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Antifungal activity of single and combined extracts of medicinal plants from Southern Highlands of Tanzania

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Abstract
This study investigated the antifungal activities of single and combined extracts of traditionally used Tanzanian medicinal plants against the selected fungal pathogens. Plants samples were collected from Southern regions of Tanzania, dried, ground and extracted with petroleum ether, dichloromethane and ethanol. Broth micro dilution method was used to determine minimum inhibition concentration (MIC) while Fractional inhibitory concentrations (FIC) index was calculated from MICs of combined extracts to determine the effect of interactions. A total of sixteen medicinal plants were evaluated for antifungal activity against *Candida albicans*, *Cryptococcus neoformans* and *Aspergillus niger*. Strong antifungal activity was demonstrated by *Bidens pilosa* (MIC value 0.078-0.313 mg/ml), *Mucuna stans* (MIC value 0.117-0.313 mg/ml), *Parinari curatellifolia* (MIC value 0.039-0.469 mg/ml), *Solanum incanum* (MIC value 0.039-0.469 mg/ml), *Strychnos spinosa* (MIC value 0.313-0.469 mg/ml), *Leonotis nepetifolia* (MIC value 0.156-0.313 mg/ml) *and Diospyros usambarensis* (MIC value 0.059 mg/ml). FIC analysis indicated that, most of the combinations had additive effect while only the combination between *Bidens pilosa* and *Leonotis nepetifolia* demonstrated synergistic interaction against *Candida albicans* pathogen. This study demonstrated the importance of using combined plant-based antifungal therapy to increase efficacy. Findings also revealed plants potential candidates for drug discovery studies.

Keywords: Antifungal activity, medicinal plants, fractional inhibitory concentration, synergism, additive, antagonism

Introduction
Fungi are microbes that cause a wide range of illnesses, from minor skin conditions to life-threatening diseases. Fungal infections can become fatal to people with immune compromised conditions particularly HIV/AIDS, cancer patients [1]. Among the different HIV-associated fungal infections, oral mucosal lesions caused by Candida species are by far the most frequent manifestation. Up to 90% of HIV-infected individuals experience at least one episode during the course of their disease and the incidence and severity of the episodes increase with decreasing immunity, especially when CD4+ cell counts declines to levels below 200 cells/mm^3 [2, 3]. So far, management of fungal infections has gained much complexity due to; limited number of effective antifungal agents, toxicity of the available antifungal agents, resistance to commonly used antifungals, relapse of infections, and the high cost of antifungal agents [4, 5].

Plants have been reported to be used medicinally in different countries as sources of potent and powerful antifungal therapies [6]. Use of herbal drugs for the control of opportunistic infections is considered as a promising solution and an interesting alternative to synthetic fungicides [7]. In Tanzania, several reviewed literatures support the existence of medicinal plants that are continuously used traditionally by various ethnic groups for management of fungal related ailments [8]. Traditionally, the prepared therapy involves either a single herb, combination of herbs, or combination of herb(s) and conventional drug(s). When herbs are used in combination, the effects can be complicated as various interactions can occur among the individual components. The most desired interactions in the body are those which can result in additional therapeutic benefit [9] however, they can also reduce activity or cause toxicity. Different herbal formulations available in African herbal markets/shops consist of a mix of several plants or parts of plants, however most studies have been done to test a single plant extract. Based on this evidence it seemed appropriate to examine the effect of individual plant extract and in combination for antifungal activities. This will help advising the communities the on best practices using the selected plants as a way to prolong lives for immune compromised individuals.

Materials and Methods

Reagents

Ethanol (absolute), Dichloromethane, Petroleum ether were purchased from Fluka Chemie GmbH (Sigma-Aldrich®, Zwijndrecht, Netherlands), Dimethyl sulfoxide (DMSO) was from Sigma® (Poole, Dorset, UK), Sabouraud Dextrose agar and broth from HIMEDIA® (Himedia Laboratories Pvt Ltd, Mumbai, INDIA), *p*-Iodonitrotetrazolium chloride was bought from SIGMA® (Sigma- Aldrich®, St Louis, USA), Microtitre plates were supplied by KAS medics Tanzania.

