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Antifungal activity and safety of *Maesa lanceolata* for the treatment and management of selected fungal pathogens

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Abstract
Infectious diseases affect human health. Current research focused on *in vitro* antifungal activity and safety of aqueous, dichloromethane and methanolic extracts of *Maesa lanceolata* against *Candida glabrata* ATCC 24433, *Cryptococcus neoformans* ATCC 66031, *Microsporum gypseum, Trychophyton mentagrophtes* and *Aspergillus flavus* using agar-disc diffusion and broth dilution methods. Bioassay of leaves, roots and stem bark extracts were determined at the Kenya Medical Research Institute. Root and stem bark water extracts were highly active against *C. glabrata* ATCC 24433 with zone inhibition diameter and minimum inhibitory concentration of 23.33mm, 31.25 mg/ml and 19.67mm, 125mg/ml respectively. Methanolic and aqueous root extracts were active against *C. neoformans* showing 18mm and 15.625mg/ml respectively. The phytochemicals present were alkaloids, phenols, terpenoids, anthraquinones and tannins. Extracts displayed cytotoxicity on Vero E6 cell lines with cytotoxicity concentration (CC_{50}) ranging from 206 µg/ml to 684 µg/ml. The activity of *M. lanceolata* extracts confirms its use in traditional medicine.

Keywords: *Maesa lanceolata,* fungi pathogens, antifungal activity, extracts

Introduction
The utilization of plants as medicine is an old practice common to all societies particularly the developing nations. However, scientific research on medicinal plants with the intention of developing the best medicines for therapeutic purposes has become critical. *Maesa lanceolata* Forsk also commonly known as false assegai, and belonging to the family Myrsinaceae is a sprawling shrub, 2 to 3 m tall, or a small tree with a single stem up to 9m tall, or a rounded bushy tree with branches almost at ground level. It thrives on stream banks, cliff tops in both midland and coastal areas to about 1500 m above sea level [1]. In some parts of Kenya, the plant is traditionally used for the treatment of helminthes and bacterial infections [2]. Among the Marakwet community, the stem bark is cut to pieces, boiled and used to treat individuals with itchy skin rashes and dermatophytic conditions after bathing in stem bark decoctions [3]. Other authors [4] have investigated the antimicrobial activities of *M. lanceolata* leaves using aqueous and methanolic extracts and also isolated saponins, new triterpene including triterpeneglycosides. Furthermore, biological activity studies revealed MIC values of 100µg/ml for *Vibrio cholera* and 125 µg/ml for *Salmonella typhi.* However, there were no biological activities reported for other organic extracts and also on other microbial strains particularly fungi. The recognition of traditional medicine as an alternative form of health care by the general public and the development of microbial resistance to the available antibiotics has led many scientists to examine the antimicrobial activity of several medicinal plants [5, 6, 7]. Currently, therapy and management of opportunistic fungal infections is limited. Furthermore, emerging antimicrobial drug-resistant bacterial strains such as staphylococci and enterococci, merit research into novel therapeutic alternatives and medicinal plants contemporaneous potential candidates. The current research focusses on the evaluation of *in-vitro* antifungal properties and cytotoxicity of methanol, aqueous and dichloromethane extracts of *M. lanceolata.*

Materials and Methods

Plant Materials

Maesa lanceolata roots, leaves and stem bark were collected on the basis of indigenous knowledge from Kapsowar, Elgeyo Marakwet county of Kenya. Taxonomical identification was done at University of Eldoret herbarium by a botanist and voucher specimen number MU/0038/87 deposited.

Extraction Procedure

The plant parts (roots, leaves and stem bark) were air dried at room temperature (25 °C) under shade for 14 days, pulverized using a laboratory mill (Christy &Norris Ltd., Chelmsford, England) and packed in airtight polythene bags at the Center for traditional medicine and drug research (CTMDR). Fifty grams of the powdered plant material was extracted using 500 ml distilled water in a shaking water bath set at 70 °C for two hours. The mixture was filtered and dried using a freeze dryer (Edwards freeze dryer Modulyo) then weighed and stored. Similarly, soaking of the powdered plant material was done using methanol, and dichloromethane for 24 hours each and the organic solvents evaporated to dryness by vacuum evaporation using a rotary evaporator (Büchi Rotavapor R-114). Percentage yield was calculated as follows:

$$\% \text{ yield} = \frac{\text{Weight of extract obtained}}{\text{Weight of powdered material}} \times 100$$

Fungi test cultures

The fungi strains used were *Candida glabrata* ATCC 24433, *Cryptococcus neoformans* ATCC 66031(yeast), *Aspergillus flavus* and clinical isolates of dermatophytes; *Microsporum gypseum* and *Trychophyton mentagrophytes.* All the fungal strains were obtained from the Mycology Laboratory, KEMRI culture collection and maintained as stock cultures in 50% glycerol in Eppendorf® tubes at -30 °C until when need arose.

