THE IMMUNOSTIMULATORY AND ANTIMICROBIAL PROPERTY OF TWO HERBAL DECOCTIONS USED IN THE MANAGEMENT OF HIV/AIDS IN GHANA

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Abstract

Background: HIV/AIDS is a pandemic retarding economic growth and destroying human capital globally. This study therefore investigated the perceived efficacy of *Betula alba* (BA) and *Sutherlandia frutescens* (SF) decoctions used in the management of HIV/AIDS in Ghana.
Materials and Methods: A study on the records of HIV/AIDS patients attending Habibi Herbal Clinic, Kumasi, Ghana, was conducted to obtain information on the initial viral load presented during their maiden visit and results after treatment with the herbal decoctions. The decoctions were assessed for immunostimulatory property in cyclophosphamide-induced immunosuppressed ICR mice. Total white blood cell count, as well as lymphocyte and neutrophil counts were determined and their effects compared with Levamisole. The decoctions were also screened for antimicrobial activity by the micro dilution method.
Results: The two herbal decoctions used significantly reduced ($P \leq 0.001$) the patients' viral loads (47.42 ± 17.28 % to 13.69 ± 12.42 %; n=16). BA (1, 2, and 4 mg/kg) and SF (0.4, 0.8, and 1.6 mg/kg) caused significant increment ($P \leq 0.001$) in total WBC and lymphocyte count in mice comparable to that produced by 2.5 mg/kg Levamisole. The decoctions also exhibited antimicrobial activity against gram negative and gram positive bacteria as well as *Candida albicans* (MIC 0.607 to 3.062 mg/ml). Phytochemicals present in both plants include saponins, terpenoids and coumarins.
Conclusion: The *Betula alba* and *Sutherlandia frutescens* decoctions have interesting immunostimulatory and antimicrobial properties and hence could be useful in the management of HIV/AIDS and associated opportunistic infections.

Key words: *Betula alba*, *Sutherlandia frutescen,s,*Viral load, Minimum inhibitory concentration, Micro-well dilution assay

Introduction

Human Immunodeficiency Virus (HIV) is a retrovirus that primarily infects and destroys CD4+ T cells of the human immune system directly and indirectly (Alimonti *et al.,* 2003). It is one of the most infectious pathogen with devastating consequences. HIV/AIDS is now a pandemic (Kallings, 2008) and in 2007, an estimated 33.2 million people lived with the disease worldwide. It killed an estimated 2.1 million people, including 330,000 children (UNAIDS/WHO, 2007). Over three-quarters of these deaths occurred in sub-Saharan Africa, (UNAIDS/WHO, 2007). These developments negatively affect economic growth and destroy or decrease the human resource base (Bell *et al.,* 2008). In Ghana, the current HIV epidemic estimates show that 225,478 persons made up of 100,336 males and 125,141 females are affected by the condition. Rather disturbingly, there are 12,077 new infections and 15,263 AIDS deaths annually. A large proportion of children to the tune of 30,395, are living with HIV with an annual AIDS death estimated at 2,080 (NACP, 2011).

The life expectancy of HIV/AIDS patients have improved as a result of the development of antiretrovirals drugs. The introduction of highly active antiretroviral therapy (HAART) into clinical practice in 1996 has dramatically changed the development of HIV-related diseases in industrialized countries (Tirelli, 2001; Vella, 2000). Although the antiretroviral drugs only manage the HIV infection , they have an impact on reducing morbidity and mortality of AIDS patients by suppressing viral load and reducing HIV transmission; thereby not only prolonging lives but also improving the quality of life of many people living with HIV/AIDS (WHO, 2002). However, HAART has a limited response in some patients and is also associated with some toxicity challenges. Some of the adverse effects include heart attacks and adult diabetes (Yolan *et al.,* 2007). Moreover, many people with HIV/AIDS in developing countries cannot access HAART due essentially to the high cost of the drugs, limited human and structural resources as well as social stigma. In addition, antiretroviral drugs (ARVs) require the support of expensive medical infrastructure not available in many developing countries and rarely available in rural areas of any developing country. ARVs can also produce significant side effects, as well as sometimes being ineffective.

