In-vitro Antimicrobial Properties of Methanol extracts of three Medicinal Plants from Kilifi District - Kenya

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SUMMARY

Multidrug resistant microbes are a health management challenge in immunocopromised individuals. The study aimed to evaluate antimicrobial potential and toxicity of the methanol extracts of *Hosludia opposita, Rhus natalensis* and *Combretum illairii*. The plants were collected from Kilifi District and authenticated at East African Herbarium. Samples collected were extracted in methanol. Quantitative bioassay was done using disc diffusion method; minimum inhibition concentration was done using broth dilution methods. The isolates used for bioactivity testing were *Staphylococcus aureus, Pseudomonas aeruginosa*, *Escherichia coli, Candida albicans* and *Trichophyton mentarophyte*. Phytochemical screening was done using thin layer chromatograpy and cell toxicity was done using human embryonic lung cells. The *H. opposita* and *C. illairii* had terpenoids, flavonoids and anthaquinones. All the extracts were safe to the mammalian cells. *Combretum illairii* plant extracts had good activity against *S. aureus* and *P. aeruginosa* with inhibition zones diameters of 15.60 mm and 17.00 mm respectively. *Rhus natalensis* had an MIC of 6.25mg/ against both *S. aureus* and *P. aeruginosa*. The plant extracts were active against both bacteria and fungi. The result indicates that's the plants extracts have potential for managing infections caused by the tested microbes. Isolation of compounds present and determination of their bioactivity should be done together with conservation initiatives.

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Introduction

Opportunistic infections are a major challenge to people with HIV as susceptibility is increased due to weakened immune defenses. According to W.H.O clinical HIV-staging, some of the opportunistic infections characterizing clinical stage 2-4 are caused by bacterial and fungal infections. These include oral and Vulvovaginal candidiasis, cryptococcosis, and non-typhoid *Salmonella* septicemia among others [17]. Emergence of microbes that are resistant to cheap first-choice or "first-line" drugs is threatening the gains made by the discovery of antimicrobial agents during the 20th Century. Antibacterial and antifungal drug resistance is becoming a major problem in immunocompromised patients. People infected with HIV in whom drug resistance of the agent causing oropharyngeal candidiasis is a major problem.

Until recently, available treatment for serious fungal infections comprised of amphotericin B and azoles, which have limitations [11]. Renal toxicity is a major concern with amphotericin B, while drug-drug interactions, hepatotoxicity, and skin rashes are the primary concerns with azoles [15]. Mainstream medicine is increasingly receptive to the use of antimicrobials and other drugs derived from plants. Traditional medicine has been an important source of products for the developing countries in treating common infections. For example, in Kenya about 400 plant species have been recorded as being used in traditional remedies [8, 11]. Traditional medicine is accepted by a majority of people because it is affordable and easy to access. While the benefits are appreciated, most of the traditional medicine used lack documented evidence of therapeutic efficacy, safety and quality. Numerous plants have been reported to possess antifungal activity. Here in Kenya, the petroleum ether fraction of` the aerial parts of *Ajuga remota* have been reported to have antifungal activity [7]. The ethanolic extract of the bark of *Bersama lucens* and *Harperpyhullum caffrum* have been reported to have activity against *Candida albicans* [8].

