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Research Article

Antimicrobial properties and toxicity of *Hagenia abyssinica* (Bruce) J.F.Gmel, *Fuerstia africana* T.C.E. Fries, *Asparagus racemosus* (Willd.) and *Ekebergia capensis* Sparrm.

Lilian C. Ngeny [a,*], Esther Magiri [b], Charles Mutai [a], Nicholas Mwikwabe [a], and Christine Bii [c]

[a] *Center for Traditional Medicine and Drug Research, Kenya Medical Research Institute, Nairobi, Kenya*
[b] *Department of Biochemistry, Jomo Kenyatta University of Agriculture and Technology, Nairobi, Kenya*
[c] *Center for Microbiology Research, Kenya Medical Research Institute, Nairobi, Kenya*

* **Corresponding author:** Kenya Medical Research Institute, Centre for Traditional Medicine and Drug Research, P.O. Box 54840-00200 Nairobi, Kenya. **Tel**: +254-20-2722541 Ext 3326; **Fax**, 254-20-2720030; **Email**: Lngeny@Kemri.org

Background: The world health organization (WHO) estimates that 80% of population in Africa relies on traditional remedies for their healthcare. However, very few studies have been carried out to establish the therapeutic effects of these remedies.

Objective: Four medicinal plants were investigated for antimicrobial activity and toxicity.

Materials and Methods: Plants were collected from their natural habitat, dried, and extracted with organic and aqueous solvents. Antimicrobial activity was determined by the disc diffusion assay technique. *In vitro* cytotoxicity studies were carried out on extracts using MTT assay on Vero cell lines while acute toxicity in Swiss mice.

Results: Extracts from *H. abyssinica*, *F. africana* and *A. racemosus* exhibited antibacterial activity with minimum inhibitory concentration of ≤ 6.25mg/ml against *S. aureus*, MRSA and *P. aeruginosa*. However, the plants studied had weak antifungal activity. *H. abyssinica* and *F. africana* extracts were found to be cytotoxic with CC_{50} of < 90 µg/ml. These extracts were tested for acute toxicity and found to be safe at 5000 mg/kg body weight per day.

Conclusion: The results of the study support the medicinal use of these plants and indicate that useful compounds from *Hagenia abyssinica* and *Fuerstia africana* can be isolated for further exploitation.

Keywords: Medicinal plants, Antimicrobial activity, Cytotoxicity, Acute toxicity

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1. Introduction

Antimicrobial resistance is a major problem globally with the emergence of superbugs that are resistant to multiple classes of antibiotics such as methicillin-resistant *Staphylococcus aureas*, *Mycobacterium tuberculosis*, Enterobacter species, *Klebsiella pneumoniae*, *Acinetobacter baumanii* and *Pseudomonas aeruginosa* (Levy and Marshall, 2004). The most recent case is the superbug New Delhi metallo-beta-lactamase 1 (NDM-1), a gene carried by some bacteria that is resistant to most antibiotics. NDM-1 was initially isolated from *K. pneumonia* but has spread to organisms in the Enterobacteriaceae family (Moellering, 2010). NDM-1 is resistant to all classes of antibiotics except polymyxins, necessitating search for new antimicrobial agents.

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A number of plants have been documented to be used as antimicrobial agents in folkloric practises (Kokwaro, 1993; Beentje, 1994; Gachathi, 1989). Studies on a number of these plants have proved that plants can be a source of new antimicrobial agents (Charles Mutai et al, 2009; C Bii et al, 2010; Matu et al, 2012).

Hagenia abyssinica (Bruce) J.F. Gmel, *Fuerstia africana* T.C.E. Fries, *Ekebergia capensis* Sparrm and *Asparagus racemosus* (Willd.) that are traditionally used to treat diarrhea, tongue infections, sores, among other infections (Kokwaro, 1976; Beentje, 1994), were selected for the study.

2. Materials and Methods

2.1 Collection and processing of plant material

Plants were collected from their natural habitats in Olenguruone in Nakuru county and Cheptenye in Kericho county in 2009. Authentication of the plant species was done by a botanist from University of Nairobi, and samples were assigned voucher specimen numbers 2009/001-004, then deposited at the University of Nairobi Herbarium (**Table 1**).

