively. Also, ethylacetate showed minimum concentration of 1,5625mg/ml and maximum of 25mg/ml against *B. subtilis, P. aeruginosa* and *E. feacalis* respectively among others while n-butanol had MIC of 3.125 each against both *Staph. aureus, E.coli, B. subtilis* and *P. aeruginosa* and maximum of 6.25mg/ml each against *E. feacalis, Sal. typhi* and *Klebsiella pneumonia*, similarly, n-hexane showed highest MICs against the tested organisms with minimum concentration of 3.125mg/ml against *P. aeruginosa* and 25mg/ml against *E. feacalis.* The above range of the minimum inhibitory concentration against the test organisms used confiremd the report of ethanolic leaf extract and n-hexane fraction of Combretum species on antibacteria, anthelmintic and anti-amoebic activity of medicinal plant in South Africa[33] which showed MIC of 0.049mg/ml against *B. subtilis* and *Staph. aureus* and 12.5mg/ml against *K. pneumonia* and *E. coli* as well as n-hexane fraction of 12.5mg/ml against both *B. subtilis, E. coli, K. pneumonia* and *S. aureus* respectively

3.3 Toxicity

LD50 >5000 mg/kg because there was no death recorded at all dose level.

3.4 Percentage diarrhea inhibition of crude extract of *C. dolichopetalum*

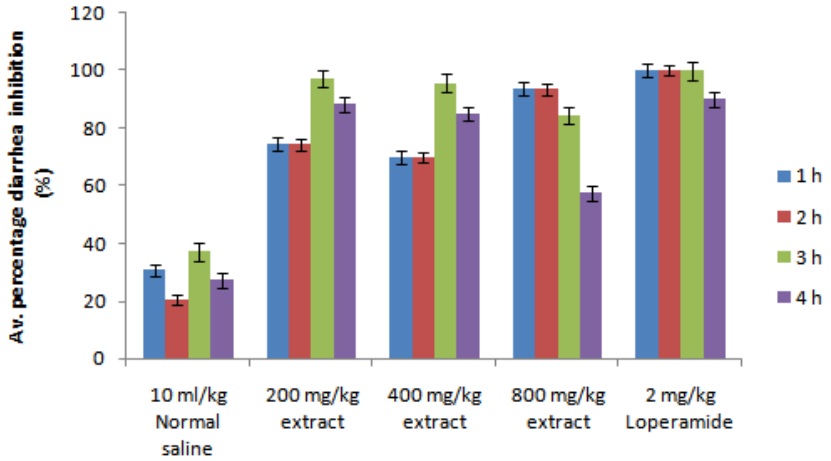


Fig. 4.0 Percentage diarrhea inhibition of crude extract of *C. dolichopetalum*

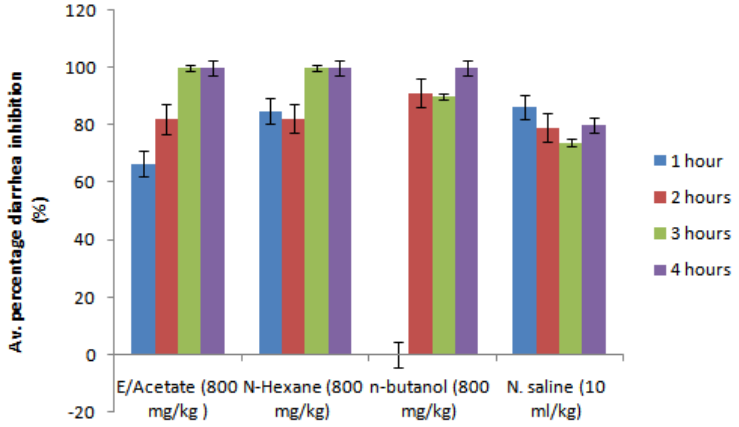


Fig. 5.0 Percentage diarrhea inhibition of *C. dolichopetalum* fractions

The alcoholic extract of *C. dolichopetalum* is used in folklore medicine to relieve stomach ache, blood in the stools, diarrhea, cramps and related gastrointestinal disorders [34]. The percentage diarrhea inhibition of crude extract and fractions in Figure 4 and 5 respectively of the *C. dolichopetalum* showed that within 3 to 4 hours, the