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IN VITRO ANTIMICROBIAL ACTIVITY OF EXTRACTS OF SOME PLANT SPECIES USED IN THE MANAGEMENT OF HIV/AIDS IN TANZANIA

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ABSTRACT

Six plants species (Harungana madagascariensis, Sapium ellipticum, Teclea nobilis, Pseudospondias microcarpa, Rauvolfia vomitoria and Psorospermum febrifugum) which are used in managing HIV/AIDS in Bukoba, Tanzania were screened for their antimicrobial effect on the selected representatives of infectious bacterial and fungal species. Gram-positive bacteria Streptococcus lactis, Gram-negative bacteria Klebsiella pneumoniae and a fungus Candida albicans were tested for their sensitivity to the treatment with selected plants extracts using disc diffusion and broth dilution techniques. Extracts of S. ellipticum, P. febrifegum and P. microcarpa demonstrated growth inhibition of S. lactis with the MIC ranging from 3.2 to 100 mg/ml. The extracts of S. ellipticum, P. febrifegum and T. nobilis demonstrated growth inhibition of K. pneumonia with the MIC ranging from 6.3 to 25 mg/ml. On the other hand, extracts of H. madagascariensis, P. microcarpa and R. vomitoria inhibited growth of C. albicans with the MIC ranging from 12.5 to 50 mg/ml. These results are very promising towards discovery of new antimicrobial drugs especially against neglected S. lactis, K. pneumoniae and invasive Candida infections and could explain the routine use of the plants' infusions in traditional medicines in Bukoba against HIV/AIDS related illnesses.

Key words: Bukoba Tanzania, HIV/AIDS, opportunistic infections, plant extracts, traditional medicine

INTRODUCTION

HIV/AIDS is a devastating syndrome accompanied by a severe decline in immunity, which gives rise to emergence of multiple opportunistic infections. Opportunistic infections are those infections that emerge when people have a compromised immunity such as in the case of HIV/AIDS infections (Mastromarino et al. 2013, Africa et al. 2014).

The use of combination antiretroviral therapy (cART) in recent years has contributed greatly in the suppression of the virus and therefore increases in survival rates of patients (Greenberg et al. 2009, Buchacz et al. 2010, Tan et al. 2012, Iroezindu et al. 2013). cART targets the HIV life cycle leading to suppression of viral load and increase in CD4 cell count, helping the body in fighting opportunistic infections (Mocroft et al. 2013). Unfortunately, the use of cART does not eradicate the virus or the opportunistic infections from the infected host completely (Van Rie et al. 2011, Auld et al. 2013). For this reason, opportunistic infections such as *Streptococcus lactis*, *Klebsiella pneumoniae* and *Candida* species infection have remained a serious challenge in HIV/AIDS patients (Tan et al. 2012,

Mocroft et al. 2013). These microorganisms are usually found on different body parts of healthy persons, however in cases of immunocompromised individuals, the microorganisms can cause several serious conditions including liver abscess, empyema (pus in lungs), endocarditis and chronic diarrhea, pneumonia, urinary tract infections, wound infections and blood infections (Burkitt and Watson. 2009, Kim et al. 2010, Arnold et al. 2012, Tzouvelekis et al. 2012, Duin et al. 2013, Munoz-Price et al. 2013, Karaaslan et al. 2016)

There has been an increased drug resistance by many of the causative agents of opportunistic infections against the available standard antibiotics, giving rise to the use of alternative medicine to manage them. Several literature reports have indicated the use and importance of plant and natural products in managing opportunistic infections (Arekemase et al. 2011, Mathur et al. 2011, Dubey et al. 2011, Savithramma et al. 2011, Moshi et al. 2012, Sen and Barta 2012,Kengni et al. 2013, Al-Terehi et al. 2015,Tsobou et al. 2015). The great diversity in therapeutic properties make plant products to act as alternatives to synthetic antibiotics which over the years have faced resistance from super bugs (Jebashree et al. 2011).

