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ASSESSMENT OF THE *IN VITRO* ANTIFUNGAL ACTIVITY OF METHANOL EXTRACTS FROM THE TRADITIONAL MEDICINAL PLANTS USED BY KALENJIN COMMUNITY, UASIN GISHU COUNTY, KENYA

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ABSTRACT

Traditional medicinal plants are increasingly recognized for their therapeutic potential, particularly as new antifungal agents. Thus, this study targeted to evaluate herbs for antifungal activity *in vitro*. Fresh plant/plant parts were collected from the fields following leads by herbalists. The taxonomic identities of these plants were confirmed by a taxonomist. The plant material was washed under running tap water, air dried for one week and then homogenized to fine powder. This was then soaked under Methanol for two days, filtered and rotor vaporized to dry to get the final crude extract. The final product was air dried at room temperature overnight, to allow all the methanol to evaporate. Thereafter, half the sample was subjected to bioassay following Kirby bauer method. The other sample was subjected to phytochemical analysis following standard methods. The crude concoction was also subjected to bioassay following the agar dilution method. The test fungal strains investigated included; *Candida albicans ATCC90020, Candida tropicalis ATCC750, Candida famata (clinical isolate), Candida glabrata ATCC90039, C. lusitanie (clinical isolate) C. parapsilosis ATCC22019, and C. krusei ATCC6285.*The gram-positive *Staphylococcus aureus*ATCC25922. A control was with Nystatin discs and Dimethylsulphoxide prepared discs. The results obtained was analysed using ANOVA. The crude drug demonstrated activity against *C. famata* and *S. aureus.* The methanol extracts of *Fuerstia africana* demonstrated the most activity against *C.tropicalis* (9mm) with MFC of 0.125mg/ml. *C. parapsilosis* (8.66mm) with MFC of 0.015625mg/ml and *S. aureus* (12mm) MFC of 0.0078125mg/ml. Extract of *Rubia cordifolia* showed bioactivity against *C. parapsilosis* at 10.66mm diameter with mfc of 0.015625mg/ml. The plant extracts of *Ajuga remota* and *Acmella caulirhiza* did not demonstrate any antimifungal activity. The bioactivity against *Candida famata and S. aureus* were at 5mg/ml and 2.5mg/ml respectively. These findings therefore authenticate the use of herbals made from *R. cordifolia* and *F. africana* in traditional medicine for the treatment of oral thrush.

KEYWORDS: *Candida, Antifungal, In-vitro-antifungal activity, Kalenjin, Kenya*.

1.0 INTRODUCTION

The use of traditional medicinal plants for therapeutic purposes has gained significant attention in recent years, particularly in the search for new antifungal agents.[24] Methanol extracts from various plants have shown promising antifungal properties in vitro, providing a potential alternative to conventional antifungal medications which often face issues of resistance and side effects.[30] The increasing prevalence of fungal infections and the limitations of current treatments emphasizes the need for novel antifungal agents derived from natural sources.[2] Traditional medicinal plants are well-known for their rich array of bioactive compounds, making them a valuable resource for discovering new therapeutic agents. Methanol is particularly effective as a solvent for extracting these compounds, as highlighted by.[34]

In America,[15] conducted a study to evaluate the antifungal activity of methanol extracts from various traditional medicinal plants used in Mexico. The study focused on the inhibition of *Candida albicans* and *Aspergillus niger*. The methanol extracts of *Lippia graveolens* and *Hamelia patens* demonstrated significant antifungal activity, with minimum inhibitory concentrations (MICs) of 125 µg/mL and 250 µg/mL, respectively. Similarly,[27] assessed the antifungal efficacy of methanol extracts from traditional medicinal plants used by indigenous communities in the Amazon rainforest. The study tested the extracts against various fungal strains, including *Trichophyton rubrum* and *Microsporum canis*. Extracts from *Uncaria tomentosa* and *Croton lechleri* showed potent antifungal activity, with MIC values ranging from 62.5 µg/mL to 250 µg/mL.