Microorganisms

Authentic pure cultures of human pathogenic fungi: *Candida albicans* (ATCC 13803), *Candida albicans* (clinical isolates), *Cryptococcus neoformans* (ATCC 90112) and *Aspergillus niger* (AZN 8240) were obtained from the Department of Microbiology and Immunology, Muhimbili University of Health and Allied Sciences (MUHAS). The fungal species were maintained on sabouraud dextrose agar at 4 °C. The pure strains of fungal species were sub cultured in a sabouraud dextrose agar and incubated at 35 °C prior to antifungal testing.

Collection of plant materials

Plant samples were collected from several villages in Southern regions of Tanzania particularly Iringa and Njombe regions in Tanzania. Collection of the plants was done with the help of a botanist from the department of Botany, University of Dar es Salaam, Tanzania. Decision of the plant and/or part of plant to be collected was mainly influenced by ethnomedical survey [10]. Voucher specimens were kept at the Herbarium of the Institute of Traditional Medicine, Muhimbili University of Health and Allied Sciences.

Extraction and concentration

Plant materials from the field were air-dried, cut into small pieces, ground using a machine grinder and consequently soaked sequentially at room temperature for 48 hours using different solvents (Petroleum ether, Dichloromethane and Ethanol). The crude extracts were filtered and the filtrate concentrated in *vacuo* using a rotary evaporator with the bath temperature maintained at 40 ºC to prevent thermal decomposition of labile compounds. The obtained crude extracts were subjected to freeze drier to remove any remains of solvents. All dried extracts were stored in a -20 ºC refrigerator until the time of bioassay.

Selection of plant extracts for combination assay

Six (6) plant extracts were selected for fractional inhibitory concentration analysis from the initial screening assay results (Table 1). The basis for selection of each extract was the activity level reported in the first screening test as well as the medium of solvent used in extraction process. In this case, the most active ethanol extracts were picked for combination and because the expected most active extracts will be considered for herbal formulation.

A concentration of 2mg/ml was prepared from individual selected plant extracts. The extracts were combined in a 1:1 v/v ratio and depending on the number of combinations made, equal volume of each extract was taken and combined to make a working solution. This solution was used according to microdilution method described below.

Determination of Minimum Inhibitory Concentration by broth dilution method

The MIC was determined by fold microdilution tests performed in a sterile flat bottom 96- well polystyrene microtiter plates. The extracts were tested against standard and clinical isolates of fungi including *Candida albicans*, *Cryptococcus neoformans* and *Aspergillus niger*. The microorganism's inocula were prepared from 24 hours grown cultures. The serial fold dilution method was performed as follows; 50 µl of broth (Sabouraud's dextrose broth) were pipetted into each well. To each well of the first row 50 µl of the extract solution or fluconazole or solvent was added. Each of the test materials were tested in duplicate. After thorough mixing, 50 µl of the mixture was drawn and transferred to the second well in the row and after mixing well again 50 µl of the mixture was drawn and transferred to the third well in the row. This procedure was repeated until the last well in the row. Then 50 µl of the mixture was discarded from each last well of the row. One additional row was used as growth control, in which no drug was added; instead a blank culture medium was added. The inoculated microtiter plates were incubated at 37 ºC for 24 hours.

MIC values were detected using tetrazonium salt indicator, INT (Iodonitrotetrazolium chloride) which changes color from pink to yellow colour by viable microbes. In the MIC assay plate, a volume of 20 µl of a 0.2% INT was pipetted into each well and incubated at 37 ºC for 2 hours. The lowest concentration at which no growth observed was taken as MIC.

Determination of Fractional Inhibitory Concentration (FIC Index)

An algebraic equation to determine synergy by means of the ΣFIC is a widely accepted means of measuring interaction. The ΣFIC is expressed as the interaction of two agents where the concentration of each test agent in combination is expressed as a fraction of the concentration that would produce the same effect when used independently [11]. The interactions between the combined extract is analyzed by using measurements of the MIC to calculate the fractional inhibition concentration index (FIC_{Index}) according to the formulas defined by Davidson and Parish (12) with few modifications;

$FIC_{Index} = FIC_A + FIC_B + FIC_N = (C_A/MIC_A) + C_N/MIC_N)$

Where; MIC_A and MIC_B are the Minimum Inhibitory Concentrations of drugs A and B alone, respectively, and C_A and C_B are the concentrations of the drugs in combination.