The plant species *Harungana madagascariensis, Sapium ellipticum, Teclea nobilis, Pseudospondias microcarpa, Rauvolfia vomitoria* and *Psorospermum febrifugum* are among the most widely used herbal medicines used in managing HIV/AIDS in Bukoba, Tanzania (Kisangau et al. 2007). From the best of our knowledge, there has never before been any scientific verification of the effectiveness of the infusions from the named plants against the bacteria *S. lactis* and *K. pneumoniae*.

Therefore, in the current study, extracts were screened for their growth inhibition activity against *S. lactis, K. pneumoniae* and *C. albicans*. The findings of this study provide scientific verification of the therapeutic potential of the studied plants' infusions in the management of opportunistic infections linked with HIV/AIDS in traditional medicines in Bukoba.

MATERIALS AND METHODS

Collection of plant material

The plants samples which were collected and used in this study included *Harungana madagascariensis* (Stem bark), *Sapium ellipticum* (Stem bark), *Teclea nobilis* (Stem bark), *Pseudospondias microcarpa* (Stem bark), *Rauvolfia vomitoria* (Stem bark) and *Psorospermum febrifegum* (Leaves). Field collection and identification of the plants was done in April 2014. Voucher specimen (Table 1) were coded and kept in the herbarium of the Department of Botany at University of Dar es Salaam (UDSM).

Processing and extraction of plant material

The collected plant material was chopped into small pieces and then air dried for a period of up to twenty one days. The dried plant materials were ground into fine powder using NATIONAL SUPER® mixer grinder [MX-119] (Emerging planet India Ltd., Coimbatore 641011, India).

To prepare aqueous extracts; the powder (400g) was soaked in distilled water for 48hr at room temperature with stirring after every 12 hr. The mixture was then decanted and filtered using Buchner flask (Vacuubrand GMBH^{+}CO Germany) and filtrate was centrifuged at 1000rpm for 5 minutes to remove any remaining particulates. This filtrate was then freeze dried using ScanVavCoolsafe, LaboGene™ to obtain crude aqueous extract. The aqueous extracts were put in clean glass bottles and stored at 4^{o}C until the day of use.

To prepare ethyl acetate extracts; the obtained powder of dried plant materials

was soaked in ethyl acetate for a period of 48 hours. The mixture was then filtered using Buchner flask (Vacuubrand GMBH^{+}CO Germany) and filtrate was then concentrated on the rotary evaporator (R-210 BUCHI, Switzerland) at 45^{o}C, 100mbar pressure. The obtained ethyl acetate crude extracts were put in clean and labeled pre-weighed glass bottles and stored at 4^{o}C until the day of use.

Test microorganisms

Three test microorganisms used were Gram-positive bacteria *Streptococcus lactis (DSM 20481)*, Gram-negative bacteria *Klebsiella pneumonia (DSM 681)* and the yeast *Candida albicans (DSM 1665)*, all obtained from the microbial strains library of the Department of Molecular Biology and Biotechnology (MBB), UDSM

Preparation of the microbial cultures

Nutrient broth and Malt Extract broth were used to prepare media cultures of bacteria and yeast test microorganisms respectively. The Agar media were prepared according to the manufacturer's instructions. Approximately 10ml of autoclaved broth was aseptically transferred to sterile capped test tubes and then pure isolates of sub cultured bacteria and yeast colonies were aseptically transferred to the respective broth. By using the 0.5 McFarland standards, the bacteria and yeast number was adjusted to 1 x 10^{6}c.f.u/ml. The bacteria or yeast cultures in respective tubes mixed with broth media were incubated for an overnight at 37± 1 °C for bacteria and for 48 hours at 30°C for *C. albicans*.

Antibacterial and antifungal activity tests

Antibacterial activity of the crude extracts was determined by the Disc diffusion assay as described by Bauer et al. (1966).