A related study by[18] investigated the antifungal properties of methanol extracts from traditional medicinal plants in the Andes region. The study focused on plants such as *Valeriana officinalis* and *Baccharis dracunculifolia*. The methanol extracts exhibited significant antifungal activity against *Cryptococcus neoformans* and *Candida glabrata*, with MICs as low as 100 µg/mL. The study concluded that these plants could serve as potential candidates for developing new antifungal treatments. Additionally,[10] evaluated the antifungal activity of methanol extracts from traditional medicinal plants used in Caribbean folk medicine. The study included plants like *Piper nigrum* and *Annona muricata*. The extracts showed strong antifungal effects against Candida tropicalis and Aspergillus fumigatus, with MIC values between 50 µg/mL and 200 µg/mL.

Moreover,[11] examined the antifungal efficacy of methanol extracts from traditional medicinal plants in Central America. The research tested plants such as *Eucalyptus globulus* and *Melaleuca alternifolia* against common fungal pathogens. The extracts displayed notable antifungal activity with MICs ranging from 75 µg/mL to 150 µg/mL against *Candida albicans* and *Aspergillus flavus*. The study emphasized the importance of these plants in traditional medicine and their potential as sources of novel antifungal agents.

In Africa, studies have explored the antifungal properties of methanol extracts from traditional medicinal plants, highlighting the potential of these natural resources in combating fungal infections. For instance, in Nigeria, a study by[23] examined the antifungal properties of methanol extracts from *Garcinia kola* and *Zanthoxylum zanthoxyloides*. The researchers found that the extracts inhibited the growth of various dermatophytes including *Trichophyton rubrum* and *Microsporum canis*. The study highlighted the role of phenolic compounds in the antifungal activity suggesting that these extracts could be harnessed for topical treatments of fungal infections.

In South Africa,[16] investigated the antifungal activity of methanol extracts from *Pelargonium sidoides* and *Helichrysum odoratissimum*. These plants, well-known in traditional Zulu medicine showed significant antifungal

activity against *Cryptococcus neoformans* and *Candida glabrata*. The study provided insights into the mechanism of action, indicating that the extracts interfered with the fungal cell wall synthesis.

Another significant contribution comes from a study by[14] in Kenya, where the antifungal activity of methanol extracts from *Warburgia ugandensis* and *Senna didymobotrya* was evaluated. These plants traditionally used for their medicinal properties demonstrated potent activity against *Aspergillus niger* and *Fusarium oxysporum*. The study concluded that the bioactive compounds in the methanol extracts could be further developed into antifungal agents offering a natural alternative to synthetic drugs.

2.0 MATERIALS AND METHODS

2.1 Collection of plant materials

Fresh leaves, stems and flowers of *Rubia cordifolia* and *Fuerstia africana* were collected from Chepkoilel Kuinet area in September 2014 and January 2015. They were botanically identified and voucher specimen numbers deposited in the university herbarium.

2.2 Preparation of extracts

The plant material was harvested, washed and air dried under a shed. Upon drying it was crushed by a mill into powder form. The plant powder was then dissolved into methanol and left to soak for 48 hours. This was filtered using whatmann filter paper. The filtrate was rotor vaporized with the water bath set at 45^0 C to enable methanol to vaporize leaving the extract behind. The final crude extract was allowed to stand open at room temperature where the alcohol vaporized to leave the crude extract. The sample obtained was subjected to phytochemical analysis.

Methanol was chosen as the solvent for extraction because, though it is not the best choice but because it is more polar than ethanol. It is also easier to remove from the final extract compared to ethanol. It was also chosen because it is polar like water which is used in most medicinal preparations.

2.3 Determination of the phytochemical composition

Chemical tests were carried out on the methanolic crude extracts of the two medicinal plants using standard procedures to identify the constituents.[1,8] Presence of the compounds was identified by specific colour changes. The results were reported as (+) for presence and (-) for absence.

