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Research Article
Evaluation of Lemon and Black Seed Oils on Bacteria Isolated from HIV Positive Patients

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Abstract

Background and Objective: Opportunistic bacterial infections are one of the predominant cause of morbidity and mortality amongst HIV infected patients. In this study, the potency of Black seed and Lemon oil against bacteria isolated from HIV seropositive samples of patients attending a selected specialist hospital in Akure, Nigeria and their antibiotics sensitivity profile were investigated. **Materials and Methods:** A total of 12 blood and urine samples were analyzed following their $CD4^+$ count determination. The bacteria isolates were identified using standard microbiological techniques. The antibiotics profile was carried out using Kirby-Bauer's disc diffusion method while the antibacterial activity of the essential oils were done using agar well diffusion method. **Results:** About 66% had a $CD4^+$ count between 200-600 cells μL^{-1} while 34% had $CD4^+$ count in the normal range >600 cells μL^{-1}. Out of the 7 isolated bacterial species, *B. cereus* and *P. aeruginosa* had the highest percentage of occurrence in the blood and urine samples, respectively Pefloxacin and Ciprofloxacin showed a broad spectrum of activity against all the test isolates. Both Lemon and Black seed oils were effective at 10% concentration on all the isolates and compared favorably with the commercial antibiotics. Black seed oil was observed to possess higher antibacterial activity against the isolates compared to lemon oil, with higher zones of inhibition values of above 13.00 ± 0.40^d mm, which was the highest observed on *B. cereus* with lemon oil, while the highest potent activity of black seed oil was observed on *C. xerosis* (18.67 ± 0.88^e mm). **Conclusion:** The essential oils showed better antibacterial activity against the co-infected bacterial pathogens and can serve as potential candidate for therapeutic purposes.

Key words: HIV, $CD4^+$ count, blood, urine, antibiotics, essential oils

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Human Immunodeficiency Virus (HIV) is the causative agent of Acquired Immune Deficiency Syndrome (AIDS) which was first identified in 1983 and are known to target special types of T cells often referred to as $CD4^+$ cells[1]. The HIV type 2 strain is predominant in West Africa, currently without cure although anti-retroviral drugs are available to slow down the multiplication of the virus and as such slow down the loss of $CD4^+$ T cells that the virus destroys. Bacterial opportunistic infections do lead to predominant morbidity and mortality amongst HIV seropositive patients, whose risk factors are mostly attributed to a decline in the $CD4^+$ count below a critical level[2]. Antibiotic resistance has been observed to spread between humans, animals and the environment and excessive or inappropriate usage of these antibiotics have led to a rise in their resistivity[3,4]. Immuno-compromised individuals particularly those living with HIV are at high risk of Urinary Tract Infections (UTI)[5]. The prevalence of asymptomatic bacteriuria in HIV-positive individuals varies depending on the population studied[6-8]. The most common causes of bacteremia in Western countries are *Escherichia coli*, *S. aureus*, some *Bacillus* sp. and *Streptococcus pneumoniae*[9]. However, their distribution is closely related to place of acquisition and focus of infection and linked with healthcare-associated and nosocomial infection[10].

Essential oils are complex natural mixtures containing compounds such as terpenoids, phenylpropanoids, aromatic and aliphatic constituents; which are responsible for its biological properties[11]. Lemon oil is extracted from *Citrus limonum* of the Rutaceae family, possessing many bioactive components such as citric acid, Ascorbic acid, minerals, flavonoids and essential oils[12]. The antimicrobial and antioxidant potentials of lemon leaf essential oil have been previously reported[13]. Lemon essential oil is composed of many natural compounds, including terpenes, sesquiterpenes, aldehydes, alcohols, esters and sterols. Lemons and lemon oil are popular because of their refreshing scent and invigorating, purifying and cleaning properties[14]. Research showed that lemon oil contains powerful antioxidants and helps to reduce inflammation, fight bacteria and fungi, boost energy levels and ease digestion[15]. Black seed (*Nigella sativa*) oil belonging to the family Ranunculaceae, has been used extensively in folk medicine for therapeutic purposes[16]. The ripe fruit contains numerous tiny seeds, dark black in color, with the seed and oil frequently used in ancient remedies. *In vivo* and *in vitro* studies revealed many pharmacological actions for *N. sativa*[17]. The identification and characterization of opportunistic bacterial isolates associated with HIV positive samples have been carried out including their antibiogram[18,19]. However, the antibacterial potency of lemon and black seed oil against these opportunistic bacterial pathogens is unknown. Therefore, it is necessary to provide information about these natural compounds as alternative to the use of antibiotics against co-infecting bacteria associated with HIV infection.

