

***In vitro* antibacterial activity of four plant species used in traditional medicine practices of south Omo zone, southern Ethiopia**

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Summary

Screening of plants used in traditional medicines could provide valuable information regarding antimicrobials. The present study determines the antibacterial activity of crude extracts of four medicinal plants, *in vitro*, against a panel of American Type Culture Collection (ATCC) (two Gram-positive and seven Gram-negative bacteria) and clinical isolates of Multi-Drug Resistant (MDR) bacteria (two strains of Gram-positive and three Gram-negative bacteria), by employing agar well diffusion assay. Based on the ethnobotanical data, four plants were chosen and collected from different areas of south Omo. Leaves (*Aloe pirottae*, *Kosteletzkya begoniifolia*, and *Uvaria leptocladon*) and root (*Grewia schweinfurthii*) of plants were subjected to the extraction process

using six different organic solvents. The plants that showed the highest activity indices were further screened against MDR bacterial isolates. Minimum inhibitory concentration (MIC) and minimum bactericidal concentrations (MBC) were estimated in the case of the most active plant extract. The results of primary screening revealed that two plants (*K. begoniifolia*, and *U. leptocladon*) were highly active against ATCC strains. Ethyl acetate extract of *U. leptocladon* produced the highest zone of inhibition ranging between 20 ± 1.15 mm to 40 ± 1.45 mm against Gram-negative bacteria and 21 ± 0.58 mm to 28 ± 2.03 mm against Gram-positive bacteria. Likewise, extracts of *K. begoniifolia* in the same solvent produced an inhibitory zone in the range of 10 ± 0.33 mm and 20 ± 1.15 mm corresponding to Gram-positive and Gram-negative type culture bacteria respectively. The results of the comprehensive screening showed that ethyl acetate extract of *U. leptocladon* efficiently inhibited the growth of MDR bacterial isolates. The overall findings of this study demonstrated that all four plants have antibacterial activities in varying degrees. The ethyl acetate extract of *U. leptocladon* showed the widest and highest spectrum of antibacterial activities in the range of 15.7 ± 0.3 to 23.7 ± 0.7 mm as per agar well diffusion assay, whereas the MIC values of *U. leptocladon* against the Gram-negative bacteria ranged between 7.8 and 125 $\mu\text{g/ml}$ and the corresponding MBC values were found to be in the range of 15 and 500 $\mu\text{g/ml}$. MIC and MBC values were found to be the least, 125 $\mu\text{g/ml}$ and 500 $\mu\text{g/ml}$ respectively in the case of Gram-positive bacteria. Overall results substantiate the traditional uses of *U. leptocladon* as an antibacterial agent.



Key words

Antibacterial activity, Medicinal plants, south Omo, *U. leptocladon*, *A. pirottae*, *G. schweinfurthii*, *K. begoniifolia*

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Introduction

The search for novel antimicrobials needs to be intensified due to the dearth of safe and effective antibiotics. According to the World Health Organization, plants represent a gold mine of novel leads for drug development. In many regions, particularly in Ethiopia, the vast majority of traditional medicines used for the treatment of infectious diseases are plant-based.¹ It is reported that whole plants and plant extracts constitute 95% of such medications and cures.^{2,3} Lit-

erature indicates that these plants were the backbone in providing treatments to all types of illnesses, from a minor cold to life-threatening diseases, such as tuberculosis and malaria.²⁻⁴ The uses of plants in Ethiopian traditional medicines as well as in the food and nutritional sector are well-known.² However, the antimicrobial activities of several medicinal plants have not yet been completely investigated. Research on scientific validation of plants used in traditional medicines of Ethiopia has been instigated only very recently. Among 7,600 vascular plants in the country, merely 144 are

screened for their antimicrobial activity in the last decades.⁵ The majority of therapeutic evidence attributed to Ethiopian herbal medicines are based on folk medicine inherited from the predecessors, rather than from the scientific data derived from laboratory experiments. Unfortunately, traditional ways of healing illnesses tend to disappear due to a lack of support and promotion from the scientific community.⁶