The use of medicinal plants in the management of diseases has always been an integral part of the healthcare delivery system in developing countries. Motivated by a study to clinically assess the efficacy of a South African traditional medicine by monitoring viral load and CD4 counts (Tshibangu *et al.,* 2004) which described an improvement in the immune system and general well-being of patients, due to increase in CD4+ T cell and decrease in viral load, when these markers were monitored for 12 months in HIV/AIDS participants, two plants (*Betula alba* and *Sutherlandia frutescens*) used at the Habibi Herbal Clinic in Ghana in the management of HIV/AIDS were investigated for their immunostimulatory and antimicrobial properties. Other folklore uses of *B. alba* include treatment of eczema, gout, rheumatism and edema (Grieve, 2013), while *S. frutescens* is also employed to treat fever, chicken pox, flu, rheumatism, hemorrhoids, diarrhea and cancer (Katerere and Eloff, 2005).

Materials and Methods
Plant collection

Fresh leaves of *Betula alba* (family: Betulaceae) and *Sutherlandia frutescens* (family: Fabaceae) selected through a survey at a Herbal clinic was collected in October, 2012 and authenticated in the Department of Pharmacognosy, KNUST, Kumasi, Ghana where a voucher specimen with number KNUST/HM1/2012/S012 S012 and KNUST/HM1/2012/B012 respectively has been kept.

Preparation of decoctions

A 3.3 kg quantity each of the fresh leaves of the *Betula alba* and *Sutherlandia frutescens* were washed and boiled separately in water for 4h and strained to obtain 5 liters each of decoction. The decoctions were freeze dried and weighed: BA 61.23 g and SF 24.30 g. The extracts were reconstituted in distilled water for use in this study. Dosing of the preparation was done based on the manufacturer's recommendations and was once daily by gavage throughout the experimental period at a volume of 10 ml/kg. Individual dose volumes were calculated based on the animal's most recent recorded body weight. The oral route of administration was used because it is the intended human exposure route.

Preliminary phytochemical screening

BA and SF were phytochemically screened using the standard techniques as described by Sofowora (1993), Harbone (1998), and Trease and Evans (1989).

Ethical and biosafety considerations

Permission was obtained from the Administrator of Habibi Herbal Clinic before the survey was undertaken. Laboratory study was carried out in a level 2 biosafety laboratory. Protocols for the study were approved by the Departmental Ethics Committee. All activities during the studies conformed to accepted principles for laboratory animal use and care (EU directive of 1986: 86/609/EEC). All the technical team observed all institutional biosafety guidelines for protection of personnel and laboratory.

Animals and husbandry

Hundred and twenty imprinting control region (ICR) mice (20–26 g) were obtained from the Animal House in the Department of Pharmacology, Faculty of Pharmacy and Pharmaceutical Sciences, KNUST, Kumasi, Ghana. All animals were housed in stainless steel cages with wood shaving as bedding. The animals were kept under ambient conditions of light, temperature, and relative humidity, and fed with normal pelleted mice chow (GHAFCO-Tema, Ghana) and water *ad libitum*. All activities during the studies conformed to accepted principles for laboratory animal use and care (EU directive of 1986: 86/609/EEC).

Drugs used

Cyclophosphamide (Baxter Pharmaceuticals, Illinois, USA) was used to induce immunosuppression. Levamisole (Letap Pharmaceuticals Limited, Accra, Ghana) was the reference immunostimulatory drug. Ciprofloxacin (Medreich plc, Kew Gardens, England) and fluconazole (Pfizer, New York, USA) were the reference antibacterial and antifungal respectively.

Immunostimulatory activity

Two hundred ICR mice were put into ten groups labeled A-J, (n=20). The initial total WBC as well as lymphocyte and neutrophil counts of 5 mice of each group were determine using the KX-21 N Automated Hematology Analyzer (Sysmex Corporation, Chuo-ku, Kobe, Japan) after taking blood from the jugular vein into MediPlus K3 EDTA tubes (Sunphoria Co. Ltd., Taiwan). Animals were then treated with 4 mg/kg Cyclophosphamide for four consecutive days. The hematological parameters were again determined for 5 animals from each group. The various groups were treated as shown in Table 1. All the animals were treated daily for 21 days after which blood samples from 5 mice each from each group were again taken for hematological analysis. Data on the total WBC as well as the lymphocyte and neutrophil counts were recorded and analyzed for significant changes.