Cassia species have been found to have good antimicrobial activity. The leaves and barks of *Cassia alata* have been reported to have antimicrobial activity *in vitro*, as potent as standard antimicrobial drugs against certain microorganisms. The water extracts of the bark had inhibition zones of 14.5 ± 0.3mm at a concentration of 30 µg/µl, while the inhibition zone of Triconazole was 17.1± 1.9 mm at the same concentration, against *C. albicans* [15]. Methanol extracts of *C. fistula* had a 100% inhibition against *C. albicans* [1]. Methanol extracts of *Aframomum melegueta* seeds, *Piper guineense* seeds, *Xylopia aethiopica* fruits, and *Zingiber afficinale* rhizomes were found to be active against, *Candida albicans* and *Aspergillus niger* [9]. Fractions of *Zygophyllum fabago* showed very strong antifungal activity, achieving 95% growth inhibition while *Vincetoxicum stocksii* exhibited 85% growth inhibition against human pathogen *C. albicans,* as compared to its control, miconazole which showed a 100% growth inhibition at the same concentration [13]. Ground fresh leaves of *H. opposita* are soaked in water and the extract douched to treat vaginitis and drunk for treatment of hypertension. The roots are boiled and taken orally to cure children fever and convulsions [10]. Leaves are used for skin diseases and herpes zoster while the whole plant is used for liver cancer [2]. The plant is also used for the management of gonnorrhoea, blenorrhoea cystitis, liver disease, chest pain, cough, fever, hookworm, stomach disorders, wounds and mental disturbances [16]. The roots are used to treat malaria, epilepsy, convulsions and measles like swellings on the skin [6]. Antimalarial activity of *R. natalensis* collected in Meru district (Kenya) has been confirmed by Muthaura [14]. The plant was found to be safe both *in vitro* and in animal model [14]. Many plant species in the tropics still remain unexplored for their potential as medicinal agents, yet several reports indicates their use in traditional medicine [8, 4]. Due to HIV/AIDS pandemic and emerging resistance, toxicity and high cost of conventional drugs and their implication on mortality and morbidity we explored the potential use of *H. opposita, R. natalensis* and *C. illairii* as antimicrobial agents. To evaluate antimicrobial potential and toxicity of the methanol extracts of *H. opposita, R. natalensis* and *C. illairii we* collected, authenticated and extracted the three selected medicinal plants from Kilifi district (Kenya). Phytochemical screening, antimicrobial activity and toxicity of the methanolic extracts was done so as to determine their potential as antimicrobial agents.

Materials and Methods

Study Site

The study was carried out at the Center for Traditional Medicine and Drug Research (CTMDR) and Center for Microbiology Research (CMR), Kenya Medical Research Institute (KEMRI). Extraction and *in vitro* toxicity was carried out at CTMDR while the bioassay was carried out at CMR.

Collection and Preparation of Plant Samples

The plants were collected from Kilifi District and authenticated at the East African Herbarium, Nairobi. Voucher specimen was deposited at the herbarium for future reference number KIF 019 *H. opposita*, KIF 025 *R. natalensis* and KIF 039 *C. illairii*. Samples of roots stem and leaves were dried under shade, ground, catalogued and stored. Ground material (50-100 g) were extracted in methanol and concentrated by use of a rotary evaporator. Percentage yield of the dry materials was determined and the material preserved for use in biological assays. The extracts obtained were stored in airtight containers at room temperature for the bioassays.

Antibacterial Bioassay

The disc-diffusion assay was used to determine the growth inhibition caused by the extracts against the pathological bacterial strains *Pseudomonas aeruginosa* (ATCC 2785), *Escherichia coli* (ATCC 25922) and *Staphylococcus aureus* (ATCC 25923). The bacterial strains were obtained from the Center for Microbiology Research, KEMRI and standard isolates were used. The bacteria were maintained at 4 ^{0}C on nutrient agar (NA) plates. Base plates were prepared by pouring 20ml Mueller-Hinton (MH) agar into sterile Petri dishes and allowed to set. Molten MH agar held at 48 ^{0}C were inoculated with a broth culture of the test organism and poured over the base plates forming a homogenous top layer. Filter paper discs (Whatman No. 3, 6mm diameter) were sterilized by autoclaving. Ten µl of each extract (100mg/ml) were applied per filter paper disc so that each disc contained 100 mg/ml of extract. The discs were air dried and placed onto the seeded top layer of the MH agar plates. Each extract were tested in tripricate, at a concentration of 100mg/ml, with gentamicin (30 µg/ml) disc as reference or positive control. Air-dried Dimethylsulfoxide saturated discs were used as negative controls. The plates were evaluated after incubation at 37 ^{0}C for 24 hr after which the zones of inhibition were measured. The size of the inhibition zone in (mm) produced by the plant extract and the inhibition zone around the gentamicin reference (mm) were used to express antibacterial activity.