Determination of antifungal activity

Disc diffusion method [8] was used to evaluate the antimicrobial activity of *M. lanceolata* against *C. glabrata* ATCC 24433, C. *neoformans* ATCC 66031, *A. flavus, M. gypseum* and *T. mentagrophytes* cultures at 30 °C for 72 hours on Sabouraud Dextrose Agar (SDA, Oxoid). The SDA test plates were set and inoculated on their surface with a cell suspension of the test fungi (1.5 x 10^8 cfu/ ml) in sterile normal saline. All the test assays were carried out in a Class II Biological Safety Cabinet. Sterile Whatman's No.1 (6mm diameter) discs were impregnated with 20 µl of the extracts from the stock solution of 100 mg/ml and utilized for the disc diffusion assay. The discs were then aseptically placed on the SDA. Fluconazole discs (25µg) were used as the reference drug while discs containing sterile distilled water were used as negative controls. The test plates were incubated at 30 °C for 72 hours. Each assay was done in triplicates. The zones of inhibition diameters were measured in millimeters and the findings expressed as mean inhibition zones ± standard deviation.

The minimum inhibitory concentration (MIC) was determined for extracts exhibiting inhibition zone diameter of ≥10 mm against the test microorganism using the disc diffusion technique. Serial dilutions of the extract was carried out using distilled water culminating in a working concentration range from 500 mg/ml to 3.90625 mg/ml. Sterile filter paper discs containing 20µl of the dissolved The extracts were positioned on the surface of SDA media with inoculated test fungi. MIC was described as the lowest concentration of the extract that displayed clear zone of inhibition [9].

Phytochemical Screening

Phytochemical screening was done on the active extracts to pinpoint the phytocompounds present. The phytochemical constituents of the different extracts were separated by thin layer chromatography (Kieselgel 60 F254 0.2 mm, Merck). Thin layers chromatography (TLC) plates were developed with Ethyl acetate: petroleum spirit (3:7) as the solvent system for dichloromethane extracts while dichloromethane: methanol (9.5: 0.5) solvent system was utilized for methanol extracts [10]. The separated constituents were visualized under ultra violet light (254 nm and 365 nm) then sprayed with visualizing agents for colorimetric view.

Cytotoxicity Assay

The most active plant extracts were examined for *in vitro* cytotoxicity according to the modified rapid calorimetric assay [11] using Vero E6 cancer cell lines obtained from American Type Culture Collections (ATTC). The Vero cells were maintained in Eagle's Minimum Essential Medium (MEM) supplemented with 10 % fetal bovine serum (FBS) and 2 mM L-glutamine. Approximately 2 x 10^5 cell/ml suspensions were seeded on 96-well microtiter plates and incubated in a humified atmosphere with 5 % CO_2 at 37 °C for 12 hours. Test extracts were added to the cultured cells over a concentration range of 1000 µg/ml to 1.23 µg/ml. The plates were incubated at 37 °C, 5% CO_2 for 48 hours following which 10µL of (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) MTT dye was added into each well. Incubation of the plates was carried out for another 4 hours and subsequently the media was removed from the wells and 100µL of Dimethlsulphoxide (DMSO) were added [11]. The plates were read (color absorbance) using an ELISA scanning Multiwell spectrophotometer (Multiskan Ex Labssytems) at 562 nm and 620 nm as reference. The percentage cell viability (CV) was calculated using the formula [12]:

$$\% \text{ CV} = \frac{\text{Average abs of duplicate drug wells} - \text{Average abs of blank wells}}{\text{Average abs of control wells}} \text{ x } 100\%$$

Data was entered onto a worksheet (Microsoft Office Excel 2007) and expressed as percentage of the untreated controls. The cytotoxic concentration responsible for lysis and death of 50% of the cells was determined by linear regression analysis.

Results

Extraction of the plant

The percentage yields derived from different solvent extracts are summarized in Table 1.

Table 1: Percentage yields for plant extraction

	Percentage yield (%)		
Plant part	**Dichloromethane**	**Methanol**	**Water**
Leaves	2.2	2.65	4.6
Roots	1.4	5.1	5.2
Stem bark	1.8	4.8	12.1

Out of the 200g of each powdered plant material the percentage extract yield was calculated. Aqueous stem bark presented the highest yields of 12.1 % while dichloromethane leaves presented the least yield 2.2 % (Table 1).