Calculating the FIC value (Table 1) for either substance A or B then requires determination of the MIC for the individual components. FIC_{Index} results are interpreted as synergistic if $FIC_{Index} \leq 0.5$, additive if $0.5 > FIC_{Index} < 4$, or antagonistic if $FIC_{Index} > 4$ (13).

Results and Discussion

Results

Antifungal activity of individual and combined plant extracts

The study of antimicrobial activity of plant extracts was conducted and the MICs of both individual and combined extracts were obtained as indicated in (Table 1 and 2). The MIC values and the fractional inhibitory concentrations (FICs) for the activity of the combined extracts and type of interactions are summarized in Table 3. Plant extracts exhibited antifungal activity against *Candida albicans*, *Cryptococcus neoformans* and *Aspergillus nige*r with varied potencies as indicated by the minimum inhibition

concentration (MIC) in Table 1. MIC values reported in the table are interpreted according to Sartoratto [14] classification criteria, as follows; 0.05-0.5 mg/ml (strong activity), 0.6-1.5 mg/ml (moderate activity) and MIC >1.5 mg/ml (weak activity). Based on this classification, 7 (47%) species out of 16 plant species demonstrated a strong antifungal activity against the microbial pathogens tested. These include plant extracts from *Bidens pilosa*, *Mucuna stans*, *Parinari. curatellifolia*, *Solanum incanum*, *Strychnos spinosa*, *Leonotis lepetifolia* and *Diospyros usambarensis*. Other plant extracts exhibited either moderate to weak antifungal activity.

Among all the tested plant extracts, it was interesting to note that, dichloromethane leaf extracts of *Bidens pilosa*, had lower MIC value as compared to other extracts ranging from MC 0.078 mg/ml for *Candida albicans* to 0.339 mg/ml for *Aspergillus niger* which is higher compared to standard drug used in the experiment**.**

Table 1: Minimum Inhibitory Concentration, MIC (mg/ml) for the individual tested plant extracts

Crude extract (mg/ml)		**Test organisms**			
		***C. albicans* ATCC 13803**	***C. albicans* clinical**	***C. neoformans* ATCC 90112**	***A. niger* AZN 8240**
Ketoconazole		0.339	0.078	0.3125	0.339
Bidens pilosa	LD	0.078	0.156	0.078	0.313
	LE	0. 938	1.25	0.469	0. 625
Brachystegia speciformis	LP	2.50	2.50	0.469	0. 938
	LE	1.875	1.875	0. 625	0.469
	LD	2.50	2.50	0. 625	0. 625
Croton macrostachys	LE	1.875	1.875	0.313	0. 938
	LD	0. 625	0.156	0.156	0.469
	LP	5	1.25	_	_
Commiphora africana	RE	1.875	2.50	0. 625	1.25
Diospyros usambarensis	LE	2.50	2.50	0. 938	_
	RE	1.875	0.625	0.625	0. 939
	LP	1.25	1.25	0. 625	2.50
	RD	0. 625	2.50	0.059	2.50
	RP	2.50	0.938	1.25	1.25
Eleadendron buchananii	SP	5	5	0. 938	0.625
	SE	1.938	1.25	1.25	0.938
Kigelia africana	SD	0. 625	0. 625	0. 625	0.625
	SP	5	5	5	5
	SE	2.50	2.50	1.25	1.25
	RP	5	5	1.25	0.313
	RD	1.875	2.50	0.313	1.25
	RE	2.50	2.50	0. 625	1.25
Lantana vibrinoides	SD	2.50	2.50	0.313	1.25
	SE	2.50	1.875	0. 625	2.50
	SP	_	1.25	_	_
Leonotis nepetifolia	LE	1.875	0.313	0. 625	0. 938
	LP	2.50	2.50	1.25	2.50
	LD	1.875	2.50	0.313	0. 625
Mucuna stans	LD	0. 938	0.156	0. 156	0. 625
	LP	2.50	2.50	0. 625	2.50
	LE	1.25	0.625	0. 625	0.313
Parinari curatellifolia	RE	2.50	2.50	0. 625	2.50
	RP	2.50	0.625	0.469	0.625
	LP	2.50	0.117	_	1.25
	LD	0. 625	0.313	0. 625	0. 625
	LE	2.50	1.875	0.469	0.313
	SD	2.50	2.50	0. 625	0. 625
	SP	1.469	1.25	0. 625	0.625
	RD	1.25	0.313	0.039	0.039
	SE	2.50	1.875	1.25	2.50
Psorospermum febrifugum	LE	2.50	-	2.50	-
	RE	5	-	5	-
Solanum incanum	LE	2.50	0.039	0.469	2.50
	LD	2.50	1.875	0.469	1.25
	LP	0.313	1.25	0.313	2.50
Sorindeia madagascariensis	LE	2.50	0.625	0. 625	1.875
Strychnos spinosa	LE	2.50	0.313	0. 625	3.75
	LD	0.313	0.313	0.469	0.313
	LP	0. 938	2.50	1.25	1.25
Terminalia sericea	LE	2.50	2.50	0. 938	0.469
	RE	0.156	2.50	0.625	-