Table 1: Plants collected, parts collected and voucher specimen numbers

Plant species	Family	Parts collected	Voucher Number
Hagenia abyssinica (Bruce) J.F. Gmel	Rosaceae	Leaves, Stem bark	2009/004
Fuerstia africana T.C.E. Fries	Lamiaceae	Aerial Parts	2009/003
Asparagus racemosus (Willd.)	Asparagaceae	Roots	2009/001
Ekebergia capensis Sparrm	Meliaceae	Stem Bark	2009/002

2.2 Extraction procedure and phytochemical screening

Plant samples were air dried under shade at room temperature for 2 weeks, then ground to fine powder using a laboratory mill. Powders were packed in air tight bags, weighed and stored in the dark.

Organic and water extracts were prepared for each sample. Organic extracts were prepared by extracting each sample successively with hexane, dichloromethane and methanol respectively. The filtrate after each successive extraction was concentrated under reduced pressure at 40ºC using a rotary evaporator. The resultant extract was weighed and stored in airtight sample bottles. For the water extracts, plant powder of each sample was soaked in distilled water and placed in a water bath set at 60ºC for 2 hours. The filtrate was freeze dried, weighed and stored until required for bioassays.

100 mg of each extract was weighed into a sterilized sample bottle and dissolved in DMSO (Sigma) to make a concentration of 100 mg/ml.

The major secondary metabolites classes such as alkaloids, anthraquinones, terpenoids, phenolics and flavonoids were screened according to the common phytochemical methods described by Harborne, 1973.

2.3 Microorganisms

Microorganisms used in the experiment were both standard reference strains sourced from American Type Culture Collection (ATCC) and clinical isolates obtained from KEMRI Centre for Microbiology Research (CMR) laboratories. The following bacteria and fungi were used in the experiment. Bacteria: *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureas* ATCC 25923, *Eschericha coli* ATCC 25922, *Klebsiella pneumoniae* (clinical isolate), Methicillin-resistant *Staphylococcus aureas (*clinical isolate). Fungi: *Trichophyton mentagrophytes* (clinical isolate), *Microsporum gypseum (*clinical isolate), *Candida albicans* ATCC 90028, *Candida krusei* ATCC 6258, *Candida parapsilosis* ATCC 22019, *Cryptococcus neoformans* ATCC 66031.

2.4 Antimicrobial activity

The agar diffusion method (Singh et al, 2006) was used to evaluate the antimicrobial activity. Bacteria were cultured overnight at 37 °C in Mueller Hinton Agar (MHA, Oxoid) and fungi at 30 °C for 72 h in Sabouraud Dextrose Agar (SDA, Oxoid) and used as inoculum.

Test plates were prepared with MHA and SDA and the plates and inoculated on the surface with a cell suspension in sterile normal saline. In all cases, the concentration of the inoculum was adjusted to 1.5×10^8 CFU/mL.

Paper discs prepared from Whatman No 1 and sterilized in an autoclave were used for the disc diffusion assay. The disc (6 mm in diameter) was impregnated with 10 µl of extracts from a stock solution of 100 mg/ml and placed on agar. Discs of Chloramphenicol (30 µg) was used as standard for bacteria while Fluconazole discs (25 µg) for fungi. Discs containing DMSO were used as negative controls.

The test plates were incubated at 37º C for 24 h for bacteria and at 35° C and 30 °C for 72 hr for both yeasts and dermatophytes respectively. Triplicate assays were carried out for each extract. Inhibition zones were measured in millimeters and results expressed as mean inhibition zones and standard error of means (SEM).

The Minimum Inhibitory Concentrations (MIC) was determined for extracts which had inhibition zones of ≥ 8 mm against the test microorganisms using the agar diffusion technique. Two fold serial dilutions of the extracts was performed in DMSO resulting in a concentration range from 100 mg/ml to 0.195 mg/ml.