Nutrient Agar and Malt Extract Agar were prepared for bacteria and fungi respectively according to the manufacturer's instructions. Immediately after autoclaving, the media was allowed to cool in a 45°C water bath. The freshly prepared and cooled media was mixed with suspension of microorganisms then poured into sterile polystyrene, flat-bottomed Petri dishes placed on a level, horizontal surface (FASTER® Laminar flow to give a uniform distribution. The agar media was allowed to cool and solidify at room temperature.

The Petri dishes were marked with spots placed almost equidistantly respective for each extract used in the test. Spots for positive and negative controls were also included in each petri dish.

The discs were soaked into the dissolved crude extracts for a minimum of one hour. Standard discs of Ampicillin/Sulbactam (HiMedia Laboratories Pvt. Limited, Mumbai, India) for Gram-positive bacteria, Gentamicin (HiMedia Laboratories Pvt. Limited, Mumbai, India) for Gram-negative bacteria and Clotrimazole (HiMedia Laboratories Pvt. Limited, Mumbai, India) for *C. albicans* were used as positive controls at 10µg/ml. Negative control discs were soaked in dimethyl sulfoxide (DMSO) (Sigma, St. Louis, MO, USA) and distilled water for ethyl acetate and aqueous extracts respectively.

Discs made of sterile 5 mm-Whatman No. 1 filter paper (WHATMAN®, Springfield Mill, Maidstone, Kent, England) were used in the disc diffusion method according to Faiza et al. (2011) and Lekshmi et al. (2012). By use of a sterile forceps, the discs of the plant extracts and controls were placed equidistantly onto each on the inoculated petri dishes. The petri plates treated with extracts and/ or controls were stored in a refrigerator (DAEWOO®, Daewoo Electronics, Europe GmbH, Germany) at 4°C for six hours and thereafter transferred to incubator for 24 hours at 37°

C for bacteria and for 48 hours at 30°C for *C. albicans*.

The test was carried out in duplicates for each plant extract and repeated three times. Antimicrobial activities were determined by measuring the diameters of zones of inhibition in mm. The means, Activity index and standard deviations (±SD) of the diameters of zones of growth inhibitions for treatments were recorded.

Activity index (AI) was calculated as = Inhibition zone of the test sample / Inhibition zone of a standard drug

The minimum inhibitory concentration (MIC) is the lowest concentration of antimicrobial agent that completely inhibits growth of the organism in tubes or microdilution wells as detected by the un aided eye (Balouiri et al. 2016). The MIC was determined using standard broth dilution methods (Balouiri et al. 2016). Two-fold dilutions (0.05, 0.1, 0.2, 0.4, 0.8, 1.6, 3.12, 6.2, 12.5, 25.0, 50.0, 100.0 mg/ml) of each of the plant extracts were prepared in a liquid growth medium and 100 µl dispensed in wells of a 96-well microtitration plate. Then, each well was inoculated with a 100 µl microbial inoculum prepared in the same medium after dilution of standardized microbial suspension adjusted to 0.5 McFarland scale. After well-mixing, the inoculated 96-well microtitration plate were incubated without agitation at 37^{o}C for 24 hours. The extract blank (200 µl) was also included for each concentration. The MIC endpoint was determined by using SPECTROstar Nano® plate reader from BMG LABTECH to facilitate reading microdilution tests and recording results with high ability to discern growth in the wells.

Data analysis

Kruskal-Wallis analysis followed by Dunn's pair-wise comparison test using GraphPad Prism software Version 6.0 were applied to determine the differences in sensitivities of microbes towards aqueous or ethyl acetate extracts treatments. A student *t* test was used to compare means of zones of inhibition for treatment by aqueous and ethyl acetate extracts.

RESULTS

Six aqueous and five ethyl acetate plant crude extracts were tested for their growth inhibition activities on *K. pneumoniae*, *S. lactis* and *C. albicans* by using disc diffusion method. Positive controls were standard discs of Ampicillin/Sulbactam for *S. lactis* (Gram positive bacterium), Gentamicin for *K. pneumonia* (Gram negative bacterium) at a concentration of 10 µg/ml and Clotrimazole for *C. albicans.* Negative control was DMSO for ethyl acetate extracts and distilled water for aqueous extracts.