LE = Leaves Ethanol extract, LD = Leaves Dichloromethane extract, LP= Leaves Petroleum ether extract, SE = Stem barks ethanol extract, SD = Stem barks Dichloromethane extract, SP = Stem barks Petroleum ether extract, RE = Roots Ethanol extract, RD = Roots Dichloromethane extract, RP = Roots Petroleum ether extract, Ket. = Ketokonazole, (–) =Not active

Variation in antifungal activity was observed between clinical and standard fungal pathogen. This can be attributed by development of resistant genes in clinical strain due to exposure to various antibiotics in patient's body. Least or no activity was exhibited by *P. febrifugum* and most of the petroleum ether extracts had shown very low antifungal activity against fungal pathogens. Minimum Inhibitory Concentration of combined crude plant extracts and fractional inhibitory concentration.

Interactions analysis for combined extracts against fungal pathogens

The interactions were observed between two to five combinations (Table 3 and Table 2). Synergistic interaction was demonstrated by the combinations of *Bidens pilosa* and *Leonotis nepetifolia* (BPLE/LNLE) extracts against *Candida albicans* pathogen (FIC value of 0.5). Additive interaction was exhibited by several combinations including *Parinari curatellifolia* extract and *Mucuna stans* extract (PCLE/MSLE), *Bidens pilosa* and *Leonotis nepetifolia* (BPLE/LNLE), *Bidens pilosa* and *Parinari curatellifolia* (BPLE/PCLE), *Mucuna stans* and *Leonotis nepetifolia* (MSLE/LNLE) and *Mucuna stans* and *Bidens pilosa* (MSLE/BPLE). Antagonism interaction of extracts was demonstrated by *Parinari curatellifolia* and *Mucuna stans* (PSLE/MSLE), against the standard *Candida albicans* while *Leonotis nepetifolia* and *Mucuna stans* (LNLE/MSLE) combination was exhibited against both *Candida albicans* standard and clinical.

Table 2: Minimum Inhibitory Concentration of combined crude plant extracts against tested pathogenic fungi

Combination and MIC (mg/ml)	*C. albicans* ATCC	*C. albicans* clinical	*C. neoformans* ATCC	*A. niger* clinical
Clotrimazole	0.03	0.03	0.01	0.01
BPLE /LNLE	0.25	0.25	0.5	0.5
LNLE /MSLE	0.25	1	0.5	0.25
MSLE /BPLE	0.25	0.25	0.5	0.5
BPLE /PCLE	0.25	0.25	0.5	0.25
PCLE /MSLE	0.25	0.5	0.5	0.5
MSLE/DURE	0.41	0.21	0.83	1.65
LNLE/PCLE	0.05	0.01	0.83	1.65
LNLE/DURE	0.83	0.05	0.83	1.65
MSLE/TSLE	-	-	0.83	1.65
DURE/PCLE/MSLE	0.06	0.06	-	-
LNLE/PCLE/MSLE	0.5	1	-	1
DURE/LNLE/PCLE	-	-	-	-
DURE/LNLE/MSLE	1.65	0.21	0.41	1.65
DURE/LNLE/TSLE	-	0.83	-	1.65
MSLE/LNLE/TSLE	-	0.83	0.43	1.65
DURE/LNLE/PCLE/MSLE	1	1	0.5	1
DURE/LNLE/PCLE/TSLE	1	1	0.5	0.13
DURE/PCLE/MSLE/TSLE	1	1	0.5	0.13
LNLE/PCLE/MSLE/TSLE	0.5	1	0.5	0.13
DURE/LNLE/PCLE/MSLE/TSLE	1	1	0.5	0.5