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Table 2: Antimicrobial activity and percentage yield of extracts of selected plant species

Plant species (part)	Test Extract	Extract % yield	Mean Inhibition zones (mm) ± SEM							
			Sa	Mrsa	Pa	Kp	Ck	Cn	Tm	Mg
H. abyssinica (SB)	Hexane	0.09	9.00±1.00	9.50±0.96	-	-	-	-	7.00±0.33	-
H. abyssinica (L)	Hexane	0.47	16.67±0.67	19.33±1.33	13.00±1.00	-	-	-	6.83±0.17	-
F. africana (A)	Hexane	0.65	9.33±0.33	9.33±0.83	8.5±1.50	-	-	-	7.83±1.09	-
E. capensis (SB)	Hexane	0.09	-	-	-	-	-	-	-	-
A. racemosus (R)	Hexane	0.1	-	-	-	-	-	-	-	-
H. abyssinica (SB)	DCM	0.33	8.67±0.33	9.33±0.67	-	-	-	-	-	-
H. abyssinica (L)	DCM	1.35	20.00±1.15	19.50±1.50	15.5±1.50	-	-	-	7.17±0.44	-
F. africana (A)	DCM	1.23	10.67±0.33	10.50±0.29	9.67±0.33	-	-	-	7.17±0.44	-
E. capensis (SB)	DCM	1.02	7.50±0.50	-	-	-	-	-	-	-
A. racemosus (R)	DCM	0.4	-	-	-	6.83±0.17	-	-	-	-
H. abyssinica (SB)	Methanol	4.07	11.00±0.41	10.75±0.25	12.25±1.75	-	-	-	-	7.33±0.33
H. abyssinica (L)	Methanol	3.58	7.75±0.25	7.63±0.24	12.00±0.82	-	-	-	-	-
F. africana (A)	Methanol	3.43	6.75±0.25	-	7.75±1.25	-	-	-	7.33±0.33	-
E. capensis (SB)	Methanol	3.38	10.50±0.50	11.50±0.87	10.67±1.33	-	-	-	12.33±0.88	8.00±0.58
A. racemosus (R)	Methanol	9.57	-	-	11.67±0.33	-	7.38±0.36	11.25±4.75	12.33±0.88	10.67±0.58
H. abyssinica (SB)	Water	5.22	-	-	11.33±0.67	-	-	-	-	-
H. abyssinica (L)	Water	10.57	-	-	-	-	-	-	-	-
F. africana (A)	Water	9.42	-	-	7.00±0.00	-	-	-	-	-
E. capensis (SB)	Water	5.32	-	10.50±0.50	7.50±0.76	-	-	-	-	7.67±0.67
A. racemosus (R)	Water	27.96	-	-	-	7.00±0.00	-	-	-	-
Chloramphenical			17.33±0.33	15.00±0.00	16.50±0.29	29.67±0.33				
Fluconazole							10.67±0.33	15.00±0.00	24.33±0.33	25.00±0.58

Key: Inhibition diameters inclusive of the disc diameter of 6 mm. **Sa**, *Staphylococcus aureus ;* **Mrsa** Methicillin-resistant *Staphylococcus aureus;* **Pa**, *Pseudomonas aeruginosa ;* **Kp**, *Klebsiella pneumoniae*, **Ck**, *Candida krusei*; ; **Cn**, *Cryptococcus neoformans*; **Tm**, *Trichophyton mentagrophytes*; **Mg**, *Microsporum gypseum* . **–** Represents no inhibition zones observed

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Sterile filter paper discs containing 10 µl of dissolved plant extracts were placed on the surface of appropriate medium inoculated with test microorganism. MIC was defined as the lowest concentration of extract that showed clear zones of inhibition. Extracts with MIC of < 8mg/ml are considered to have antimicrobial activity (van Vuuren, 2008).

2.5 Cytotoxicity assay

The extracts of the most active plants were tested for *in vitro* cytotoxicity following a modified rapid calorimetric assay of Mosmann, 1983 using Vero E6 cancer cell lines sourced from American Type Culture Collection (ATCC). Cells were maintained in Eagle's Minimum Essential Medium (MEM) supplemented with 10 % fetal bovine serum (FBS) and 2 mM L-glutamine. 5 x 10^3 cells/well suspensions were seeded on 96- well micro titer plates and incubated at 37 °C / 5 % CO_2.