The results showed that indeed the six Tanzanian indigenous medicinal plant species tested under the current study have therapeutical potentials against different opportunistic infections in HIV/AIDS patients. Determination of the AI values (Table 1) and MIC values (Table 2) helped to form the basis of distinguishing active from non-active extracts.

Streptococcus lactis: S. lactis was the most sensitive microorganism tested in this study. *S. lactis* growth was strongly inhibited by crude extracts from four different plant species, namely, ethyl acetate and water extracts of *P. febrifugum, S. ellipticum, P. microcarpa* and aqueous extract of *T. nobilis* with MICs ranging from 3.1 mg/ml to 100 mg/ml (Table 2) and zones of inhibition as shown in Figures 1 and 2

Klebsiella pneumonia: *K. pneumonia* growth was strongly inhibited by extracts from three plant, namely, ethyl acetate and aqueous extracts of *S. ellipticum*, ethyl acetate extract

of *P. febrifugum* and aqueous extract of *T. nobilis*, with MICs ranging from 6.2 mg/ml to 25 mg/ml (Table 2) and zones of inhibition as shown in Figures 1 and 2

It is worthy noting that the activity of *T. nobilis* against both *S. lactis* and *K. pneumonia* was only concentrated in the ethyl acetate extracts.

Candida albicans: Extracts from three plant species showed activity against *C. albicans*, these were: the ethyl acetate extracts of *P. microcarpa* and *R. vomitoria*, and the aqueous extract of *H. madagascariensis* with MICs ranging from 12.5 mg/ml to 50 mg/ml (Table 2) and zones of inhibition as shown in Figures 1 and 2

Note that extracts from *R. vomitoria* and *H. madagascariensis* showed activity only against the fungi *C. albicans*.