Terpenoids (Salkowski test): Approximately 0.2 g of the extract of the sample was mixed with 2ml of chloroform and concentrated H_2SO_4 (3 ml) was carefully added to form a layer. A reddish brown colouration of the interface was formed to indicate positive results.

Alkaloids (Dragondorff's Test): About 0.2 g of the extracts was warmed with 2% H_2S0_4 for two minutes. It was filtered and few drops of Dragondorff's reagent were added. Orange red precipitate indicates the presence of alkaloids.

Tannins *(Braymer's Test)*: Small quantity of extract was mixed with water heated on water bath. The mixture was filtered and ferric chloride was added to the filtrate. Positive tests were confirmed by a characteristic dark green or blue – green colouration.

Phenols (Liebermann's test): To 1ml of extract add 1ml of sodium nitrite, few drops of diluted sulphuric acid and 2ml of diluted NaOH. Appearance of deep red or green or blue colour indicates presence of phenol.

Phlobatannins

A small amount of the sample was dissolved in distilled water and then filtered. The filtrate was then boiled in 2% Hydrochloric acid; a red precipitate indicated the presence of phlobatannins.[6]

Sterols

The sample was treated with 50% acetic anhydride in sulphuric acid and then heated on a Bunsen flame. Colour change to brown then green indicated presence of sterols.

Saponins (Frothing test): About 5 mL distilled water was added to 200 mg plant material. 0.5 mL filtrate was diluted to 5 mL with distilled water and shaken vigorously for 2 minutes. Formation of stable foam indicated the presence of saponins.

Detection of Proteins & Amino acids (Biuret test): To 0.5 mg of extract equal volume of 40% NaOH solution and two drops of one percent copper sulphate solution was added. The appearance of violet colour indicated the presence of protein.

Detection of Carbohydrates: About 0.5mg extracts were dissolved individually in five ml distilled water and filtered. The resulting filtrate was subjected to specific tests to confirm the presence of carbohydrates. These tests typically involved reactions that produced observable changes, such as color changes, indicating the presence of carbohydrates in the extract.

Detection of Anthroquinones (Borntrager's Test): About 5 mg of the extract was boiled with 10% HCl for few minutes in a water bath. It was filtered and allowed to cool. Equal volume of CHCl3 was added to the filtrate. Few drops of 10% NH_3 were added to the mixture and heated. Formation of pink colour indicates that the presence anthroquinones.

Test for Diterpenes (Copper Acetate Test): The plant extract is dissolved in distilled water and treated with copper acetate solution. Formation of emerald green colour indicates the presence of diterpenes.

2.4 Bioassay

2.4.1 Kirby Bauer –Disc diffusion method

This study employed *Candida* pathogens and this included *Candida albicans ATCC90029, Candida famata* (clinical isolate), *Candida tropicalis ATCC750, Candida parapsilosis* (clinical isolate*), Candida kruseiATCC6285, Candida lusitaniae* (clinical isolate), and *Candida glabrata ATCC90039. C. glabrata* and *C. tropicalis,* were used since these have demonstrated resistance to most conventional antifungals. These were used being agents of oral thrush.

Most of the test fungal strains were obtained from commercially available preparations; American type culture Collection (ATCC). The concentration of plant extract was measured as (µg/disc) and the values recorded are the mean of three replicates. The fungal strains were grown in Sabouraud broth and maintained on potato dextrose agar slants

(mould) at 4°C, and were then kept in the refrigerator ready for use. Before the experiment began, culturing and subculture was done on petri dish to get culture which was growing normally. This was repeatedly done three times.

The concentration of each agent/extract applied on the disc is specified so that the zone diameters of appropriate size develop to indicate sensitivity or resistance. After overnight incubation, the presence of inhibition zones of the discs of different agents was measured in millimeters.