Samples were added to the cultured cells over a concentration range of 100 µg/ml to 0.14 µg/ml. The plates were incubated for 48 hours at 37 °C / 5 % CO_2, after which 10 µL of MTT (Thiazoil Blue Tetrazolium Bromide) dye was added and incubated for another 4 hours. Media was removed from all wells and 100 µl of DMSO added. The plates were then read (colour absorbence) on an ELISA scanning multiwell spectrophotometer (Multiskan Ex labssystems) at 562 nm and 690 nm as reference. Data was analysed as follows:

$$\% \text{ Cell viability} = \frac{[OD_{sample562} - OD_{620}] \times 100}{OD_{control562} - OD_{620}}$$

Where OD = optical density

Data was transferred onto a graphic program (MS Excel 2003) and expressed as percentage of the untreated controls. The concentration that kills 50% of the VERO cells (CC_{50}) was evaluated by linear regression analysis. Extracts were classified as cytotoxic if their CC_{50} < 90 µg/ml (Irungu et al, 2007).

2.6 Determination of acute toxicity

Authorization to use mice for *in vivo* acute toxicity was granted by the KEMRI Animal Care and Use Committee (ACUC). Healthy Swiss female mice, 8 weeks old weighing 20 ±2 g were randomly divided into groups of five in each cage. The extracts were dissolved in a solution of 10% Tween 80 in double distilled water and administered by gavage at a dose of 2500 and 5000 mg/kg body weight of mice/0.2ml. The mice had access to tap water and food, except for a short fasting period (12 h) before oral administration of 0.2 ml of the extract to each mouse (Muthaura et al, 2007). The general behavior of mice was observed continuously for 1 h after the treatment and then intermittently for 4 h, and thereafter over a period of 24 h. The mice were further observed for up to 14 days following treatment for any signs of toxicity such as food intake, activity, tremors, and the latency of death.

3. Results

3.1 Extraction and phytochemical screening.

Percentage yields of the different extracts of each plant are summarized in **Table 2**.

The trend in yield showed increase in percentage yield with increase in polarity of extracting solvent. Water extract of *A. racemosus* had the highest yield of 27.96 % while hexane extracts of *Hagenia abyssinica* (SB*)* and *Ekebergia capensis* (SB) recorded the lowest yields of 0.09%.

Preliminary screening for phytochemicals revealed presence of terpenoids in *H. abyssinica* (L, SB) and *F. africana*, phenolics in *H. abyssinica* (L, SB), *E. capensis* and *A. racemosus*, while anthraquinones was present in *H. abyssinica* (L). Flavones and alkaloids were absent in all plants tested.

3.2 Antimicrobial assays

The crude extracts of the four plants selected had inhibitory effects on several test organisms (**Table 2**).

In the antibacterial assays, the dichloromethane and hexane extracts of leaves of *H. abyssinica* were the most active extracts with the highest zones of inhibition against *S. aureus*, MR*Sa* and *P. aeruginosa*.

In the antifungal assays, the methanol extracts of *A. racemosus* and *E. capensis* were the most active with inhibition zone diameters of 12.33 mm against *T. mentagrophytes* for both plants. Water extracts of all plants were the least active*. E. coli* and *C. parapsilosis* were not sensitive to all plant extracts.

The MIC results of extracts are shown in **Table 3**. The lowest MIC value of 0.195 mg/ml was exhibited in hexane and dichloromethane extracts of *H. abyssinica* (L) against *S. aureus*, MR*Sa* and *P. aeruginosa*. Similar MIC values were observed for the methanol extract of *A. racemosus* against *P. aeruginosa*. The results indicate that the plants selected for the study had antibacterial activity, with the exception of *E. capensis* that had MIC for all extracts > 8mg/ml.

3.3 Toxicity studies

Cytotoxicity against VERO cell lines and acute toxicity was determined for extracts having low MIC values.

Four extracts were found to be cytotoxic with CC_{50} of <90µg/ml (**Table 3**). This correlated to the extracts with the lowest MIC values. The remaining 6 extracts tested were not cytotoxic having CC_{50} values of >90 µg/ml.