The study area, south Omo is considered one of the richest zones of plant diversity in Ethiopia. A preliminary survey showed that more than one hundred and fifty species of plants were widely used for the preparation of various traditional medicines. People in the Omo valley heavily rely on these plant-based traditional medicines for the treatment of infectious and non-infectious diseases. A comprehensive literature review indicated that the uses of plants in traditional medicines in the Omo valley are documented only by ethnobotanical studies.⁶⁻⁹ The antibacterial activity of plants used in traditional medicines in this area has

seldom been explored. Besides, the plant diversity of the Omo valley is waning due to climate change, industrialization, and excessive exploitation. There exist some endemic species. The present study is intended to evaluate the *in vitro* antibacterial activities of four medicinal plants (*Aloe pirottae*, *Kosteletzkya begoniifolia*, *Uvaria leptocladon* and *Grewia schweinfurthii*) which are used in traditional medicines against ATCC and MDR clinical isolates of bacteria.

Material and Methods

Plant material selection, collection & transportation

The selection of four medicinal plants for the antibacterial study was exclusively based on their ethnobotanical usage against diverse infectious diseases in the study area, Omo valley. The ethnobotanical use of selected plants is indicated in Table 1. Four plants were collected from different expanses of the Omo

Table 1 Ethnobotanical data of medicinal plants selected for antibacterial screening.

Scientific name and family	Parts used	Ethnobotanical usage	MOP & ROA	Geographical location (Omo valley)	Voucher No.
<i>Grewia schweinfurthii</i> Burret (Tiliaceae)	Root, leaves	Strong cough, cold and malaria ^{53,54,55}	Fresh root chopped and boiled for longer time, then administered orally Leaf juice	Alt. 433 m. Long. N05°11110 E036°12330	SAM001
<i>Aloe pirottae</i> Berger (Aloaceae)	Latex, rhizome	Wound healing ⁵⁶	Applying latex from fresh leaves on wound	Alt. 1117 m. Long. N05°08371 E036°31179	SAM002
<i>Uvaria leptocladon</i> Oliv. (Annonaceae)	Leaves, stem bark and fruit	Intestinal problems, diarrhea and vomiting, chronic wound, UTI, chest pain ^{6,57}	Fresh or dried leaves and stem bark boiled in water or squeezed to take out the juice then administered orally	Alt. 1290 m. Long. N05°43 558 E036°46 405	SAM003
<i>Kosteletzkya begoniifolia</i> (Ulber) Ulber (Malvaceae)	Leaves, stem	High fever, dermatologic infection, Gastrointestinal infections, Toothbrush ^{6,58}	Fresh leaves squeezed and mixed with coffee to be administered orally for fever, Leaf and stem paste used for the dermatological and gastrointestinal infections	Alt. 1290 m. Long. N05°43 558 E036°46 405	SAM004
MOP- mode of preparation, ROA- route of administration; Some of the information pertaining to the ethno-botanical uses were compiled through face-to-face interview with traditional healers using a questionnaire.					

valley (between September and October 2018) with the help of traditional healers. The geographical locations of collection sites and plant parts used are indicated in Table 1. Plant samples were collected by local digging tools as well as by using an industrial pruning shear (Outils Wolf) in sterile conditions. The collected plant specimens were transported to the laboratory in zip-locked polythene and isothermal bags. Taxonomic identification of plants were done and voucher specimens and photographs were retained in the National Herbarium, Addis Ababa University, Ethiopia for future reference.

Processing of medicinal plants

Prior to extraction, healthy plant materials of the respective species (the root part of *G. schweinfurthii* and leaves of *A. pirottae*, *U. leptocladon*, and *K. begoniifolia*) were thoroughly washed and rinsed with distilled water to remove associated debris. Cleaned samples were then dried under shade for one week at room temperature ensuring the prevention of photolysis and thermal degradation of metabolites. The leaf part of *A. pirottae* was shade dried for six weeks. The completely dried material was ground finely with an electrical grinder (Moulinex turbo mix 350W). Finely ground plant materials were then weighed using a digital balance (Ormade DB-300, 300X0.1g- Milano, Italy) and stored in a cool dry place until use.