Minimum Inhibition Concentration

Broth micro dilution method was used to determine minimum inhibitory concentration for the active crude extracts against the test microorganisms. The procedures were done as recommended by the National Committee for Clinical Laboratory Standards now Clinical Laboratory Standard Institute [18]. The tests were performed in 96 well-micro-titer plates. Plants extracts dissolved in respective solvents were transferred into micro-titer plates to make serial dilutions ranging from10^1, 10^2, 10^3……..10^{10}. The final volume in each well was 100 µl. The wells were inoculated with 5µl of microbial suspension. The yeast and bacteria were incubated at 37^0C for 24 hours while molds were incubated at 25^0C for 3-7 days in ambient air. The MIC was recorded as the lowest extract concentration demonstrating no visible growth as compared to the control broth turbidity [7, 12]. Wells that were not inoculated were set to act as control. All the experiments were done in triplicates and average results were recorded.

Antifungal Bioassays

Antifungal activity was performed on *Candida albicans* (ATCC 90028) and *Trichophyton mentagrophyte* (clinical isolate). *In vitro* activity was performed as described for bacterial assays except that Sabourands dextrose agar was used for antifungal bioactivity testing and incubation was done at 30 ^{0}C for 24 hr for yeasts and 72 hr for the filamentous fungi. All the bioassays were done in triplicate and results given as average results for three experiments.

***In-vitro* Cytotoxicity Assay**

Human embryonic lung fibroblast (HELF) cells were used for the cytotoxicity assay. Cells were maintained in Minimum Essential Medium (MEM) containing 10 % fetal bovine serum (FBS). 2 x 10^5 cell suspensions were seeded on 96- well microtiter plates and incubated at 37 ^{0}C / 5 % CO_2 for 12 hours and different concentration of the drugs were added serially. Cells without drugs served as negative controls while wells with no cells or drug served as blanks. The plates were incubated for 48 hours at 37 ^{0}C, 5% CO_2, followed by addition of MTT reagents to each well. Plates were incubated for another 4 hours. Media was removed from the wells and 100 µl of Dimethyl sulfoxide (DMSO) added. The plates were read on a scanning multi-well spectrophotometer at 562 nm and 620 nm as reference. Etoposide was used as a positive control/reference. The CC_{50} was determined using a dose-response curve

Phytochemical Evaluation

Phytochemical screening was done on the active extracts to determine the possible chemical principles present. Based on the initial bioassays the plant extracts exhibiting biological activity were screened for groups of chemical constituents. Thin layers chromatography plates were developed with Chloroforms: Methanol (98:2) with five drops of glacial acetic acid before spraying with TLC visualization reagents giving specific reactions.

Data Analysis

The results were presented in table forms. Test was done in triplicates and average results were computed using Excel computer package. The cytotoxic concentration causing 50% cell lysis and death (CC_{50}) were determined for the extracts and analyzed using excel computer package. The data was presented inform of tables, graphs and photographs.

RESULTS

Phytochemical Compounds Present

According to the results of the phytochemical screening study, all tested plant extracts were found to show a positive test for the presence of phenols. All the plants screened were negative for alkaloids. The *H. opposita* and *C. illairii* were positive for terpenoids, flavonoids and anthaquinones while *R. natalensis* was negative for the three compounds. The results are as shown in table 1.