crude extract and fractions exert better diarrhea inhibition of up to 97.2 and 93.5 % of 200mg/kg and 800mg/kg respectively in the Figure 5, this can be considered good inhibitory effect as compared with the positive control (2mg/kg loperamide) which showed 90 to 100% diarrhea inhibition when used. Similarly, the n-butanol fraction also had up to 90 to 100 % inhibition as well as ethyl acetate and n-hexane 100% between 3 to 4 hours. The effect could be as a result of the present of tannin and saponin as earlier studies shows that tannins and other secondary metabolite present in most medicinal plants are responsible for antidysentric and anti-diarrheal properties [35-36]. The ethanolic extract of C. dolichopetalum has shown a gastroprotective effect in stress-induced and non-steroidal antiinflammatory (indomethacin)-induced ulcer models. The crude extract inhibited secretions induced in rats by pyloric ligation together with histamine. The crude extract inhibited the contractions induced by acetylcholine and histamine in the guinea-pig ileum in a concentrationdependent manner and also delayed gastric emptying in rats in a dose-dependent manner [37]. These results therefore suggest that C. dolichopetalum has gastric antisecretory activity, increasing gastric emptying time, and acts as a smooth muscle relaxant and spasmolytic agent [35].

3.4 *In vivo* anti-trypanosoma property of *C. dolichopetalum*

Table 3.0 *In vivo* anti-trypanosoma activity of *C. dolichopetalum*

Concentrations	Av. body weight (g)		PCV		Av. % parasitemia/ml
	Pre-infec.	Post-infec.	Pre-inf.	Av. Post inf.	
400 (mg/ml)	137	141.02	42.2	34	6.01
200 (mg/ml)	141.9	146.8	42.6	34.9	7.3
100 (mg/ml)	139	139.3	41.8	31.7	7.8
Diminazene (1.03mg/ml)	147.8	147.9	41.4	34.1	3.3
Normal saline	142.1	142.4	40.4	22.2	8.8