Preparation of plant extract

Plant materials were then subjected to extraction by the maceration method described earlier with slight modifications.¹¹ Five grams of each plant material was macerated separately with 50 ml of six different extraction solvents of increasing polarity (petroleum ether, chloroform, ethyl acetate, methanol, ethanol,

and distilled water) separately in 100 ml capacity reagent bottles. Plant specimens in the respective solvent were placed on a shaker (Labtech RS-12 R) at 120 rpm for 24 hours at room temperature. After the stipulated time, extracts were filtered using a Whatman filter paper No. 1. This procedure was repeated thrice. Total extracts were then obtained by merging the filtrates from each of the three extractions in a conical flask (250 ml), following evaporation to dryness in a water-bath (VIGITAL serological) at 40°C. The resulting gummy extract was collected and diluted with dimethyl sulfoxide (DMSO), in order to make a concentration of 1 mg/ml aliquot and kept at 4°C until laboratory analysis.

Test microorganisms

The antibacterial activities of plant extracts were determined against a battery of nine ATCC bacterial strains obtained from the Ethiopian Public Health Institute (Table 2). Besides, a panel of five clinical isolates sourced from diverse clinical samples was used. The clinical isolates established as MDR pathogens were obtained from the Jinka Public Health Laboratory. The resistance patterns of clinical isolates were confirmed using selective antibiotics that are routinely used to combat bacterial diseases. Clinical isolates resistant to one or more antibiotic discs were considered as MDR.¹² All the bacterial strains were maintained on nutrient agar slants (Hi-media) at 37°C (Table 2 & 3).

Antibacterial assay

Organic as well as water extracts of four medicinal plants were subjected to primary antibacterial screening against ATCC strains. Afterwards, extracts with the highest activity index against ATCC strains were further subjected to a comprehensive antibacterial screening

Table 2 Panel of ATCC strains used for the antibacterial assay.

Gram positive bacteria	Gram negative bacteria
<i>Staphylococcus aureus</i> ATCC 25923	<i>Escherichia coli</i> ATCC 25922
<i>Enterococcus faecalis</i> ATCC 29212	<i>Pseudomonas aeruginosa</i> ATCC 27853
	<i>Proteus mirabilis</i> ATCC 35659
	<i>Klebsiella pneumoniae</i> ATCC7 00603
	<i>Shigella flexneri</i> ATCC 12021
	<i>Salmonella typhimurium</i> ATCC 13311
	<i>Haemophilus influenzae</i> ATCC 49766
ATCC- American Type Culture Collection	

Table 3 Panel of MDR bacteria and its antibiogram profile.

Specimen	MDR bacteria	Antibiogram profile
Stool	<i>K. pneumoniae</i>	tobramycin, ceftriaxone, doxycycline, chloramphenicol and clindamycin
Ear Discharge	<i>P. aeruginosa</i>	gentamicin, tetracycline, amoxicillin, ampicillin, chloramphenicol, trimethoprim-sulfamethoxazole and vancomycin
	<i>S. aureus</i>	gentamicin, amoxicillin, ampicillin, chloramphenicol and trimethoprim-sulfamethoxazole
Wound	<i>S. aureus</i>	penicillin, amoxicillin, ceftriaxone, and gentamicin
Urine	<i>E. coli</i>	norfloxacin, ceftriaxone, tetracycline and gentamicin

against five MDR clinical isolates. Finally, the potent plant extract that showed the highest and broadest activity was further taken up to elucidate the mechanism of antibiosis.