Table 1: Dosing of Herbal Decoctions and Levamisole to Experimental Animals

Drug/Decoctions	Groups	Dose (mg/kg)
Levamisole	A	2.5
Betula alba (BA)	B	1.0
	C	2.0
	D	4.0
Sutherlandia Frutescens (SF)	E	0.4
	F	0.8
	G	1.6
Combinations	H	2.0(BA) + 0.8(SF)
	I	2.0(BA) + 0.8(SF) + 2.5(Levamisole)
Normal Saline	J	2.0 ml/kg

Antimicrobial activity

The antimicrobial activity of BA and SF were assessed against *Staphylococcus aureus* (ATCC 25923), *Bacilus subtilis* (NCTC 10073), *Streptococcus pneumoniae* (clinical isolate), *Klebsiella pneumoniae* (clinical isolate), *Pseudomonas aeruginosa* (ATCC 4853), *Escherichia coli* (NCTC 25922), *Salmonella typhi* (NCTC 8385), *Enterococcus faecalis* (NCTC 775) and *Candida albicans* (NCPF 3179). These test organisms were from the stock kept at the Microbiology laboratory of the Department of Pharmaceutics, KNUST, Kumasi, Ghana. The bacterial and fungal growth-inhibition of the herbal products were determined using micro dilution technique (Gbedema *et al.,* 2010)

Two-fold serial dilutions of the herbal decoctions were made in sterile 96 well flat-bottomed micro-titre plates: Mueller-Hinton (MH) broth (100 µl) was placed in each well of the 96-microtiter plate except the first column. 100 µl quantities of the decoctions were placed in columns A and B. The two-fold serial dilution was made from column B to H in each row. A 100 µl MH broth inoculated with 24 h culture of the test microorganisms (2×10^4 cfu/ml) was then added to each well (to make a final volume of 200 ul) and mixed thoroughly. A row for control (MH broth

+ herbal products) and another for blank (MH broth only) were included. Plates were incubated for 24 h at 37°C. Bacterial growth was determined after addition of 50 µl p-iodonitrotetrezolium violet (0.2 mg/ml, Sigma) (Annan *et al.,* 2009): wells with microbial growth appeared bluish-black while those with no growth were yellow in colour.

Data analysis

Data presented and plotted were mean ± SD. The significance in the difference in viral load before treatment and after treatment with the preparation was established using the unpaired t-test (two-tailed). Significant differences between measured hematological parameters compared to the initial values were determined using One-Way Analysis of Variance followed by Dunnett's Multiple Comparisons Test *post hoc*. $P \leq 0.05$ was considered statistically significant.

Results
Survey

The 16 patients (7 males and 9 females) studied were aged between 21 and 50 years; 21-30 years (2 patients), 31-40 years (9 patients) and 41-50 years (5 patients). Viral load decreased significantly per individual. In total, viral load decrease from 47.42 ± 17.28 % to 13.69 ±12.42 % (Figure 1)
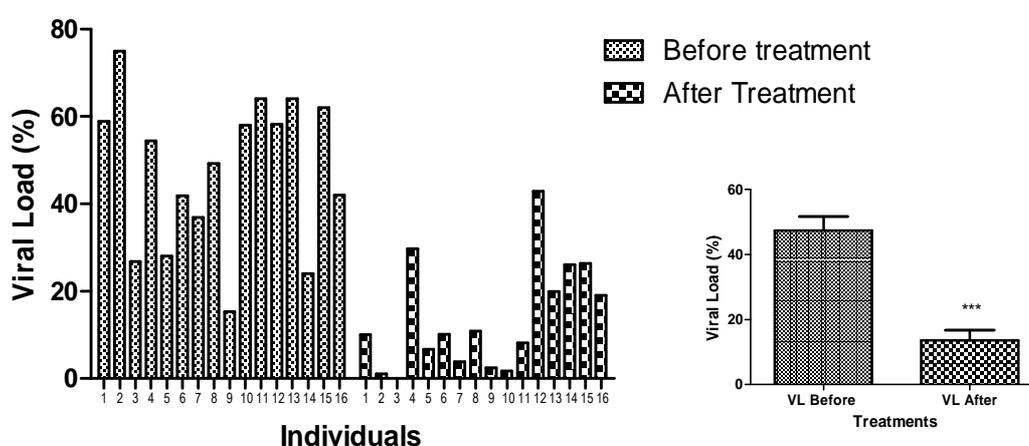

Figure 1: Viral load presented by 16 HIV/AIDS patients and that after treatment with two herbal preparations at Habibi Herbal Clinic, Kumasi, Ghana. The significance in the difference in total viral load before treatment and after treatment was established using the unpaired t-test (two-tailed). *** implies $P \leq 0.0001$.