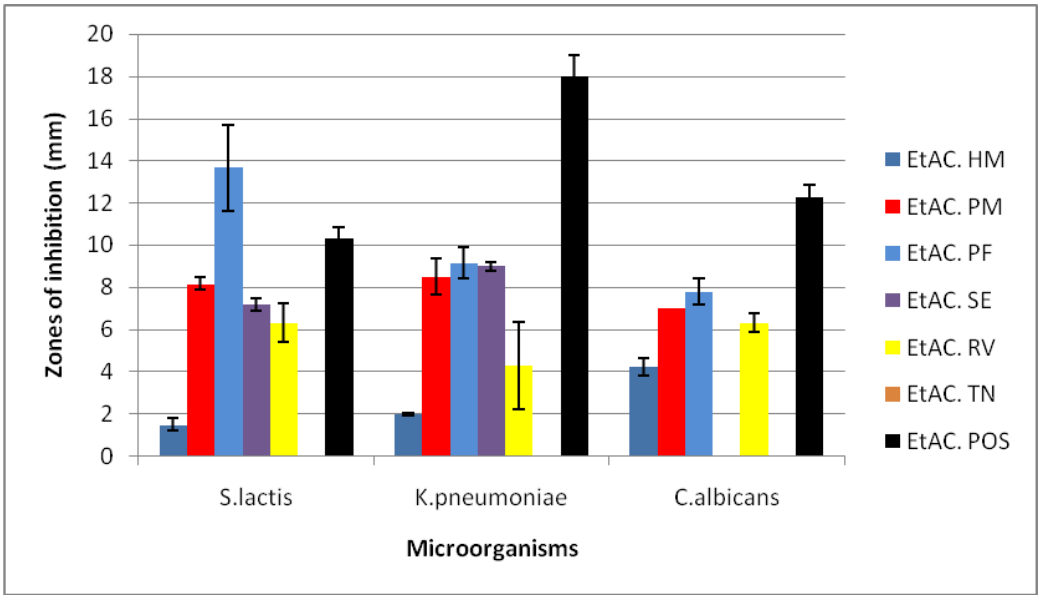

Figure 1: Growth inhibition of microbes by ethyl acetate extracts. Number of repeats n=3 and error bars represent standard deviation. EtAc – ethyl acetate; *HM- Harungana madagascariensis, SE- Sapium ellipticum, TN- Teclea nobilis, PM- Pseudospondias microcarpa, RV- Rauvolfia vomitoria,* and *PF- Psorospermum febrifugum,* Pos- Positive control were standard discs of Ampicillin/Sulbactam for gram positive bacteria, Gentamicin for gram negative bacteria at a concentration of 10 µg/ml and Clotrimazole for *C. albicans.*

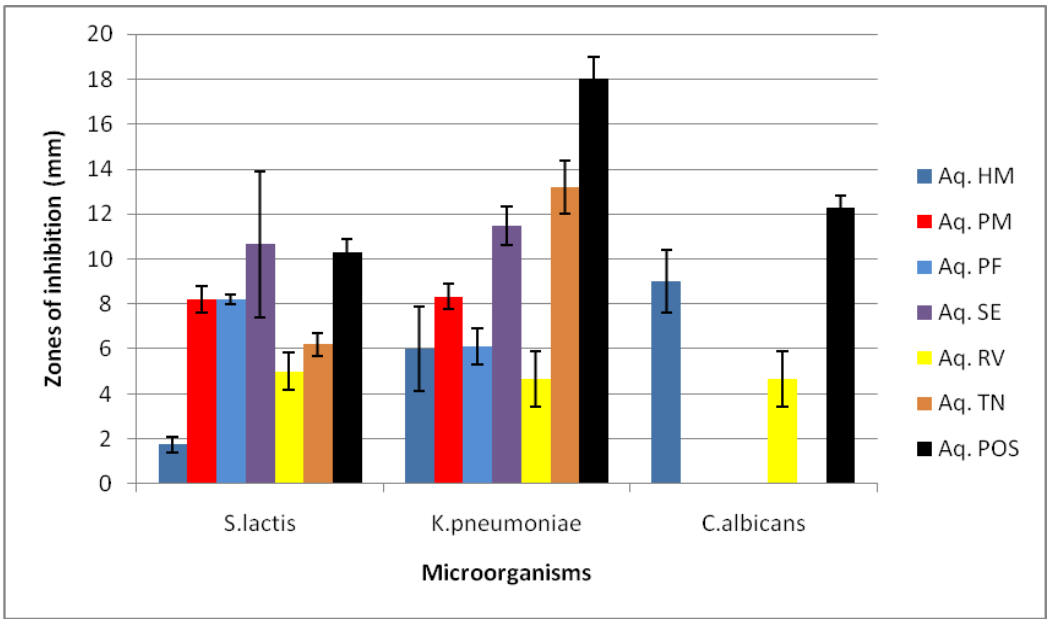

Figure 2: Growth inhibitions of microbes by aqueous extracts. Number of repeats n=3 and error bars represent standard deviation. *Aq – aqueous extracts; HM- Harungana madagascariensis, SE- Sapium ellipticum, TN- Teclea nobilis, PM- Pseudospondias microcarpa, RV- Rauvolfia vomitoria,* and *PF- Psorospermum febrifugum,* Pos- Positive control were standard discs of Ampicillin/Sulbactam for gram positive bacteria, Gentamicin for gram negative bacteria at a concentration of 10 µg/ml and Clotrimazole for *C. albicans*.

Table 1: Activity index (AI) of crude extracts in the disc diffusion method.