MATERIALS AND METHODS

Sample collection: The study was conducted among HIV infected individuals attending a Specialist Hospital in Akure, Nigeria, where a total of 12 blood samples were collected from HIV patients on retroviral drugs who also seek medical attention from this selected hospital. About 5 mL of blood samples were collected from the HIV patients by venous puncture into sterile EDTA bottles and transported to the laboratory immediately for bacteriological analyses.

$CD4^+$ cell count determination: About 20 µL of the whole blood (EDTA anti-coagulated) was mixed with 20 µL of fluorochrome-labeled antibodies, mixed gently and incubated for 15 min in the dark at room temperature. Afterwards, about 800 µL of lyse buffer was added to the mixture, swirl gently for even distribution and ran on the flow cytometry machine. The $CD4^+$ was displayed and recorded.

Bacteriological analyses: Culture media including Nutrient Agar (NA), Cystine Lactose Electrolyte Deficient agar (CLED), MacConkey agar, Blood agar and Chocolate agar were prepared according to manufacturer's specification and sterilized. The samples were each pour plated on the culture medium and the isolated bacteria were identified and characterized using standard protocols[20].

Antibacterial sensitivity test: The isolates were tested for their sensitivity to commercial antibiotics disc using the Kirby Bauer disc diffusion method[21] on Mueller Hinton agar plate. The commercial antibiotics disc includes Augumentin (25 µg), Gentamycin (10 µg), Tarivid (30 µg), Streptomycin (30 µg), septrin (30 µg), Chloramphenicol (30 µg), Sparfloxacin (10 µg), Ciprofloxacin (10 µg), Amoxicillin (30µg), Pefloxacin (10 µg) for Gram-negative isolates and Erythromycin (10 µg), Septrin (30 µg), Ciprofloxacin (10 µg), Streptomycin (30 µg), Zinacef (20 µg), Amoxicillin (30 µg), Pefloxacin (10 µg), Gentamycin (10 µg), Ampiclox (30 µg), Rocephin (25 µg) for Gram-positive isolates. Zones of inhibition were determined and interpreted[22].

Antibacterial activity of essential oils: The bacterial cultures were tested for the inhibitory effect of essential oils of Black seed and Lemon for different concentration (10, 30, 50, 75 and 100 %) using 10% tween-80 as a miscible diluent by using agar well diffusion method[23]. The zones of inhibition (mm) were measured with graduated scale after the period of incubation and the result compared with ciprofloxacin which served as a positive control. The MIC and MBC of the essential oils were determined using broth dilution method[24]. The lowest concentration at which no visible growth occurs in each culture tubes was taken as MIC, while the lowest concentration where no growth occurred on the agar surface is considered as the MBC.

Ethical approval: Ethical approval was obtained from the State Health Research Ethics Committee (SHREC) of Ondo State, Nigeria. Patients consent were sought before the collection of samples.

RESULTS

Percentage occurrence, distribution and their relative $CD4^+$ count: A total of 12 samples were obtained from 2 males and 10 females with a percentage occurrence of 16.67 and 83.33%, respectively. About 33.3% of the HIV seropositive patients each had a $CD4^+$ T-cell counts of 200-400 and 400-600 cells μL^{-1}, while the remaining 34% had a $CD4^+$ T-cell counts in a normal range, greater than 600 cells μL^{-1} as shown in Table 1.