Phytochemical screening

Both BA and SF showed the presence of saponins, triterpene nucleus, reducing sugars and coumarins. However, SF had additional phytochemicals namely; sterols and tannins (Table 2).

Table 2: Phytochemicals present in decoctions of *Betula alba* and *Sutherlandia frutescens*

Secondary Metabolites	BA	SF
Saponins	+	+
Triterpene nucleus	+	+
Sterols	-	+
Anthraquinones	-	-
Flavonoids	-	
Reducing Sugars	+	+
Alkaloids	-	-
Tannins	-	+ (condensed)
Coumarins	+	+
Purine bases	-	-

+/- indicates presence or absent

Immunostimulatory effects

There was very significant reduction in initial WBC (5.8 ± 0.62 ×10³/ to µl 3.7 ± 0.65 × 10³/; $P \leq 0$ µl.001; n=50) and lymphocyte count (4.39 ± 0.21 ×10³/ to µl 2.8 ± 0.36, $P \leq 0.01$; n=50) when ICR mice were treated with Cyclophosphamide (4 mg/kg, p.o). Change in neutrophil count was however not significant (1.09 ± 0.67 ×10³/ to µl 1.29 ± 0.8; $P > 0.05$; n=50). Treatments with BA (1-4 mg/kg; p.o), SF (0.4-1.6 mg/kg; p.o), BA + SF (2 mg/kg + 0.8 mg/kg; p.o), as well as BA + SF + Levamisole (2 mg/kg + 0.8 mg/kg + 2.5 mg/kg; p.o) caused significant increments ($P \leq 0.05$ –

0.001; n=5) in WBC and lymphocytes; similar to that caused by Levamisole (P ≤ 0.01; n=5). These treatments did not result in significant changes in neutrophil counts. Normal saline treatment did not result in significant increments (P > 0.05, n=5) in WBCs (Figures 2, 3, and 4).
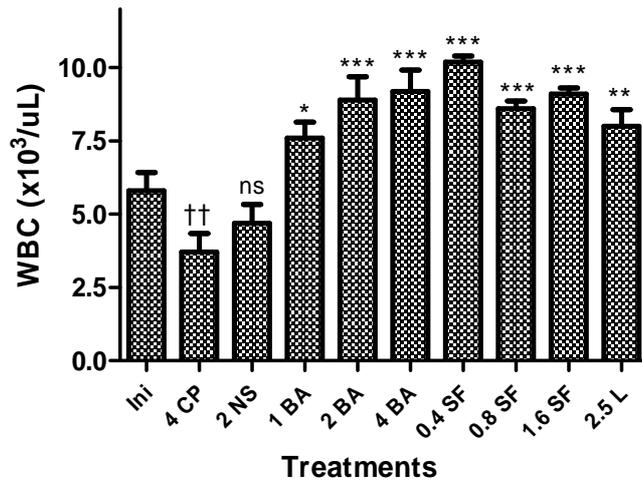

Figure 2: WBC count of ICR mice taken initially, after immunosuppression with 4 mg/kg cyclophosphamide (CP), and onward treatment with 1, 2, and 4 mg/kg BA, 0.4, 0.8, and 1.6 mg/kg SF, 2.5 mg/kg Levamisole (L), or 2ml/kg Normal saline (NS). Values plotted are means ± SD, n=3. Differences in mean values between treatments and the initial (Ini) were determined using One Way Analysis of Variance (ANOVA) followed by Dunnets multiple comparisons test. ns implies P > 0.05. Increments: * implies P ≤ 0.05, ** implies P ≤ 0.01; *** implies P ≤ 0.001. Decrement: †† implies P ≤ 0.01.
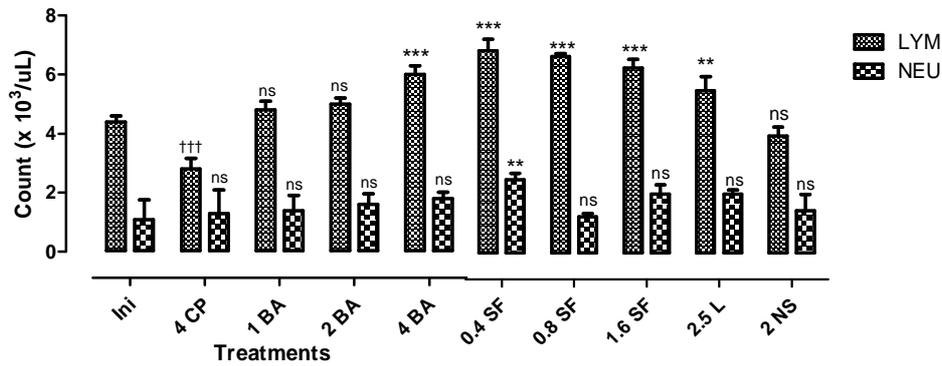