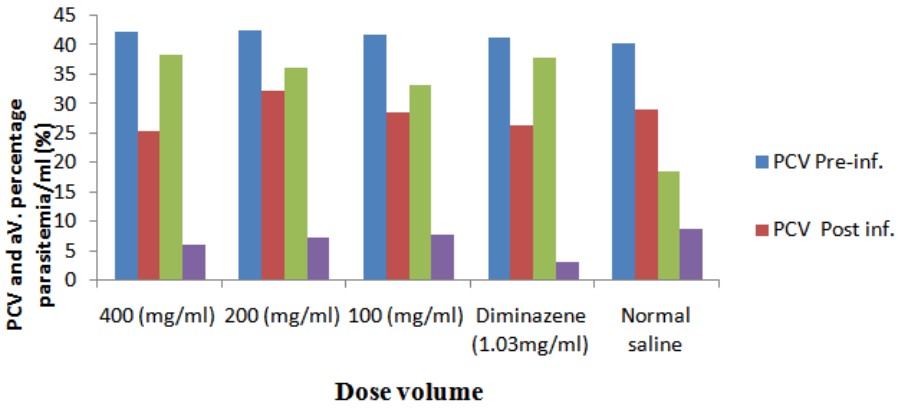


Fig. 6.0 *In vivo* anti-trypanosoma activity of *C. dolichopetalum*

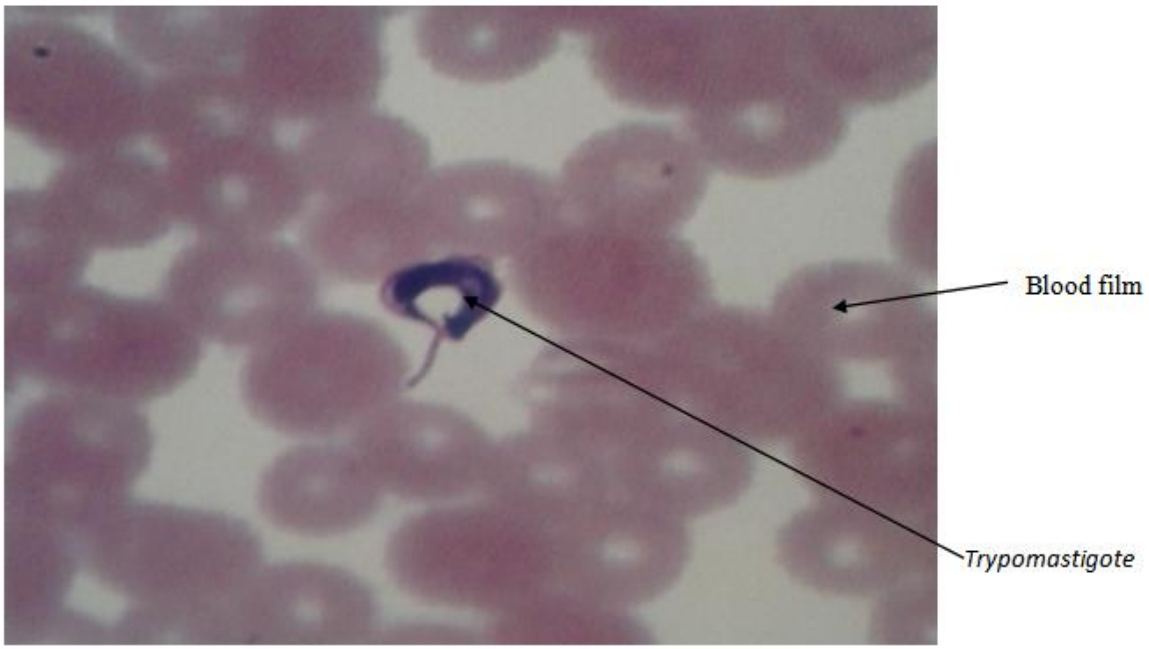


Fig. 7.0 Trypomastigotes of T. cruzi

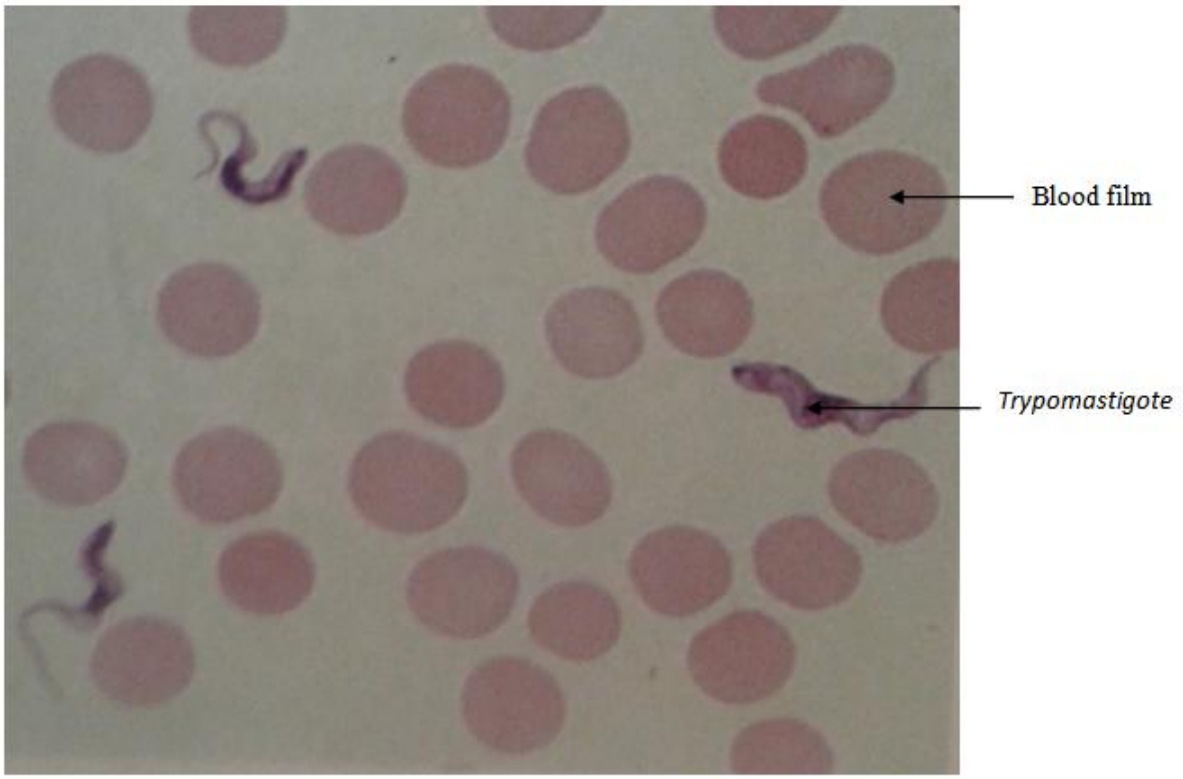


Fig. 8.0 Trypomastigotes of T. brucei

Anaemia is one of the most important clinical signs of trypanosomiasis. It has now been definitely established that the measurement of the pack cell volume (PCV) gives a reliable indication of the disease status of trypanosome infected cattle [38]. The PCV has also been shown to be a reliable index of anaemia as no evidence of haemodilution has been found in trypanosome infected cattle [39]. The anti-trypanosoma activity of *C. dolichopetalum* was evaluated *in vivo* to estimate the effect of the plant extract on the pack-cell volume (PCV) as well as the survival rate of the protozoa in the systemic circulation (blood cell) in the presence of the extract as percentage parasitemia. The result gotten from this research showed that there was establishment of

the protozoa infection which affect blood cell (red blood cell) especially the pack-cell volume ranging from 36 to 48% as normal [40]. Table 3 and figure 7 and 8 as well showed the shift of PCV from normal range to abnormal before and after infecting the animals with the protozoa with initial PCV between 40.4 and 42.6% to 25.4 and 34.9 % after infection. The PCV and other clinical indices after 4 to 8 days treatment with the extract showed significant improvement by increasing from 25.4 to 33.2 % as least PCV recorded among the test animals which confirm the report of *Combretum dolichopetalum* PCV improvement among the tropical medicinal plants. [41] The percentage parasitemia which showed the level of parasite in the blood cell had significant reduction of 6.01 and 7.3%/ml with the extract concentration of 400mg/ml and 200mg/ml respectively as a report that the extracts of *Combretum dolicopetalum* showed significant ($P < 0.05$) trypanocidal activity at high dosage levels which can be considered effective when compared with the positive control drug (diminazene) (1.03mg/ml) which had 3.3 %/ml.

3.5 *In vitro* anti-protozoa and cytotoxicity of *C. dolichopetalum* crude extract and fractions