In acute toxicity studies, the water extract of *H. abyssinica* (SB) caused 20% mortality at 5000 mg/kg. All other extracts did not cause any mortality to mice indicating that the median lethal dose (LD_{50}) of all extracts is >5000 mg/kg.

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Table 3: Cytotoxicity and Minimum Inhibitory Concentration of selected plant extracts

Plant species (Part)	Test extract	CC_{50}(µg/ml)	MIC (mg/ml)					
			Sa	Mrsa	Pa	Cn	Tm	Mg
H. abyssinica (SB)	Hexane	>100	6.25	6.25	-	-	-	-
	DCM	-	100	100	-	-	-	-
	Methanol	>100	25	50	6.25	-	-	-
	Water	31.56	-	-	6.25	-	-	-
H. abyssinica (L)	Hexane	7.84	0.195	0.195	3.125	-	-	-
	DCM	7.89	0.195	0.391	0.195	-	-	-
	Methanol	>100	-	-	12.5	-	-	-
F. africana (A)	Hexane	92.34	6.25	6.25	3.125	-	50	-
	DCM	7.91	3.125	3.125	50	-	-	-
E. capensis (SB)	Methanol	>100	25	25	25	-	50	100
	Water	>100	-	25	100	-	-	100
A. racemosus (R)	DCM	-	-	-	50	-	-	-
	Methanol	>100	-	-	0.195	100	50	50
Chloramphenical			0.002	0.008	0.008			
Fluconazole						0.002	0.008	0.016

Sa, *Staphylococcus aureus ;* **Mrsa** Methicillin resistant *Staphylococcus aureus;* **Pa**, *Pseudomonas aeruginosa ;* **Cn**, *Cryptococcus neoformans;* **Tm**, *Trichophyton mentagrophytes;* **Mg**, *Microsporum gypseum;* **-,** Not determined

4. Discussion and conclusion

The study indicated a considerable difference in antimicrobial activity between extracts obtained with different solvents. Hexane and dichloromethane extracts were more active than other extracts against Gram- positive bacteria and only one Gram- negative bacteria (*P. aeruginosa*). No activity was observed against *K. pneumoniae* and *E. coli*, both Gram-negative bacteria. The reason for the difference in activity between Gram- positive and Gram- negative bacteria possibly lies in their morphological differences. The Gram- negative bacteria have an outer phospholipid membrane making their cell wall impermeable to lipophilic solutes. On the other hand, the Gram-positive bacteria lack this membrane and are thus more permeable (Nostro et al, 2000).

Hagenia abyssinica is a plant commonly found in high altitude areas in East Africa. It's an important medicinal plant with the flower reported as antihypertensive, taenicidal, (Belachew Desta, 1995), antihelminthic, trypanocidal (Nibert and wink, 2010), and in treatment of eye disease (Abebe and Ayehu, 1993). The bark has been reported to be used for stomachache and diarrhea whiles the root for malaria and general illnesses (Kokwaro, 1976). The antibacterial activity observed in this study could be attributed to the presence of phenolics, terpenoids and anthraquinones found in the plant. In the study, the leaves were more potent antibacterials and cytotoxic compared to the stem bark. Previous studies have reported presence of phloroglucinols (ά-kosin, kosotoxin and protokosin) which exhibited cytotoxic activity in vitro and in vivo against MAC tumour cells (Woldemariam et al, 1992). The antibacterial and cytotoxicity activity observed in this study may be attributed to presence of kosins which could be in high quantity on the leaves compared to the stem bark.

The dichloromethane extract of *Fuerstia afric*ana had antibacterial activity, but the extract was quite cytotoxic. One compound, known as Ferruginol, has been previously isolated from this plant and it presented cytotoxic activity (Koch et al, 2006). This substance may contribute to the cytotoxicity observed in this study. The plant has other therapeutic properties and has been reported to have antimicrobial and antimalarial activity (Koch et al, 2005; Muthaura et al, 2007; Muganga et al, 2010; Matu et al, 2012). Traditionally, it's reported to be used in treating conjuctivities, malaria, gonorrhoea, skin complaints, colds and in preventing diarrhea (Githinji and Kokwaro, 1993; Koch et al, 2005; Kokwaro, 1976; Muganga et al, 2010).