BPLE= *Bidens pilosa* leaves ethanol extract, MSLE=*Mucuna stans* leaves ethanol extract, DURE=*Diospyros usambarensis* roots ethanol extract, LNLE= *Leonotis nepetifolia* leaves,ethanol extract, PCLE= *Parinari curatellifolia* leaves ethanol extract, TSLE= *Terminalia sericea* leaves ethanol extract, (-) =No MIC determined

Table 3: Fractional Inhibitory concentration index of combined crude plant extracts against tested pathogenic fungi

FIC Index	*C. albicans* ATCC	*C. albicans* clinical	*C. neoformans* ATCC	*A. niger* clinical
BP+LN	0.53	1.00	2.66	1.33
LN+MS	6.68	4.80	2.40	1.07
MS+BP	6.68	0.60	1.87	2.40
BP+PC	0.47	0.33	2.13	1.20
PC+MS	6.61	1.07	1.87	3.20
MS+DU	10.81	0.66	2.64	7.03
LN+DU	1.32	0.25	3.96	3.52
LN+PC	0.096	0.05	4.40	7.03
DU+LN+PC	1.618	0.224	N. c	N. c
LN+PC+MS	13.754	5.328	N. c	7.456
DU+PC+MS	1.618	0.224	N. c	N. c
DU+LN+MS	44.947	1.317	2.638	8.788
DU+LN+TS	N. c	4.286	9.671	N. c
MS+LN+TS	N. c	4.396	9.67	2.637
DU+LN+PC+TS	2.799	5.728	3.997	0.970
DU+LN+PC+MS	28.04	6.928	4.264	8.521
DU+LN+PC+TS	2.799	5.728	3.997	0.970
DU+PC+MS+TS	27.374	4.133	3.199	1.246
LN+PC+MS+TS	4.467	7	3.333	1.196
DU+LN+PC+MS+TS	28.44	7.328	4.797	5.327

BP= *Bidens pilosa* leaves ethanol extract, **MS**=*Mucuna stans* leaves ethanol extract, **DU**=*Diospyros usambarensis* roots ethanol extract, **LN**= *Leonotis nepetifolia* leaves ethanol extract, **PC**= *Parinari curatellifolia* leaves ethanol extract, **TS**= *Terminalia sericea* leaves ethanol extract, N. c = Not calculated

Discussion

Antifungal activity of single plant extracts against fungal pathogens

Fungal infections are a persistent and increasingly important public health problem, particularly for vulnerable populations such as cancer and HIV patients [15]. The increasing resistance to available antifungal drugs calls for search for therapeutic alternatives among traditionally used medicinal plants [16]. Plants used in this study were collected from Southern Tanzania where traditionally they are used for treatment of conditions such as skin infections, oral infections, abscesses, carbuncles, sepsis of wounds, treatment of venereal diseases [10]. Further ethnomedical support for these plants species in the treatment of various microbial infections were reported by various authors in the previous studies [10, 18, 19].