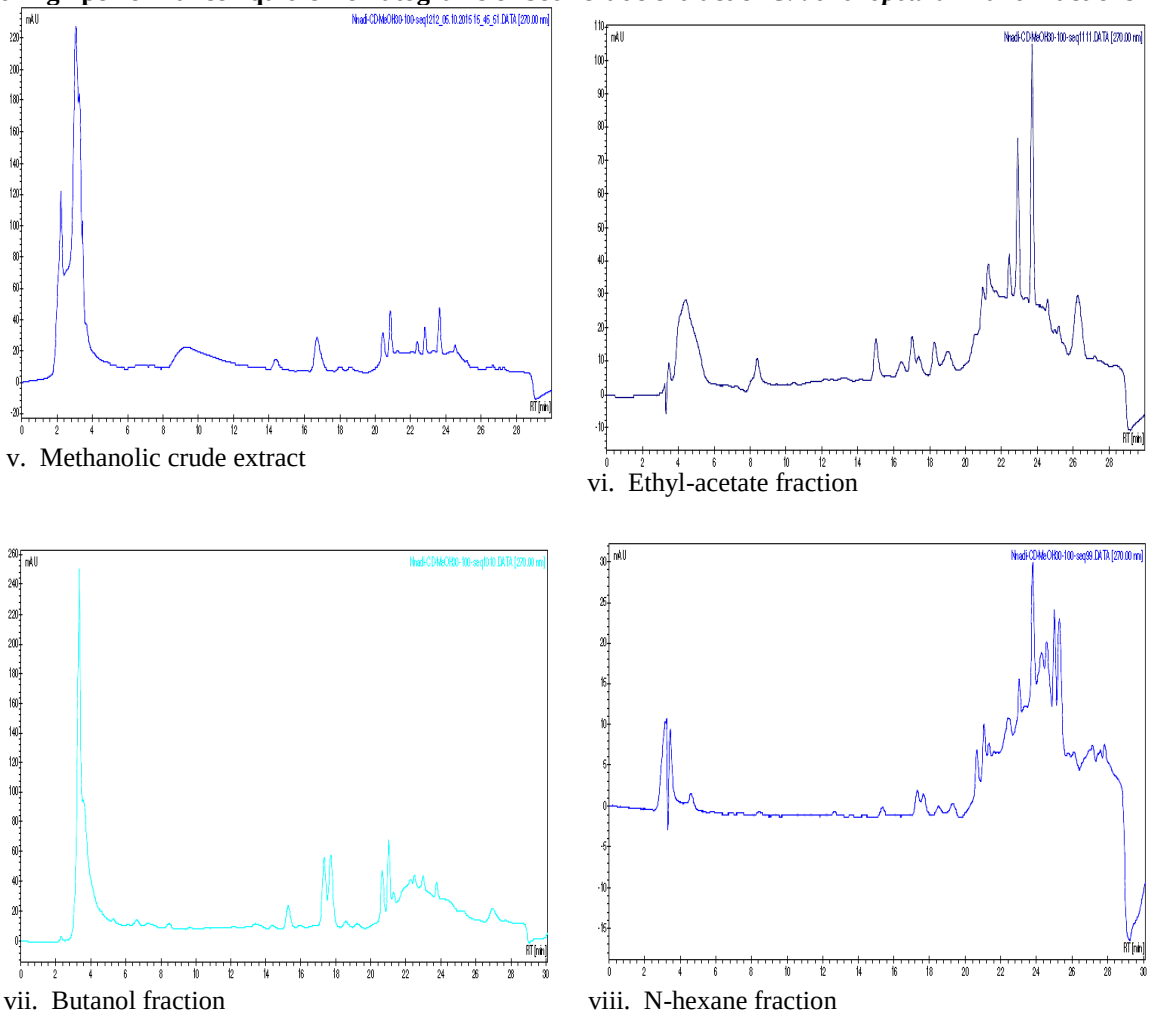
Table 4.0 *In vitro* anti-trypanosoma, anti-plasmodia, anti-Leishmani and cytotoxicity properties of *C. dolichopetalum* crude extract and fractions.

Sample codes	*T.b. rhod.*		*T.cruzi*		*L. don. ax am*		*P.falc NF54*		*Cytotox. L6*
	Inhibitory concentration (µg/ml)								
	IC_{50}	S.I	IC_{50}	S.I	IC_{50}	S.I	IC_{50}	S.I	IC_{50}
Melarsoprol	0.003		NU		NU		NU		NU
Benznidazole	NU		0.500		NU		NU		NU
Miltefosine	NU		NU		0.053		NU		NU
Chloroquine	NU		NU		NU		0.001		NU
Podophyllotoxin	NU		NU		NU		NU		0.005
Crude extract	>100	0.51	3.61	14.02	>100	0.51	4.12	12.3	50.60
CD N-H	>100	NT	>100		>100		>100		NT
CD n-But	>100	0.31	4.028	7.6	>100	0.31	10.48	2.91	30.47
CD EtAce	89.506	NT	>100		>100		>100		NT
CD MeOH	>100	NT	>100		>100		>100		NT

Key: NU = Not used, NT = Not tested, S.I = Selectivity index, IC = Inhibitory conc.