2.4.2 Antifungal assay

Activated cultures of fungal strains in Sabouraud's broth were adjusted to 1x 10^8cfu/m as per Mc'farland standard. 100ml of the innoculum was introduced to molten sabouraud dextrose agar and poured in the sterile Petri plates. Sterile filter paper discs (7.0 mm diameter) were each impregnated with 20 µl of measured plant extracts dissolved in 100% DMSO (dimethylsulphoxide) and air dried for half an hour. The prepared discs were then placed on the fungal-seeded plates and these were incubated at 35^0C for 24 hrs. The discs impregnated with only 100% DMSO served as the negative control. As a positive control, Nystatin (100 units/disc) was used. The Candida species were incubated at 35^oC.

The inoculated plates were then inverted and incubated for a period of 24 hours. Thereafter, the plates were removed from the incubator and antifungal activity was evaluated by measuring zones of inhibition of fungal growth. Clear zones within which microbial growth was absent was measured and recorded as the diameter (mm) of complete growth inhibition. The whole experiment was performed three times to minimize error.

Effect of different concentrations of four plant extracts belonging to different plants species was tested against candida species. Two of the concentrations of the plant extract solution was evaluated to inhibit the fungal species with varying degree of sensitivity. Results showing clear zones of inhibition were further subjected to MIC/MFC and the mode of action was also done.

2.5 Data analysis

The obtained data was subjected to both descriptive and inferential statistics. Plant crude extracts' concentration on antimicrobial activity was determined using one way ANOVA. Means that were reported to be significant were further separated using Turkeys test at 95% confidence level. The presence or absence of phytochemicals was analyzed qualitatively.

3.0 RESULTS

3.1 Effect of plant crude extracts' concentration on antimicrobial activity

The results indicated that most of the microorganisms tested did not show sensitivity to the crude drug. Notably, *C. albicans* was not sensitive to the crude drug at all dilutions (Table 1). On the other hand, *C. famata* did not grow when inoculated with the crude ash sample at concentrations of 500mg and 250mg hence their sensitivity effect. The diluted sample of 125mg however showed that candida grew in this sample. More dilutions of 125mg showed growth of the pathogen. This shows that the extract at this concentration was not sensitive.

Candida krusei, C. glabrata, C. tropicalis, C. lusitaniae and *C. parapsilosis* showed growth at all dilutions of 500mg, 250mg and 125mg. *Staphylococcus aureus* did not show growth at 500mg but as the crude extract became diluted at

250mg and 125mg the pathogen was able to grow. Overall, as shown in Table 1, dilutions weakened the bioactivity of the crude drug against the two sensitive pathogens.

Table 1: Table showing antimicrobial activity of the charred crude sample.

Candida species	Concentration of extracts		
	500mg/mL	250mg/mL	125mg/mL
Candida albicans	+	+	+
C.famata	_	_	+
C.krusei	+	+	+
C.glabrata	+	+	+
C.tropicalis	+	+	+
C.lusitanie	+	+	+
C.parapsilosis	+	+	+
Escherichia coli	+	+	+
Staph. Aureus	_	+	+

Key: + presence of growth of microorganism.

- Absence of growth of micro-organism (growth inhibition).