Table 1: Phytochemical Profile of the Plants extracts

Plant species	Solvents	Plants compounds				
		Terpenoids	Alkaloids	Flavonoids	Phenolics	Anthraquinones
H. opposita	MeOH	+	-	+	+	+
R. natalensis	MeOH	-	-	-	+	-
C. illairii	MeOH	+	-	+	+	+

*Key: + Present, -Absent, MeOH Methanol extracts

Bioactivity Testing

Antibacterial Results

The *C. illairii* plant extracts had good activity against *S. aureus* and *P. aeruginosa* with inhibition zones diameters of 15.60 and 17.00 mm respectively. The *R. natalensis* extracts had moderate activities with inhibition zone diameters of 11.6 mm while *H. opposita* had the least activities judged by the inhibition zones diameters of

6.60 and 7.60 mm against *S. aureus* and *P. aeruginosa* respectively. All the plants tested had no activities against *E. coli*. The activity of the test plants expressed in inhibition zones diameters average for the triplicate tests are tabulated as shown in table 2.

Table 2: Antimicrobial Activities of the three Plants Extracts against Bacterial Isolates

Plant species	Organisms	Inhibition zone diameters in mm			Average in mm	Positive control (X)	Negative control (DMSO)
H. opposita (Aerial parts)	*Staphylococcus aureus*	7	7	6	6.6	25.3	6
	Pseudomonas aeruginosa	8	7	8	7.6	18	6
	Escherichia coli	6	6	6	6	18	6
R. natalensis (Roots)	*Staphylococcus aureus*	11	12	12	11.6	25.3	6
	Pseudomonas aeruginosa	12	11	12	11.6	18	6
	Escherichia coli	6	6	6	6	18	6
C. illairii (Leaves)	*Staphylococcus aureus*	16	15	16	15.6	25.3	6
	Pseudomonas aeruginosa	18	17	16	17	18	6
	*Escherichia co*li	6	6	6	6	18	6

*Key: Positive Controls –**X** (gentamicin): Negative control – **DMSO** (Dimethlysulfoxide)

Antifungal Results

All the plant extracts tested against fungal isolates were least active judged by the presence of the inhibition zones diameters. The average zones were 6.00 and 7.30 mm. the results are as shown in table 3.

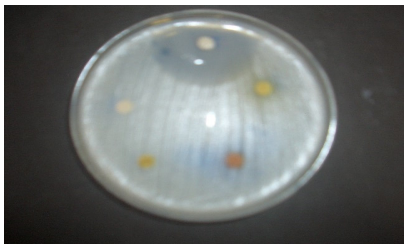
Table 3: Antimicrobial Activities of the Plants Extracts against Fungal Isolates

Plant species	Organisms	Inhibition zone diameters in mm			Average	Positive control (Y)	Negative control (DMSO)
H. opposita (Aerial parts)	*Candida albicans*	7	7	7	7	25	6
	Trichophyton mentagrophyte	6	6	6	6	24	6
R. natalensis (Roots)	*Candida albicans*	7	7	7	7	25	6
	Trichophyton mentagrophyte	6	6	6	6	24	6
C. illairii	*Candida*	7	7	8	7.3	25	6

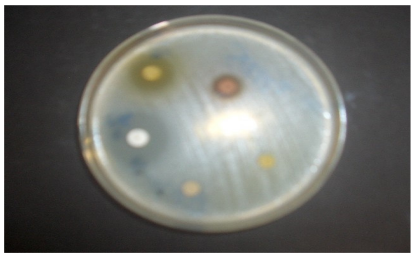
(Leaves)	*albicans*						
	Trichophyton mentagrophyte	6	6	6	6	24	6

*Key: Positive control - **Y** (Fluconazole), Negative control – **DMSO** (Dimethlysulfoxide)