Antifungal Bioassay

Table 2: *In-vitro* activity of *M. lanceolata* and minimum inhibitory concentration against selected fungi

Fungi	Part	ZD (mm) ± SD and MIC (mg/ml)			
		Methanol	DCM	Water	Fluconazole
C. glabrata	L	6.3±0.6 (*)	7.3±0.6 (*)	8.0±1.0 (*)	23.0±1.0 (25)
	R	7.0±0.0 (*)	7.0±1.0 (*)	23± 1.0 (31.25)	22.7±0.6 (25)
	SB	7.3±0.6 (*)	6.7±0.6 (*)	19.7±1.5 (31.25)	23.3±1.2 (25)
M. gypseum	L	7.3±1.2 (*)	7.3±0.6 (*)	7.3±0.6 (*)	16.0±1.0 (25)
	R	8.0±1.0 (*)	9.7± 1.5(*)	8.0±1.0 (*)	17.0±0.0 (25)
	SB	11±1.0 (62.5)	8.0± 1.0(*)	10.3±1.5 (125)	16.7±0.6 (25)
T. mentagrophytes	L	9.0±2.7 (*)	7.0± 1.0(*)	7.0±1.0 (*)	15.0±1.0 (25)
	R	11.0±1.0 (62.5)	10.7± 1.5(62.5)	10.0± 2.0(62.5)	16.0±0.0 (25)
	SB	14.7±0.6 (1.25)	9.0±1.0 (*)	9.0± 2.0 (*)	16.3± 0.6(25)
C. neoformans	L	6.0±0.0 (*)	7.0±1.0 (*)	7.0±1.0 (*)	20.0±0.0 (25)
	R	18.3±0.6(15.63	9.0±2.0 (125)	19.7±0.6 (15.63)	19.7±1.5 (25)
	SB	17.3±1.2 (62.5)	14.7± 2.1(62.5)	11.0± 1.0(62.5)	20.0± 0.0 (25
A. flavus	L	6.7± 0.6 (*)	6.0± 0.0 (*)	6.0± 0.0 (*)	14.7± 0.6 (25
	R	7.0± 1.0 (*)	6.0± 0.0 (*)	6.0± 0.0 (*)	13.0± 1.0(25)
	SB	7.0± 1.0 (*)	6.3± 0.6 (*)	6.7± 0.6 (*)	14.0± 0.0(25)

Key: L- leaves, R- roots, SB- stem bark. (ZD) zone diameter of microbial inhibition, (MIC) Minimum inhibitory concentration, (*) MIC value not considered for extracts with ZD<10mm (little or no activity against the selected pathogens); 6mm is the diameter of the disc (no activity).

Generally, in all extracts stem bark showed higher degree of activity against the selected fungi. Root and stem bark extracts of water were highly active against *C. glabrata* with a zone diameter and minimum inhibitory concentration of 23mm, 31.25 mg/ml and 19.7 mm and 19.7 mm, 25 mg/ml respectively. Moderate activity was observed in other fungi (*C. neoformans, M. gypseum* and *T. mentagrophytes*) bearing zone inhibition diameter range between 8.0 mm to 15 mm. Major activity was detected with water and methanolic extracts of roots presenting zones of inhibition diameter of 18 mm with MIC value 15.625 mg/ml and 19 mm with MIC value 15.625 mg/ml respectively against *Cryptococcus neoformans*. It was noted all the herbal extracts were not active against *Aspergillus flavus* as depicted by the little or no activity; 7 mm or 6 mm zone inhibition diameter. Fluconazole was used as the standard (Table 2).

Phytochemical Screening

Preliminary screening for phytochemicals revealed the abundant presence of flavonoids, phenols, terpenoids, anthraquinones, saponins and alkaloids on the leaves, roots and stem bark extracts derived from water and dichloromethane while their presence were moderate with regard to methanol extracts. Tannins were absent in methanol derived extracts of the leave, roots and stem bark (Table 3).

Table 3: Phytochemical constituents present in *M. lanceolata* extracts.

Solvent	Plant part	Tannins	Flavonoids	Phenols	Saponins	Terpenoids	Alkaloids	Anthraquinones
Water	L	++	+++	+++	+++	+++	+++	+++
	R	+++	+++	+++	+++	+++	+++	+++
	SB	++	++	+++	+++	+++	+++	+++
DCM	L	++	+++	+++	+++	+++	+++	+++
	R	++	++	++	++	+++	+++	++
	SB	+	++	+++	+++	+++	+++	+++
Methanol	L	-	++	++	+++	+++	++	+++
	R	-	+	+	++	++	++	++
	SB	-	++	++	++	+	++	++

Key: L -Leaves, R - Roots, SB - Stem bark, DCM - Dichloromethane. +++ Abundant, ++ Moderate, + Trace, - Absent

3.4 Cytotoxicity Studies

Cytotoxicity against Vero cell lines were determined for extracts displaying higher antifungal activity as indicated by their MIC value of ≤125 mg/ml. Four extracts of *M. lanceolata* were determined giving their cytotoxicity concentration (CC_{50}) values. Dichloromethane leave, methanolic leave and dichloromethane stem bark extracts were lowly toxic with CC_{50} values of 684.995 µg/ml, 546.86 µg/ml and 322.08 µg/ml respectively. Aqueous stem bark extract was moderately toxic with CC_{50} value of 206.45 µg/ml (Table 4).