Figure 3 Lymphocyte and neutrophil count of ICR mice taken initially (Ini), after immunosuppression with 4 mg/kg cyclophosphamide (CP), and onward treatment with 1, 2, and 4 mg/kg BA, 0.4, 0.8, and 1.6 mg/kg SF, 2.5 mg/kg Levamisole (L) or 2 ml/kg Normal saline (NS). Values plotted are means ± SD, N=3. Differences in mean values between treatments and the initial were determined using One Way Analysis of Variance (ANOVA) followed by Dunnets multiple comparisons test. ns implies P > 0.05. Increments: ** implies P ≤ 0.01; *** implies P ≤ 0.001. Decrement: ††† implies P ≤ 0.001.
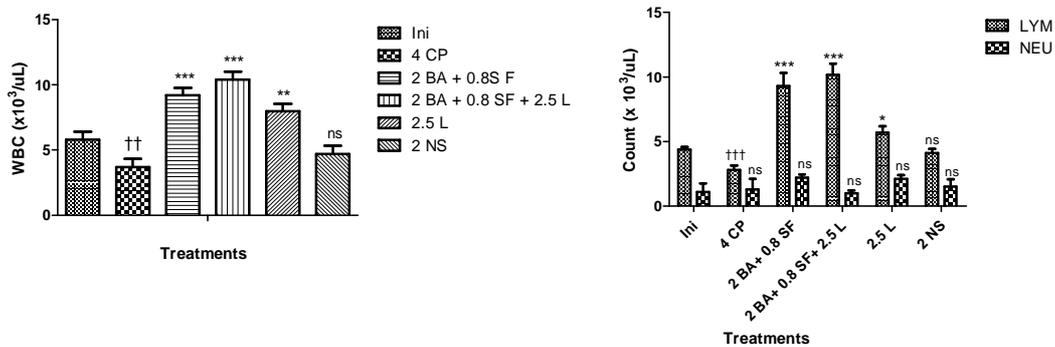

Figure 4: The WBC, Lymphocyte, and Neutrophil counts of ICR mice taken initially (Ini), after immunosuppression with 4 mg/kg cyclophosphamide (CP), and onward treatment with 2 mg/kg BA + 0.8 mg/kg SF, 2 mg/kg BA + 0.8 mg/kg SF + 2.5 mg/kg Levamisole, 2.5 mg/kg Levamisole (L) only, or 2 ml/kg Normal saline (NS). Values plotted are means ± SD, N=3. Differences in

mean values between treatments and the initial were determined using One Way Analysis of Variance (ANOVA) followed by Dunnets multiple comparisons test. ns implies P > 0.05. Increments: :* implies $P \leq 0.05$; ** implies $P \leq 0.01$; *** implies $P \leq 0.001$. Decrement: ††† implies $P \leq 0.01$.

Antimicrobial activity

BA and SF exhibited various degrees of antibacterial and antifungal activities. SF showed higher antimicrobial activity (with MIC ranging between 0.607 and 1.215 mg/ml) against the test organisms than BA (with MICs between 1.531 and 3.062 mg/ml) (Table 3).