Antibacterial compounds either kill the cells (bactericidal) or inhibit its growth (bacteriostatic). The cytotoxicity observed in the most active extracts in this study could influence the antibacterial activity as cytotoxic compounds destroy living cells either selectively or indiscriminately (Kigondu et al, 2009). However, toxicity observed either *in vitro* or *in vivo* in a drug candidate is not a basis for disqualification as structural modifications can be made on the compounds in order to synthesize new drug analogues to improve the safety profile (Phillipson, 2007). Examples include Podophyllotoxin from *Podophyllum* species which was

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too toxic for clinical use. However chemical modifications yielded semi-synthetic analogues Etoposide and Teniposide which are safer and more soluble drugs used for cancer treatments (Kinghorn and Balandrin, 1993).

Asparagus racemosus methanolic extract was active against *P. aeruginosa*, but had no antifungal activity. This differs from previously reported anticandidal activity of the extract by Uma et al, 2009. The difference in the results of both studies may be explained by the fact that the plant samples were collected in two different regions, India and Kenya. The activity of a plant varies with the region it's collected. It is a known fact that plants grown in different climatic regions contain different chemical compositions of active principles (Gilani and Atta-ur-Rahman, 2005). *A. racemosus* is a herb employed in traditional medicine in many parts of the world. Its medicinal usage has been reported in the Indian and British Pharmacopoeias and in indigenous systems of medicine.

The extracts of *Ekebergia capensis* were not active against the microorganisms tested. Similar results were reported by Rabe and Van staden, 1997. This may be attributed to the fact that the extracts tested are still in impure form, or that the active compound/s are present in very low concentrations. In an earlier study, the bark of *E. capensis* has been reported to be active against drug resistant strains of *M. tuberculosis* (Lall and Meyer, 1999). *E. capensis* has been reported to be used traditionally for treating malaria (Koch et al, 2005), heartburn, coughs and respiratory complaints (Kamadyaapa et al, 2009).

Majority of extracts assayed presented antibacterial activity. It's interesting to note that most of antibacterial activity was found in the hexane, dichloromethane and methanol extracts, indicating that active compounds are unlikely to be obtained when plants are used traditionally. Medicinal plants are traditionally taken as aqueous extracts (Gachathi, 1989; Kokwaro, 1993), and in combination with others at very high doses (Azas et al, 2004). This may explain why low activity was observed for the aqueous extracts, when tested singly at low concentrations (100 mg/ml).

In recent years there is increasing trend for using alternative system of medicine. It is argued, that such drugs are not only effective but also very safe as compared to allopathic drugs for the similar indications. The claim that natural plant products are safe should be accepted only after the plant product passes through toxicity testing using modern scientific methods (Jaykaran et al, 2009).

In the study, the hexane and dichloromethane extracts of the leaves of *H. abyssinica*, and the dichloromethane extract of *F. africana* were found to be cytotoxic. However, these extracts did not demonstrate acute toxicity *in vivo*. The difference can be explained in that cells *in vivo* have the ability to metabolize chemicals to more- or less- toxic compounds. This function is usually expressed to a small extent in cultured cell, which results in limited activation or deactivation of test chemicals or in the *in vitro* accumulation of intermediates that do not occur *in vivo* (Walum, 1998).

Acute toxicity studies in animals are of value in predicting potential toxic effects of a chemical in human beings exposed to near fatal doses. From these studies the nature of acute response in man as a result of exposure to these phytochemicals may be anticipated (Jaykaran et al, 2009).

In conclusion, this study indicated that the extracts of *H. abyssinica* (L) and *F. africana* assayed were active against gram- positive bacteria and some gram-negative bacteria. However, they were also cytotoxic and further investigations are therefore needed to clarify which compound is responsible for one or both activities. The results of this study support to a certain degree the traditional medicinal uses of the plants evaluated.

Conflict of Interest declaration

The authors declare no conflict of interest

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