Percentage occurrence of bacterial isolates from samples of HIV positive individuals: Seven bacteria isolated from the samples include: *Bacillus cereus*, *Staphylococcus aureus*, *Micrococcus luteus*, *Corynebacterium xerosis*, *Proteus mirabilis*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*. The *B. cereus* (18%) and *P. aeruginosa* (20%) had the highest percentage of occurrence in the blood and urine samples, respectively (Table 2).

Antibiogram of bacterial isolates: All the test isolates showed varying degrees of sensitivities and resistance against the antibiotics. Pefloxacin and Amoxicillin were more potent against the Gram-negative isolates while Ciprofloxacin as well as Pefloxacin were effective against the Gram-positive isolates. However, all the Gram-positive isolates were resistant to Zinacef and Amoxicillin while the Gram-negative isolates were sensitive to at least one of the antibiotics. Both Pefloxacin and Ciprofloxacin displayed a broad spectrum of activity against all the isolates as shown Table 3.

Table 1: Distribution and $CD4^+$ count of HIV positive individuals

$CD4^+$ (cells μL^{-1})	Males	Females	Total (%)
200-400	0	4	4(33.33)
400-600	2	2	4(33.33)
600-800	0	2	2(16.67)
800-1000	-	-	-
1000-1200	0	1	1(8.33)
1200-1400	0	1	1(8.33)
Total	2(16.67%)	10(83.33%)	12(100%)

Table 2: Percentage occurrence of bacterial isolates from the blood and urine of HIV seropositive individuals

Samples	Bacterial isolates	Occurrence (%)
Blood	*B. cereus*	18
	S. aureus	12
	C. xerosis	12
	P. mirabilis	8
Urine	*P. aeruginosa*	20
	K. pneumoniae	15
	S. aureus	8
	M. luteus	7
Total		100

Table 3: Antibiotics sensitivity profile of Gram-negative and Gram-positive bacterial isolates from HIV positive blood and urine samples

	Zones of Inhibition of isolates (mm)		
Antibiotics	*P. aeruginosa*	*K. pneumoniae*	*P. mirabilis*
Gram-negative bacterial isolates			
Pefloxacin	17.50±0.50^{c}	37.50±1.50^{c}	20.00±0.29^{ab}
Tarivid	19.25±0.75^{d}	0.00±0.00^{a}	17.00±0.58^{bc}
Streptomycin	11.50±0.50^{b}	0.00±0.00^{a}	15.00±0.29^{cd}
Septrin	0.00±0.00^{a}	0.00±0.00^{a}	11.00±0.58^{e}
Chloramphenicol	0.00±0.00^{a}	0.00±0.00^{a}	15.00±1.15^{cd}
Sparfloxacin	19.20±0.80^{d}	0.00±0.00^{a}	20.00±0.58^{ab}
Ciprofloxacin	11.50±0.50^{c}	10.25±0.25^{c}	14.00±1.15^{a}
Amoxicillin	21.50±0.50^{e}	27.50±2.50^{b}	17.00±1.15^{bc}
Augmentin	0.00±0.00^{a}	32.50±1.50^{bc}	0.00±0.00^{a}
Gentamycin	0.00±0.00^{a}	0.00±0.00^{a}	12.00±0.58^{de}

	Zones of inhibition of isolates (mm)			
Antibiotics	*S. aureus*	*M. luteus*	*B. cereus*	*C. xerosis*
Gram-positive bacterial isolates				
Ciprofloxacin	9.80±0.20^{b}	5.50±0.50^{a}	8.20±0.00^{a}	14.00±1.15^{b}
Streptomycin	20.00±1.00^{d}	13.50±0.50^{c}	15.00±1.15^{ab}	0.00±0.00^{b}
Septrin	11.50±0.50^{b}	0.00±0.00^{a}	15.00±0.87^{ab}	0.00±0.00^{b}
Erythromycin	13.75±0.25^{c}	0.00±0.00^{a}	19.67±2.40^{ab}	0.00±0.00^{b}
Pefloxacin	21.50±0.50^{e}	10.70±0.30^{b}	20.00±1.15^{a}	12.00±0.58^{a}
Gentamycin	0.00±0.00^{a}	10.50±0.50^{b}	11.00±0.58^{a}	12.00±1.15^{a}
Ampiclox	0.00±0.00^{a}	0.00±0.00^{a}	15.67±1.20^{ab}	0.00±0.00^{b}
Zinacef	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{c}	0.00±0.00^{b}
Amoxicillin	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{c}	0.00±0.00^{b}
Rocephin	14.65±0.35^{c}	0.00±0.00^{a}	10.67±1.33^{b}	0.00±0.00^{b}