Table 3: Antimicrobial activity of *B. alba* (BA) and *S. frutescens* (SF) decoctions

Organisms	Minimum Inhibitory Concentration		
	SF (mg/ml)	BA (mg/ml)	Ciprofloxacin (ug/ml)
Escherichia coli	0.607	1.531	0.016
Pseudomonas aeruginosa	1.215	3.062	0.032
Salmonella typhi	1.215	3.062	0.008
Klebsiella pneumonia	0.607	3.062	0.032
Enterococcus faecalis	1.215	1.531	0.016
Staphylococcus aureus	0.607	1.531	0.032
Streptococcus pneumoniae	0.607	3.062	0.032
Bacillus subtilis	1.215	3.062	0.008
Candida albicans	1.215	3.062	0.004[β]

[β] Fluconazole used

Discussion

Determination of viral load is a significantly important part of monitoring the therapy of HIV-infected and AIDS patients. Results obtained from the survey indicated that the viral load of the patients had decreased significantly after treatment with the herbal preparations at Habibi Herbal Clinic (Figure 1). Viral load is the number of HIV genetic materials (RNA) per millilitre of blood. It is a measure of the amount of HIV copies in a milliliter (copies/mL) of blood (Carter and Hughson, 2012). In HIV/AIDS patients, the viral load keeps on increasing as the immune system is compromised. Viral load measurements are used to determine the risk of disease progression, to monitor response to treatment, to detect viral breakthrough as a marker of regimen failure, and to decide when to initiate antiretroviral therapy (Vajpayee and Mohan, 2011). If the viral load measurement is high, it indicates that HIV is reproducing and that the disease will likely progress faster (Tlakula, 2011). A decrease in the viral load therefore reflects a boost of the body's immune system (Wodarz and Nowak, 1999). Viral load response is therefore used as a surrogate marker for efficacy or a strong indicator to monitor the success or failure of antiretrovirals in ART drug trials and in clinical practice (Panel on Antiretroviral Guidelines for Adults and Adolescents, 2013). The decreased viral load indicates therefore that HIV is not actively reproducing in the individuals and that the risk of disease progression is reducing which augurs well for ART at the clinic while using the herbal preparations from BA and SF.

In the determination of the immunostimulant property of the herbal preparations, mice were immunocompromised with cyclophosphamide which acts to decrease total WBC and lymphocyte count. Cyclophosphamide is bio-activated by cytochrome P450 mediated hydroxylation followed by equilibration of the 4-hydroxy metabolite with aldophosphamide and spontaneous decomposition of hydrophosphamide to yield acreolin as well as phospharmide mustard. This metabolite is the active component is responsible for its immunosuppressive action (Sladik *et al.,* 1987, LeBlanc and Waxman, 1990). It causes inhibition of proliferation of the rapidly dividing cells of the bone marrow (Clarke and Waxman, 1989). The bone marrow produces white blood cells as well as other cells and this causes a decrement in the white blood cell (B and T lymphocytes) and hence the immune system in general (Gurtoo *et al.,* 1976).

It was observed that the WBC count in general as well as the lymphocyte count was significantly increased on treatment with Levamisole. Chemically, Levamisole may form a thymopoietin-mimetic tertiary structure which stimulates lymphocyte by its imidazole component. Physiologically, thymopoietin affects many components of the immune system including lymphocytes, neutrophils and macrophages (Harris *et al.,* 1994). The therapeutically important actions of thymopoietin are probably targeting stimulation of phagocytosis and stimulation of regulatory T-cells to restore homeostasis in perturbed immune system. Throughout the experiment, Levamisole caused an increase in the WBC count of the immune compromised mice making it a standard immunostimulatory drug for comparing with the herbal preparations (Elmuataz *et al.,* 1995).

The herbal preparations (BA and SF) caused significant increases in WBC and lymphocyte counts (but not neutrophil count). This indicates that the preparations had immunostimulatory effects with SF being the most potent. The results obtained from a combination of BA and SF was less potent than the monotherapy of each of the decoctions. An increase in total WBC and lymphocyte count could be attributable to enhanced hematopoiesis, or could be working via similar mechanisms as Levamisole. The herbal preparations could have some components that could stimulate the synthesis and release of the hematopoietic growth factors to cause the proliferation and differentiation of hematopoietic stem cells of the bone marrow into WBCs.