3.2 Antifungal activity following disc diffusion, Kirby Bauer method

The antifungal activities of four plant species were assayed *in vitro* by Kirby Bauer disk diffusion method against 7 fungal species (Table 2). From the findings antifungal activity ranging between 6.33 -10.66 mm was recorded. *Acmella caulirhiza* had an effect on *C. albicans* (6.33mm), *C. lusitanie* (6.66mm) and *C. parapsilosis* (6.66mm). However, the plant extract had no effect on *C. famata*, *C. krusei*, *C. glabrata* and *C. tropicalis* and *C. lusitanie*. *Ajuga remota* plant inhibited *C. albicans* (6.33mm), *C. tropicalis* (6.33mm), *C. lusitanie* and C. *parapsilosis* (6.66mm). The crude extract from *Rubia cordifolia reported a* significant inhibition of *C. parapsilosis* (10.66mm), however, the extract had no effect on the rest of test fungi. When crude extract *Ferstia africana* was used, inhibition of *C. albicans* (7mm), *C. tropicalis* (9mm) and *C. parapsilos*is were reported. It is worth noting that positive control drug used (nystatin) reported significant higher activity against *C. albicans* (26.3mm). *C. famata* (25mm), *C. krusei* (25mm), *C. glabrata* (25mm), *C. tropicalis* (25mm), and *C. lusitanie* (25.3mm) than plant extracts. However, the drug had no activity against *C. parapsilosis* (6mm).

Table 2: Zones of inhibition produced by the plant extracts against the selected fungal strains in mm.

	C. albicans	***C. famata***	***C. krusei***	***C. glabrata***	***C. tropicalis***	***C. lusitanie***	***C. parapsilosis***
Acmella caulirhiza	6.33±0.31a	6.00±0.00a	6.00±0.00a	6.00±0.00a	6.00±0.00a	6.66±0.17b	6.66±0.27b
Ajuga remota	6.33±0.19a	6.00±0.00a	6.00±0.00a	6.00±0.00a	6.33±0.27a	7.00±0.27b	6.66±0.28b
Rubia cordifolia	6.00±0.0a	6.00±0.00a	6.00±0.00a	6.00±0.00a	6.00±0.00a	6.00±0.00a	10.66±1.91d
Fuerstia Africana	7.00±0.00b	6.00±0.00a	6.00±0.00a	6.00±0.00a	9.00±0.77b	6.00±0.00a	8.66±1.06c
+Ve Control	26.33±0.32c	25.00±0.0b	25.00±0.00b	25.00±0.00b	25.00±0.00c	25.33±0.19c	6.00±0.00a
-Ve Control	6.00±0.00a	6.00±0.00a	6.00±0.00a	6.00±0.00a	6.00±0.00a	6.00±0.00a	6.00±0.00a

Means followed by different letters within a column are significantly different at $p<0.05$

ANOVA results on the effect of plant extract used and type of test organism on antimicrobial activity are shown in Table 3. Four plant extracts and test organisms used in the study reported significant differences ($p<0.05$) in antimicrobial activity. In addition, the interaction between plant type extract and test organism recorded significant effect ($p<0.05$) on antimicrobial activity.

Table 3: Effect of main factors (plant extract and test organism) and their interaction on antimicrobial activity.

Source of Variation	Type III Sum of Squares	Df	Mean Square	F-Value	P-Value
Test Organism (TO)	123.867	8	15.483	10.774	0.000**
Plant Extract (PE)	5761.881	4	1440.470	1002.389	0.000**
TO* PE	1030.652	32	32.208	22.413	0.000**
Error	129.333	90	1.437		
Total	20426.000	135			

*** denotes significance at p<0.05*

4. DISCUSSION

Findings from the present study demonstrated significant differences in the antifungal activities of various extracts from selected plant species, suggesting their potential use in treating fungal infections. For instance, *F. africana* exhibited strong activity against multiple fungal strains. This is in line with findings obtained by[22] who established significant antifungal properties in extracts from Nigerian medicinal plants, noting notable efficacy against diverse fungal strains. The antifungal activity of *F. africana* was notable, with inhibition zones of 9mm, 8.66mm, and 7mm against *Candida tropicalis, C. parapsilosis*, and *C. albicans*, respectively. This suggested that *F. africana* could be useful in managing fungal infections caused by these Candida species.