The *in vitro* properties of *C. dolichopetalum* crude extract and fractions were done confirm the *in vivo* effect in experimental animal and some report of anti-trypanosoma, anti-plasmodia, anti-Leishmani and cytotoxicity activities about the plant. The *in vitro* anti-trypanosomiasis of crude extract and some fractions showed significant activities against *T.b. rhodonensis* and *T. cruzi* tested with the IC50 of crude extract against the two organisms 3.61 µg/ml with selectivity index of 14.02 and >100 with selectivity index of 0.51, while n-butanol fraction of the extract against *T. cruzi* was 4.028 µg/ml with SI of 7.6 and *T.b. rhodonensis* >100 with SI of 0.31 and also, the ethyl acetate fraction against *T.b. rhodonensis* had IC50 of 89.506 µg/ml. The lower the IC50 value of extracts and fractions against tested organisms the potent or more active and reflects better protective action of the plant [42]. This affirm that the plant axtract and its fractions possess good activity against trypanosomiasis as tropical diseaes which confirm the report on ethanolic extract of *C. dolichopetalum* root bark anainst *Trypanosoma brucei* and *Trypanosoma congolense* [43]. The anti-plasmodia showed that the crude extract and n-butanol fraction had IC50 of 4.12 and 10.40 with selectivity index of 12.3 and 2.91 respectively. This is in line with the report of ethanolic extract of *C. micranthum* leaf which had IC50 of 33.05 µg/ml and methanoli extract of >25 µg/ml through In vitro-Cell culture (erythrocytes) against parasite maturation of *Plasmodium falciparum* [44-45]. The cytotoxicity test of the crude extract and fractions was done to determine the toxic selectivity effect of the plant or other chemical agents on a particular cell especially mammalian cancerous cell, etc. The cytotoxic IC50 of crude extract had 50.6 and n-butanol fraction 30.47 and this can be considered safe in comparism with the positive control which showed 0.005. The high value of the crude extract could be as a result of many compounds present and this can posse better when narrow down to possible compound responsible for that as n- fraction proved that with low IC50 [46]. The cytotoxicity effect of this plant is to confirm a report other species of the *Combretum epiculatum* [47-48] while other fractions does not tested as they did not pose any threat when tested

3.6 High performance liquid chromatograms of both crude extract of *C. dolichopetalum* and fractions



v. Methanolic crude extract

vi. Ethyl-acetate fraction

vii. Butanol fraction

viii. N-hexane fraction

Fig. 9.0 The chromatogram of crude and fractions

Fig 9.0 above showed the high performance liquid chromatography (HPLC) of both the crude extract and fraction of the *C. dolichopeatlun* was conducted to estimate the present and possibly the quantity of the unknown compound found in the plant which is most likely responsible for the various activities it exhibited. The peaks observed in the crude and fractions could be as a result of presence of the following compounds: ellagic acid, 3, 3', 4-tri-O-methylellagic acid, arjunolic acid, 4'-dihydrophaseic acid, echinulin and arestrictin B as reported [33] isolated from the plant stem bark. The

IV. Conclusion

The outcome of the studies carried out, revealed that the *C. dolichopetalum* extract and its fractions exhibit good activity against the Trypanosoma species which are regarded as causative agents of tropical diseases (TDs). The crude extract of *C. dolichopetalum* had good antimicrobial activity against bacteria with lowest MIC of 0.391 mg/ml and 12.5 mg/ml against *E.coli* and fungi (yeast) respectively. The fractions of *C. dolichopetalum* also presented better activity as regards to the concentrations used when compared with the orthodox drugs. The significant percentage diarrhea inhibition possesses by the crude extract and fractions suggest that they may be used as an antimotility agents. Also, the *in vitro* and *in vivo* anti-protozoal activities also suggest that the plant can be used in the management of some TDs. The cytotoxicity studies of both the crude extract and n-butanol fraction showed that they are relatively safe.

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IOSR Journal of Pharmacy and Biological Sciences (IOSR-JPBS) is UGC approved Journal with Sl. No. 5012, Journal no. 49063.

Gugu Thaddeus Harrison." In vitro and in vivo biological evaluations of Combretum dolichopetalum leave extract and fractions in the management of some tropical diseases." IOSR Journal of Pharmacy and Biological Sciences (IOSR-JPBS) 14.1 (2019): 47-60.