Plant species	**Voucher number**	**Extract**	***Streptococcus lactis***	***Klebsiella pneumoniae***	***Candida albicans***
Harungana madagascariensis	010 CMHS	Aq	0.125	0.316	0.73
		Et	0.11	0.105	0.34
Pseudospondias microcarpa	012 CMHS	Aq	0.80	0.46	0.00
		Et	0.81	0.47	0.57
Psorospermum febrifugum	005 CMHS	Aq	0.82	0.34	0.0
		Et	1.36	0.51	0.47
Sapium ellipticum	006 CMHS	Aq	1.06	0.62	0.00
		Et	0.72	0.5	0.0
Rauvolfia vomitoria	003 CMHS	Aq	0.33	0.29	0.467
		Et	0.413	0.27	0.633
Teclea nobilis	002 CMHS	Aq	0.62	0.73	0.0
		Et	-	-	-

Although the aqueous extracts showed relatively higher activity than ethyl acetate extracts, the difference was statistically non-significant. Comparison between means of the activity of the aqueous and that of ethyl acetate extract treatments gave no significant difference between the two treatments (*t* test *p*=0.6235). Also, the difference in sensitivity of different sensitive microbes treated with aqueous extracts was found to be non-significant (Kruskal Wallis *p* value =0.082). Similarly, the difference on sensitivity of different sensitive microbes treated with ethyl acetate extracts was also not statistically significant (Kruskal Wallis *p* value =0.46).

The concentration of extracts used was 200mg/ml. Aq = aqueous, Et = Ethyl acetate. The higher the AI, the better the activity. If AI = 1, it means the activity of the extract is as good as the standard drug.

Table 2: Minimum inhibitory concentrations (MICs) of aqueous and ethyl acetate extracts

Extracts		**MIC (mg/ml)**		
		C. albicans	***K. pneumoniae***	***S. lactis***
Harungana madagascariensis	Aq	12.5	>100	>100
	EtAc	>100	>100	>100
Pseudospondias microcarpa	Aq	>100	>100	50
	EtAc	50	>100	25
Psorospermum febrifugum	Aq	>100	>100	100
	EtAc	>100	12.5	3.1
Sapium ellipticum	Aq	>100	6.2	6.2
	EtAc	>100	12.5	12.5
Rauvolfia vomitoria	Aq	>100	>100	>100
	EtAc	25	>100	>100
Teclea nobilis	Aq	>100	100	25
	EtAc	>100	>100	>100

DISCUSSION

The results showed presence of antimicrobial activity in crude extracts from six indigenous medicinal plants against some microorganisms responsible in causing sickness in HIV/AIDS patients. Determination of AI values and MIC values helped to form the basis of distinguishing active from non-active extracts. These therapeutical potentials of the six plants against different opportunistic infections in HIV/AIDS patients could explain the wide use of the plants in traditional medicine for HIV/AIDS patients in Bukoba (Kisangau et al. 2007).

S. lactis was the most sensitive microorganism tested in this study. This is the first ever study on the effect of the medicinal plants *P. microcarpa*, *P. febrifegum* and *S. ellipticum* on the probiotic food grade microorganism *S. lactis*. The extracts of these plants inhibited growth of the well-known serious opportunistic bacteria that cause life-threatening infections, which is also an important probiotic microorganism that has been shown to fight drug resistant *Staphylococcus aureus* (Wang et al. 2017). However, when such a complex combination of *S. aureus* and *S. lactis* infections happen on a victim, the later is known to be very resistant to drugs (Elliot and Facklam 1996, Mofredj et

al. 2006, Mofredj et al. 2007), and standard treatment for infections caused by this microorganism (*S. lactis)* has not been well established (Karaaslan et al. 2016).

A positive control in the present study against *S. lactis* (Ampicillin/Sulbactam) was not very active against *S. lactis* possibly because this microorganism is known to have natural resistance to some drug classes (Elliot and Facklam 1996, Mofredj et al. 2007). The inhibition of *S. lactis* by these plant extracts in the current study is a break through and again emphasizes that plants might emerge the important sources of drugs against such notorious bacterium and also as a means to confer relief to HIV/AIDS patients.