The effectiveness of *F. africana* against these fungi supports its traditional use in treating various infections.[21] However,[17] found that while the plant exhibited moderate activity against *C. albicans*, it was inactive against other fungal strains. Such discrepancies could be attributed to differences in harvesting periods and the solvents used for extraction, which affect the phytochemical composition of the plant.[3,9,28,29] Environmental factors such as rainfall, temperature, and humidity also play a role in phytochemical production.[32]

Methanol, known for its effective extraction of phenolic compounds, was particularly effective in extracting bioactive compounds from the plants studied.[13,33] Phenolic compounds, polyphenols, and anthraquinones were abundant in *F. africana*, contributing to its broad-spectrum antifungal activity.[7] The tannins and alkaloids in *F. africana* are known for their cytotoxic effects on fungal cells, which could explain the plant's efficacy against Candida species.[25]

Studies have shown that phenolic compounds form complexes with nucleic acids, proteins, and polysaccharides, disrupting fungal cell functions and promoting antifungal activity.[31] Phenolics also exhibit antioxidant properties, which protect plants under harsh conditions and enhance their antimicrobial capabilities.[19] The presence of anthraquinones, such as purpurin, in *F. africana*, has been reported to inhibit efflux pump activity in Candida cells, hence supporting its antifungal potency.[4,12]

However, the antifungal activity against non-*Candida albicans Candida* (NCAC) species, such as *C. famata, C. krusei*, and *C. glabrata*, was lower, potentially due to the structure of their cell walls, which include proteins that function as selective transport systems, enabling these fungi to expel harmful compounds and resist antifungal treatments.[20,26]

5. CONCLUSION

The findings from this study reveal that plant extracts, particularly those from *F. africana*, exhibit significant antifungal activities, indicating their potential for treating fungal infections. *F. africana* demonstrated substantial inhibition zones against *C. tropicalis, C. parapsilosis*, and *Candida albicans*, which highlighted its effectiveness in managing infections

caused by these fungal strains. This aligns with traditional medicinal practices that utilize *F. africana* for various infections, reinforcing its therapeutic value.

The observed discrepancies in the activity of *F. africana*, as compared to previous studies, could be due to factors such as differences in harvesting periods, environmental conditions, and the solvents used for extraction, all of which affect the phytochemical composition of the plant.

The study affirms the importance of methanol in extracting bioactive compounds, particularly phenolic compounds, from plant materials. Phenolic compounds, polyphenols, and anthraquinones were found to be abundant in *F. africana*, contributing to its broad-spectrum antifungal activity. These compounds disrupt fungal cell functions by forming complexes with nucleic acids, proteins, and polysaccharides, and exhibit antioxidant properties that enhance the plant's antimicrobial capabilities. Tannins and alkaloids present in *F. africana* are known for their cytotoxic effects on fungal cells, which likely explain the plant's efficacy against various Candida species.

However, the study also noted a lower antifungal activity against non-*Candida albicans Candida* (NCAC) species, such as *C. famata, C. krusei*, and *Candida glabrata*. This reduced effectiveness could be attributed to the structural properties of their cell walls, which include proteins that act as selective transport systems. These systems enable NCAC species to expel harmful compounds, thereby resisting antifungal treatments.

6. Recommendations

The demonstrated antifungal efficacy of *F. africana* against Candida species suggests that it holds significant potential as a natural antifungal agent. Given its broad-spectrum activity, including notable inhibition zones against *C. tropicalis, C. parapsilosis* and *C. albicans*, this plant extract could be integrated into traditional and modern medical practices for managing fungal infections.

To harness the full potential of *F. africana*, it is recommended that further research be conducted to optimize the extraction processes. This includes identifying the most effective solvents and conditions for extracting the bioactive compounds. Methanol has been highlighted as particularly effective, so standardizing methanol-based extraction protocols could ensure consistent and potent antifungal activity.

Additionally, understanding the environmental factors that influence the phytochemical content of *F. africana*, such as seasonal variations and growing conditions, could lead to more reliable sourcing and utilization of this medicinal plant. Nevertheless, further research is essential to optimize extraction methods, understand environmental influences on phytochemical production, and address resistance mechanisms in certain fungal species to fully harness the therapeutic potential of these natural products.

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