Values are mean zone of inhibition (mm) ± Standard deviation of three replicate, $^{a-c}$Means in the same column not sharing a common letter are significantly different ($p \leq 0.05$) by Duncan's multiple range test

Potency of black seed and lemon oil against the bacterial isolates: The natural compounds compared favorable well as compared with the commercial antibiotics used as a positive

Table 4: Antibacterial activity of lemon oil and black seed oil on bacterial isolates from blood and urine of HIV seropositive samples

	Lemon oil concentration (%)					Black seed oil concentration (%)					Ciprofloxacin concentration (µg)
Isolates	10	30	50	75	100	10	30	50	75	100	10
B. cereus	13.00±0.40[d]	11.23±0.50[a]	10.23±0.10[a]	10.00±0.22[a]	0.00±0.00[a]	10.00±1.15[a]	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]	8.20±0.00[a]
P. mirabilis	10.10±0.13[c]	10.00±0.47[b]	8.00±0.405[a]	0.00±0.00[a]	0.00±0.00[a]	10.70±1.17[b]	10.11±0.30[b]	9.50±1.2.0[a]	0.00±0.00[a]	0.00±0.00[a]	14.00±1.15[d]
C. xerosis	11.89±0.32[a]	11.00±0.67[b]	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]	18.67±0.88[e]	17.10±0.35[e]	10.70±0.80[a]	6.43±0.20[d]	0.00±0.00[a]	12.30±1.15[b]
S. aureus	10.95±0.63[b]	8.65±1.30[d]	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]	15.62±0.70[e]	13.10±0.58[c]	11.33±0.88[d]	0.00±0.00[a]	0.00±0.00[a]	9.80±0.20[b]
P. aeruginosa	12.75±0.35[b]	11.90±0.00[b]	10.33±0.00[b]	6.70±0.10[e]	0.00±0.00[a]	10.00±0.23[c]	8.6±0.54[c]	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]	11.50±0.50[c]
M. luteus	10.80±0.56[a]	10.00±1.10[a]	9.00±0.40[b]	7.20±0.60[c]	0.00±0.00[a]	12.12±1.22[b]	10.45±0.22[a]	10.10±0.88[b]	0.00±0.00[a]	0.00±0.00[a]	5.50±0.50[a]
K. pneumoniae	12.95±0.63[b]	12.65±0.30[b]	10.00±0.30[b]	8.20±0.70[c]	0.00±0.00[a]	9.60±1.40[c]	8.23±0.34[d]	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]	10.25±0.25[c]

Vales are mean zone of inhibition (mm) ± Standard deviation of three replicate, [a-c]Means in the same column not sharing a common letter are significantly different ($p \leq 0.05$) by Duncan's multiple range test

Table 5: Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of lemon oil and black seed oil on the bacterial isolates

	Lemon oil		Black seed oil	
Isolates	MIC (%)	MBC (%)	MIC (%)	MBC (%)
B. cereus	10	100	100	10
S. aureus	100	7.5	10	50
K. pneumoniae	10	100	100	100
P. aeruginosa	10	100	100	100
M. luteus	10	75	10	10
C. xerosis	100	10	10	30
P. mirabilis	10	100	10	10

control (Ciprofloxacin) as shown in Table 4. Both oils were effective at 10% concentrations on all the bacterial isolates. Black seed oil was observed to possess higher antibacterial activity against the isolates as compared to lemon oil, with higher zones of inhibition values of above 13.00±0.40[d] mm, which was the highest observed on *B. cereus* with lemon oil. The *B. cereus*, *P. aeruginosa* and *M. luteus* were mostly sensitive to lemon oil up to 75% concentration while *S. aureus* and *C. xerosis* were least sensitive at 30% concentration. However, black seed oil showed potent activity against *C. xerosis* with the highest zone of inhibition value of 18.67±0.88[e] mm and least effective against *K. pneumoniae*. The MIC and MBC values of the oils ranged from 10-100% as shown in Table 5. The isolates were found to be inhibited by lemon oil at a lower concentration compared to the black seed oil.