Plates Showing Antibacterial Inhibition Zones Exhibited by the Extracts

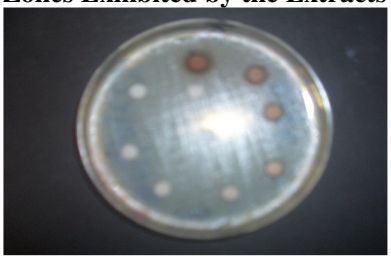


(a) *Pseudomonas aeruginosa*

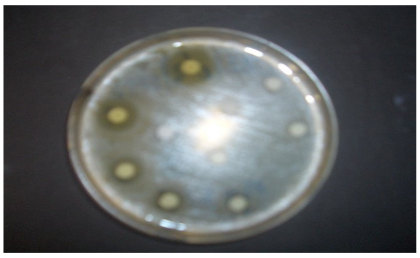


(b) *Staphylococcus aureus*

Plates Showing Antifungal Inhibition Zones Exhibited by the Extracts



(d) *Candida albicans*



(e) *Trichophyton mentagrophyte*

The MIC Values of Active Plants Extracts against Bacteria Strains

The MIC of the plant extracts which had inhibition diameters of 10 mm and above (significance activity) was determined. *C. illairii* leaves extracts had the least minimum inhibition concentration of 3.125 mg/ml against *S. aureus* and the highest MIC against *P. aeruginosa* of 12.50 mg/ml. The extracts of *R. natalensis* had MIC of 6.25 mg/ml against both *S. aureus* and *P. aeruginosa*. The results are as shown in table 4.

Table 4: Minimum Inhibition Concentration of Plants Extracts against Bacterial Isolate

Plant species	Organism	Minimum inhibitory concentration in mg/ml
R. natalensis (Roots)	*Staphylococcus*	6.25
	Pseudomonas aeruginosa	6.25
C. illairii (Leaves)	*Staphylococcus*	3.125
	Pseudomonas aeruginosa	12.5
Control	Gentamicin	0.5

Cytotoxicity

The concentration of herbal extract reducing cell viability by 50% (CC_{50}) was determined for the three plant extracts. All the plant extracts were tested for toxicity using mammalian cells. The extracts were not cytotoxic at 100 mg/ml; *H. opposita*, *R. natalensis* and *C. illairii* extracts were safe to the mammalian cells with no apparent cytotoxic effect at 100 mg/ml. The CC_{50} in table 5 at 500mg/ml and at 100mg/ml represents the number of cells remaining in each well after being subjected to cytotoxic effects of the extracts, since it is far from 50% all the extracts are not toxic as compared to the control which is below 50%. The results are as shown in table 5.

Table 5: Cell Toxicity of Plant Extracts Showing CC_{50} at 100mg/ml

Plants species	Extracts	CC_{50} at 500mg/ml	CC_{50} at 100 mg/ml
H. opposita	Methanol	99.9	100
R. natalensis	Methanol	100	100
C. illairii	Methanol	99.8	100
Dimethylsulfoxide	Negative control	100	100.0
Epotoside	Positive control	11.28	25.28

Discussion

Phytochemical Compounds Present

The extracts of *H. opposita*, *R. natalensis*, *C. illairii*, were screened for the presence of different phytochemical compounds of therapeutic interest using chromatography visualization reagents with the object of finding out the possible classes of compounds present in the respective plants. According to the results of the phytochemical screening study, all tested plant extracts were found to show a positive test for the presence of phenols. All the plants screened were negative for alkaloids. *H. opposita* and *C. illairii* were positive for terpenoids, flavonoids and anthaquinones while *R. natalensis* was negative for the three compounds. The presences of flavonoids, phenolics or terpenoids in plants have been reported to be responsible for antimicrobial activity in plants. Flavonoids and flavonoid-derived plant natural products have long been known to function as antimicrobial defense compounds. The different in vitro studies have also shown that they are effective antimicrobial substances against a wide spectrum of microorganisms. The antifungal and antibacterial activity of the plants studied was attributed to the presences of these secondary metabolites. In plants, their role is to protect plants against microorganisms and insects.