Among all the tested plant extracts, dichloromethane leaf extracts of *Bidens pilosa* demonstrated strong activity against *Aspergillus niger* which was also higher than the standard drug used in the experiment. The antifungal activity of *B. pilosa* has also been previously reported in the study done by Deba [20]. This suggests the presence of active secondary metabolites that can counteract microbial activity. Similarly, dichloromethane extracts from *Mucuna stans* had strong antifungal activity. In the other case, dichloromethane extracts from *Strychnos spinosa* demonstrated strong activity among all the tested microbes except for clinical strain of *Candida albicans* which had shown moderate activity. This can be attributed by the resistance of the clinical strain which has been isolated from patients who are under medication with conventional antifungal drugs. Significant antifungal activity of *S. spinosa* has also been reported by various authors [21-23]. Among the microbes subjected to extracts *Cryptococcus neoformans* was more susceptible compared to other microbes. A root bark extract from *Dyospyros usambarensis* was observed to have strong antifungal activity against *C. neoformans* (MC value 0.059 mg/ml) which concur with the study done by Hostettmann [24]. Stem barks from *Kigelia africana* has shown moderate antifungal activity in this study as previously reported by various authors [16, 18, 25].

In the cases where more than kind of extracts of the same plant depicted activity against the same pathogen, would probably mean that the compound responsible for the antifungal activity was present in each extract at a different concentration as similarly observed by Rojas *et al.,* [26].

Generally, among extracts tested dichloromethane extracts have shown significant antimicrobial activity compared to other extracts. These results confirm a statement that the intermediate polarity compounds usually have the highest antimicrobial activity found with many different plant species [27].

Minimum Inhibitory Concentration of combined crude plant extracts and fractional inhibitory concentration

Herbal drugs are normally prepared in combination of several plant species [28]. When combining extracts or drugs in therapy, synergism is usually the most desired effect expected because this mechanism of interaction improves the efficacy, minimizes toxicity, cures faster, offer a promising solution for the treatment of resistant microbes or provides broader-spectrum than monotherapy [28-30]. Few accepted mechanisms of antimicrobial interaction produce synergism through sequential inhibition of a common biochemical pathway, inhibition of protective enzymes and use of cell wall active agents to enhance the uptake of other antimicrobials [29]. Another mechanism of synergism can be contributed by serially blocking of metabolic pathways to facilitate the entry of drug into microorganism by the other drug or when one drug prevents the inactivation of the second drug by microbial enzymes [32, 33]. In this study, the combination of ethanolic extract from *Parinari curatellifolia* and *Bidens pilosa* leaves demonstrated the most desired synergistic interaction against *C. albicans* pathogen suggesting the alternative therapy in the future.

On the other hand, combination of extracts or drugs may also result into additive or antagonistic interaction and some undesirable effects such as toxicity or inefficacy. The combination between *M. stans* extract and *L. nepetifolia* extract had antagonist effect against *C. albicans* strains (for both clinical isolate and standard strains). Similar interaction was also shown by *B. pilosa* extract and *M. stans* extract. Usually, antagonism is discouraged for therapeutic applications and it occurs when fungistatic drug is given with fungicidal drug [29, 34].

Most interactions demonstrated by combinations of extracts was additive as shown in the table 2. Additive interaction occurs when the activity of the combined action is equivalent to the sum of the activity of each extract/drug when used independently [30]. Our results indicate that most of the two-by-two combinations displayed additive effects suggesting that similar molecular targets or metabolic pathways are involved in their biological action [35].

Furthermore, in searching for more effective antifungal therapy, a combination plan that exceeded two extracts was conducted. However, combination of more than three extract did not yield any potential synergy except for the combination of three extracts from *D. usambarensis*, *P. curatellifolia* and *M. stans* resulted into a significant antimicrobial activity against *C. albicans*.

Conclusion

All the extracts showed varying degrees of antifungal activity on the microorganisms tested. These results provide evidence of the efficacy of extracts from these plants to treat fungal infections. Some of the MIC values obtained against the microorganisms tested were higher than the positive control, since the crude extract contains many compounds, of which only a few may be active. Although the activities may not be very strong for some of the extracts, it is worth noting that some of the plants are used in combination to enhance their activity through synergism or additive effect. Some of these plants were more effective than traditional antibiotics to combat the pathogenic microorganisms studied.

These findings and those further supported by previous reports avail more plants from which new bioactive molecules can be generated to add to the existing drugs used in treatment against infectious diseases caused by fungi. Results confirm the potential of plants used by traditional healers in Tanzania as a source of bioactive compounds. Through these findings we continue with isolation characterization and testing of the compounds from these plants.

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