DISCUSSION

In this study, a total of 12 HIV seropositive individual's blood and urine samples were analyzed microbiologically and 7 bacterial isolates were identified, including *Staphylococcus aureus*, *Bacillus cereus*, *Micrococcus luteus*, *Corynebacterium xerosis*, *Proteus mirabilis*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*.

The high vulnerability of HIV infected females compared to males observed in this study might be associated with social, legal and economic factors[19]. None of the patients in this study had a $CD4^+$ cell count of less than 200 cells μL^{-1}, moreover, the prevalence of bacteremia amongst HIV positive individuals have been linked with a $CD4^+$ T-cell counts below 200 cells μL^{-1} which can pose a greater risk of opportunistic bacterial infection[19]. This showed the relevance of anti-retroviral treatment of HIV patients with relatively low $CD4^+$ count.

The bacterial isolates exhibited significant levels of multidrug resistant. The HIV seropositive individuals are prone and at high risk of bacterial opportunistic infections[25]. Colonization with *S. aureus* has been reported to be a risk factor for clinical infections in HIV positive patients[26,27]. The *S. aureus* has been identified as the most common uropathogen[28]. The occurrence of *P. mirabilis* agrees with a study on bloodstream infections amongst HIV patients under HAART in Thailand, who linked the occurrence of this organism to complicated urinary tract infections[29]. *C. xerosis* has previously been associated with clinical samples linked with animals[30,31]. Thus, the immunocompromised status of the host as a result of HIV infection gives a lot of serious concern due to an increase in multidrug resistant pathogens. The sensitivity of the isolates to Ciprofloxacin and Pefloxacin implies the effectiveness of quilonones class of antibiotics to opportunistic bacterial pathogens. The inappropriate usage of broad spectrum antibiotics might account for the multidrug resistant pattern observed in the isolates to the commercial antibiotics[3]. Lemon oil possess antimicrobial compounds such as limonene, phenols, terpenes and aromatic compounds, which enhances their antibacterial property[32]. Phenolic compounds of essential oils have been shown to lead to cell membrane disruption leading to leakage of cellular constituent[33]. Black seed oils are also rich sources of antimicrobial compounds such as thymoquinone and hydrothymoquinone[34]. Gram-positive bacteria appears to be more susceptible to essential oils compared to Gram-negative bacteria due to the nature of their cell wall. Black seed oil is reported to have strong antibacterial activity against some selected Gram-positive and Gram-negative bacterial species[35].

Antibiotics have been the most frequently prescribed medications in modern medicine and has led to various immunocompromised conditions of the host immunity. These essential oils have been shown to possess therapeutic values however, further research is needed to study their efficacy *in vivo*.

CONCLUSION

Quinolones such as ciprofloxacin and Pefloxacin showed better sensitivity and broad spectrum pattern to the bacterial isolates from HIV seropositive samples, however essential oils such as lemon and black seed oil compared favourably well with these antibiotics. Moreover, Black seed oil had better antibacterial activity compared to lemon oil. Essential oils can serve as potential candidates against opportunistic bacterial pathogens associated with immunocompromised conditions such as HIV.

SIGNIFICANCE STATEMENT

This study has provided information on some pathogenic bacteria that are associated with urine and blood samples of HIV seropositive patients. The antibiotics sensitivity profile also revealed the multi-drug resistant pattern of these isolated bacteria and the antibacterial efficacy of black seed and lemon oil has also been demonstrated. This will give a base line information on a possible alternative therapy towards eradicating opportunistic bacterial infections commonly associated with this viral disease.

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