Table 4: Cytotoxicity activity of *M. lanceolata* extracts against Vero E6 cell lines

Plant Extract	MIC value (mg/ml)	CC_{50} values ± SD (µg/ml)
DCM leave	125	684.995±0.332^{d}
Aqueous stem	125	206.445±1.874^{a}
Methanolic leave	31.25	322.08±0.679^{b}
DCM stem bark	62.5	564.86±1.249^{c}

CC 50 values; (mean ± SD) µg/ml. Means followed by the same letter within a column are not significantly different at $p<0.05$

Discussion

Antifungal activity of *Maesa lanceolata* against the fungi tested were obtained for each of the three extracts under investigation. Greater activity was observed with aqueous root and stem bark extracts of *M. lanceolata* against *Candida glabrata* ATCC 90030 all presenting MIC values of 31.25 mg/ml. Similarly, aqueous and methanolic extracts of *M. lanceolata* roots were very active against *Cryptococcus neoformans* providing proof of the claimed therapeutic value in fungal infections. *M. lanceolata* extracts have been used for the treatment of helminthes, fungal and bacterial infections [13]. In East Africa fruits of *M. lanceolata* are widely used to treat a variety of ailments such as sore throat, tapeworms, hepatitis and cholera [14]. Among the Marakwet community, stem bark is cut to pieces, boiled where individuals with itchy skin rashes and dermatophytic infections bath with it getting heal upon consistent application [3]. A study by [15] indicated that *M. lanceolata* roots are used as a purgative to remove pimples.

Phytochemical screening of the *M. lanceolata* extracts revealed the presence of phenols, tannins, flavonoids, alkaloids and anthraquinones. The antifungal activity observed in this study could be attributed to the occurrence of flavonoids, phenols, terpenoids and anthraquinones. Despite the common belief that phytochemicals are safe, many plant metabolites are very toxic and may possess the potential to cause serious side effects [16]. Toxicity studies are very critical in determining the efficacy of medicinal plants. The MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] colorimetric assay established by [11] serves to monitor for cytotoxic activity of all test samples.

Extracts that displayed MIC values of 1.25×10^5 µg/ml and below were considered potent and their cell toxicity were investigated. The *in vitro* cytotoxicity of the crude extracts of the four extracts of *M. lanceolata* are presented in Table 4. Inhibitory concentration at 50% (CC_{50}) is the concentration of the extracts that inhibits 50 % proliferation of the Vero E6 cell lines. The extracts had varying degrees of toxicity on the Vero E6 cell lines with IC_{50} values ranging from to 684.76 µg/ml. The criteria used to distinguish the activity of *M. lanceolata* extracts against Vero E6 cell lines based on CC_{50} values modified from those of National Cancer Institute (NSI) and [17, 18] as follows: IC_{50} value ≤ 20 µg/ml = extremely toxic (very active), CC_{50} 21-200 µg/ml = highly toxic (moderately active), IC_{50} 201-500 µg/ml =moderately toxic (weakly active), CC_{50} ≥501-1000 µg/ml = lowly toxic (inactive), CC_{50} ≥1000 µg/ml = particularly non-toxic. The cytotoxicity data for the *M. lanceolata* extracts are displayed in Table 4. Both DCM extracts of *M. lanceolata* stem bark and leaves were lowly toxic or inactive against Vero E6 cell lines with CC_{50} of 546.86±1.249 µg/ml and 684.0.332±0.332 µg/ml respectively. In contrast, aqueous stem extracts and methanol leave extracts of *M. lanceolata* were weakly active and that moderately toxic with CC_{50} values of 322.08±0.679 µg/ml and 206.445±1.874 µg/ml respectively. The *in vitro* cytotoxicity determined on Vero cells indicated that most of extracts were relatively non-toxic. This suggests that *M. lanceolata* extracts can be safe as antimicrobial agent. These results are in tandem with the findings of [19] who discovered that crude extracts of *M. lanceolata* possess chemotherapeutic compounds that can serve to protect other plants against fungal infections.

Conclusion

The methanol and aqueous root and stem bark extracts of *M. lanceolata* assayed exhibited a promising activity against *Cryptococcus neoformans*, *Candida glabrata,* dermatophytes and notwithstanding their relative non-toxic nature support to a certain degree their traditional uses. The need to clarify which particular phytocomponent responsible for one or both activities is crucial.

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