K. pneumonia growth was strongly inhibited by extracts from three plant, namely, ethyl acetate and aqueous extracts of *S. ellipticum*, ethyl acetate extract of *P. febrifugum* and aqueous extract of *T. nobilis*. The bacterial species had not been tested against the plants species before and so there is nothing to compare with the observation. The only two previous antimicrobial studies on the same plant species found the plant extracts to be active against bacterial species other than *K. pneumoniae* and *S. lactis* (Kisangau et al. 2007, Onyancha et al. 2012). The two studies strongly suggest that the plants contain broad spectrum bioactive compounds, whose mechanisms of action may target different biochemical pathways of Gram-positive and Gram-negative pathogens (Rao et al. 2014).

A gram negative *K. pneumoniae* which is a well-known resistant to many antibiotics on the market (Shaik 2014) has in this study portrayed sensitivity to some of the tested extracts with *T. nobilis* extracts being the most active against the bacterium with the AI of 0.73. Several studies have demonstrated the sensitivity of this bacterial species toward plants extracts (Cock and van Vuuren 2014, Araujo-junior et al. 2016, Winnett et al. 2017). The broad sensitivity of *K. pneumoniae* towards herbal medicines means that the possibility of discovering new effective drug to combat infections caused by this bacterium in plants is high.

Extracts from only three plant species showed activity against *C. albicans*, these were: the ethyl acetate extracts of *P. microcarpa* and *R. vomitoria*, and the aqueous extract of *H. madagascariensis*. The results are similar to the previous study by Kisangau et al. (2007) who also reported moderate sensitivity of *C. albicans* against these plant extracts. Though moderate, the activities of the tested plant extracts against *C. Albicans* observed in both studies suggest the presence of anti-fungal compounds in these plants.

Although the aqueous extracts showed relatively higher activity than ethyl acetate extracts, the difference was statistically non-significant and the result is similar to earlier studies (Kisangau et al. 2007, Sana and Ifra 2012, Aneja et al. 2012). On the contrary, the ethyl acetate extracts showed non significantly more anti- *Candida* activity than the aqueous extracts; this may suggest that, the active anti- *Candida* active compounds in the extracts have lower polarity compared to ones present in aqueous extract.

There is no literature available to explain the strong activity of the extracts of *S. ellipticum* and *P. febrifegum*. The finding may be interpreted that the two plant species contain strong broad-spectrum antimicrobial compounds. The aqueous extract of *S. ellipticum* and the ethyl acetate extract of *P. febrifegum* were slightly more active than the standard drugs used for *S. lactis*. And both the aqueous and ethyl acetate extracts of these two plants showed antibacterial activities, which could indicate that the

active components for the activity could be of both polar and non-polar nature.

Note that extracts from *R. vomitoria* and *H. madagascariensis* showed activity only against the fungus *C. albicans* but no activity against the tested bacteria. It is worthy noting that the activity of *T. nobilis* against both *S. lactis* and *K. pneumonia* was only concentrated in the ethyl acetate extracts. In the absence of literature on any possible previous studies, explanation for such observations needs to be supported by further studies especially on the isolation of the active compounds from these plants.

CONCLUSION

The selected plants species in this study are used in the management of HIV/AIDS by the people of rural Bukoba, Tanzania, and in this study these plants have shown a promising therapeutic potential against microbial growth *in vitro*. Inhibition of microbial growth could be one of the many undocumented mechanism of therapeutic action of these plants in the management of HIV/AIDS. This is the first study to examine the effects of these Tanzanian medicinal plants on probiotic, often-neglected opportunistic microorganism such as *S. lactis*. More studies on neglected microbes should be encouraged. The choice of water is still the best in preparation of herbal extracts for human consumption mainly due to safety issues; in addition, this study has shown that aqueous extracts exhibited more activity than organic extracts.

The findings provide a baseline information for further studies, especially *in vivo* studies in animal models, characterisation of the compounds responsible for and the mechanisms of the observed activites.

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