Bioactivity Testing

The *C. illairii* plant extracts had good activity against *S. aureus* and *P. aeruginosa* with inhibition zones diameters of 15.60 and 17.00 mm respectively. This plant can be considered to be having good antimicrobial activities as compared to the other plants. The solvents used for extraction were used as negative control while fluconazoles and gentamicin were used as positive controls. The standards drugs (gentamycin and fluconazole) inhibition zone diametres was between 16mm and 20mm which compared well with that of the *C. illairii* plant extracts. The extracts of *R. natalensis* had moderate activities with inhibition zone diameters of 11.6mm while *H. opposita* had the least activities judged by the inhibition zones diameters of 6.60 and 7.60 mm against *S. aureus* and *P. aeruginosa* respectively. All the plants tested had no activities against *E. coli*. The activity of the test plants expressed in inhibition zones diameters average for the triplicate tests are tabulated as shown previously. All the plant extracts tested against fungal isolates were least active judged by the presence of the inhibition zones diameters. The average zones were 6.00 and 7.30 mm. This results support the facts that most of this plants are used traditionally to treat and manage infections caused by bacterial pathogens as opposed to fungal infections.

The MIC Values of Active Plants Extract against Bacteria Strains

The preliminary screening assays for antimicrobial activity can largely be considered as qualitative assays and are used for identifying the presence or absence of bioactive constituents in the extracts. However, these methods of assay offer little information on these compounds. Minimum inhibitory concentration is a quantitative assay and provides more information on the potency of the compounds present in the extracts. Thus, the MIC values of crude extracts of the four medicinal plants were determined so as to demonstrate the potency of the extracts against the selected strains of bacteria. The MIC of the plant extracts which had inhibition diameters of 10 mm and above was determined. The *C. illairii* leaves extracts had the least minimum inhibition concentration of 3.125 mg/ml against *S. aureus* and the highest MIC against *P. aeruginosa* of 12.50 mg/ml. The least the MIC the better the plant extract against the isolate in question. The extracts of *R. natalensis* had an MIC of 6.25 mg/ml against both *S. aureus* and *P. aeruginosa*. The two plants extracts can be used to treat wound infection caused by *S. aureus* and *P. aeruginosa* since they have shown good activities.

Cell Toxicity

The concentration of herbal extract reducing cell viability by 50% (CC_{50}) was determined for the three plant extracts. All the plant extracts were tested for toxicity using mammalian cells. The extracts were not cytotoxic at both concentration of 100 mg/ml and 500 mg/ml respectively; *H. opposita*, *R. natalensis* and *C. illairii* extracts were safe to the mammalian cells with no apparent cytotoxic effect at 100 mg/ml and 500 mg/ml. DMSO was used as a solvent in dissolving the organic solvent and hence used as a negative control while Epotoside drug was used as a positive control. The results are in agreement with other work done elsewhere [14] on antimalaria properties of the three plants, [3], found out that the stem bark extracts from this plant had neither cytotoxicity nor brine shrimp lethality. The three plant extracts tested had low toxicity as seen by their cytotoxic concentration above 50% against HELF cells, suggesting that they may be safe as antimicrobials.

Conclusion

The plant extracts in this study has high activity against bacteria strains as compared to fungal.

Traditionally medicine plants decoction are taken in combination and at high dose, this may explain the low and moderate activity observed. In this study, all the plant extracts had no activity against *E. coli* thus treating

ailments caused by pathogenic strains of *E. coli* will not work. The results validate the ethnobotanical use of the studied medicinal plants used among the coastal people of Kenya.

Recommendation

Since the plant extracts are potent against bacterial isolates as compared to fungal then it is worth recommending the extracts be tested against a wide range of bacteria. All the plants are used traditionally for treating common colds which are caused by viral infection thus assays should be done on viral isolates. Analysis and isolation of the compounds present as well as determination of their bioactivity of the pure compounds should also be done. Such an effort could lead to identification of a new range of compounds for management of bacterial infections. Bioassay of combinations of plant extracts that exhibited moderate and low activity should be carried out to establish any synergism between them. Bioactivity on all parts of the plants for example, root, Stem bark and leaves combined so as to report conclusively that certain plant is inactive. Since some plants have good activities they should be formulated into different consumable forms as well as being incorporated into food as food supplements. The plants should be conserved in their natural environment so as to conserve our heritage.

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