In HIV patients with severe immunosuppression, opportunistic infections exert a heavy toll in terms of quality and length of life. Bacterial respiratory and enteric infections as well as candidiasis are among the most common opportunistic infections encountered by these patients. Herbs (e.g. *Melaleuca quinquenervia* and *Allium sativum*) have been reported useful in the management of even drug resistant bacterial and fungal pathogens in AIDS patients (Jandourek *et al.,* 1998). The two herbal decoctions (BA and SF) have exhibited broad spectrum of antimicrobial activity against bacteria (both gram positive and gram negative) and *Candida albicans* (Table 3). However, reports by Vinod *et al.,* (2012) showed that BA possessed a number of pharmacological activities like anti-inflammatory, anticancer, antibacterial antileishmanial and antiviral. The report also outlined several compounds such as betulin, betuligenol, polymeric anthocyanidins, kaempferol, xylenol and hyperoside, as the active principles isolated from BA. SF on the other hand has also been reported to possess antibacterial, anti-HIV and antioxidant properties (Katerere and Eloff, 2005; Harnett *et al.,* 2004). Apart from confirming the antibacterial property of the two plants, our study has shown that, *B. alba* and *S. frutescens* possess anti-candida activity. BA and SF decoctions therefore appear to play a role in controlling opportunistic infections in additions to strengthening the immune system as well as probably inhibiting the HIV retrovirus in patients treated at the Habibi Herbal Clinic.

The immunostimulatory and antimicrobial activity displayed by BA and SF could have been due to the collective presence of the saponins, triterpene, sterols, and tannins present in these herbs (Heroor *et al*., 2012). It has been established that saponins have adjuvant effects on humoral and

cell mediated immunity (Marciani, 1999). Saponin adjuvants from the bark of the *Quillaja saponaria* (Quillaja saponins) are chemically and immunologically well-characterized products (Dalsgaard, 1978; Higuchi *et al.,* 1987). Xiu-feng *et al.,* (2007) revealed that three diosgenyl saponins isolated from *Paris polyphylla* had immunostimulating activity. Saponins have been found to have activity against Gram positive bacteria e.g. *S. aureus* (Soetan et al., 2006; Avato, 2006; Khan, 2011).

It has been reported that some triterpene glycosides possess immunostimulatory property (Bedir *et al.,* 2000). Immunostimulatory effect of coumarin derivatives before and after infection of mice with the parasite *Schistosoma mansoni* showed that the compounds used have an immunomodulatory effect (which was stimulatory) at both humoral and cellular levels (Maghraby and Bahgat, 2004). The triterpenes have been found to be active against bacteria and fungi implicated in opportunistic and nosocomial infections (Mokoka *et al.,* 2013). Some pentacyclic triterpenes isolated from African Combretaceae have been shown to have antibacterial activity against *Mycobacterium fortuitum* and *Staphylococcus aureus* (Katerere *et al.,* 2003), while a new acylated triterpene exhibited interesting antimicrobial activity against *Candida albicans* (Fannang *et al.,* 2011). Significantly these compounds were found to exhibit potent activity against Gram negative bacteria and fairly good activity against Gram positive bacteria (Mallavadhani *et al.,* 2004).

Sterols have been reported to possess immunostimulatory activity (Burlando *et al.,* 2010). Sterols in *Euphorbia hirta* for example, have been found to have immunostimulating property in fish (Shih and Cherng, 2012). Sterols of *T. chebula*, *W. somnifera* and *E. hirta*, have also been found to possess activity against *P. aureginosa*, *B. subtilis*, *S. aureus*, and *E. coli* among others (Singh *et al.,* 2012).

Tannins are also known to possess immunostimulating activity (Heroor *et al.,* 2012). The well-known ayurvedic formulation, Triphala contains *Terminalia chebula, Terminalia belerica* and *Emblica officinalis*, which are rich in tannins have been reported to have immunostimulating activity (Srikumar, 2005). Several research reports have indicated that tannins and tannin rich fractions from various plants possess antimicrobial activity (Reddy *et al.,* 2007; Banso and Adeyemo, 2007; Min *et al.,* 2008; Maisak *et al.,* 2013).

Conclusion

Apart from their *in vitro* antimicrobial activity, the Herbal decoctions made from the fresh leaves of *Betula alba,* or *Sutherlandia frutescens* decreased human immunodeficiency viral load in patients and exhibited immunostimulatory activity in ICR mice. These two decoctions are useful in the management of HIV/AIDS and associated opportunistic infections.

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