Antibacterial screening against ATCC strains

The antibacterial screening of plant extracts was done *in vitro* by agar well diffusion assay.^{13,14} The assay was carried out on nutrient agar (Biomark Laboratories, India) as described elsewhere.^{15,16} The inoculums were prepared from overnight cultures by the direct colony method. Discrete colonies were picked up directly from the plate with a sterile wire loop and suspended in sterile 0.85% saline. The inoculum was prepared to a turbidity equivalent to that of a 0.5 McFarland standard. Afterwards, test organisms were uniformly swabbed over the nutrient agar (Hi-Media, Mumbai) surface. Using a sterile cork borer, a 5 mm well was made and filled with 120 µl (1 mg/ml) of the appropriate plant extract. Parallel reference standard (ciprofloxacin (30µg) (Himedia®, Mumbai) and negative controls (solvents used for the extraction) were used

to validate the inferences. Petri plates were transferred to an incubator and kept at 37°C for 24 hours. After incubation, antibacterial activity was determined by measuring the diameter of the inhibition zone including the well. The formation of a clear inhibition zone with a diameter of ≥10 mm was considered as an indication of antibacterial activity. The assays were conducted in triplicate to evaluate the findings statistically. The activity indices of each solvent extract of the respective plant species as well as the overall activity index of each plant tested in primary screening were determined using the following expressions.

Antibacterial screening against MDR clinical isolates

Based on the results obtained from primary antibacterial screening and overall activity index, the plant extracts that showed the highest and broadest spectrum of activities against the ATCC strains were further subjected to a comprehensive screening against a panel of multidrug-resistant bacteria comprising of five isolates. The methodology used for primary screening was applied here too.

$$\text{Activity Index (AI)} = \frac{\text{No. of solvent extract with zone of inhibition} \geq 10\text{mm}}{\text{No. of bacteria tested}} \times 100$$

$$\text{Overall Activity Index (OAI) (\%)} = \frac{\text{No of plant extract with antimicrobial activity}}{\text{Total No of plant extracts tested}} \times 100$$

Determination of MIC and MBC

Based on the results of the antibacterial screening against MDR isolates, the extract which showed the broadest and highest activities was taken up for further assay. The tube dilution method was used for determining MIC and MBC.¹⁷ Extracts of plants were dried, aliquoted in phosphate-buffered saline (pH 7.2) and were used as test solutions. The dosing range of extracts was computed by a factor of 2 (antilog 0.3) to obtain a final dose range between 3.9 and 1000 µg/ml. Afterwards, each tube was inoculated with 100 µL of a fresh overnight culture of appropriate bacterial suspension (10^5 CFU/mL) and incubated at 37°C for 24 hours. Among the three parallel control tubes, one contained the inoculated culture medium (culture control), another with the culture medium treated immediately with 0.5 ml of formaldehyde solution (blank), and the third one being an un-inoculated culture medium. The MIC value was determined by sub-culturing a loop full of suspension from each culture tube to the nutrient agar plates that were incubated at 37°C for 24 hours. Values correspond to the lowest concentrations inhibiting 90% of colonies with respect to the number of colonies in the initial culture. The concentrations which manifested no growth after 48 hours incubation at 37°C were considered as the MBC. Plant extract is considered as bactericidal if the ratio of MBC/MIC is ≤ 2 , and otherwise as bacteriostatic. A ratio, ≥ 16 , hints at the ineffectiveness of the extract.¹⁸

Quality control

All experiments were carried out in triplicates in order to validate the findings statistically. Temperature and plate contamination were inspected by thermometer and un-inoculated agar plates. Positive controls: an-

tibiotic disc ciprofloxacin (5 µg) was used as the positive control for all ATCC strains. The positive control exhibiting an inhibition zone of ≥ 16 , is considered as intermediate or susceptible.¹⁹ Negative controls: DMSO and solvents used for extraction were considered as negative controls in all antibacterial assays.

Statistical analysis

All the results were expressed as a mean $\pm$ standard deviation (S.D.). Mean values were assessed by means of one-way analysis of variance (ANOVA) using SPSS for Windows version 20.0 (Statistical Package for Social Services, Chicago, IL, USA).

Results

Overall antibacterial screening against ATCC bacterial strains

The results of antibacterial screening indicated that all the crude extracts of four plant species showed *in vitro* antibacterial activity against at least one of the tested ATCC bacterial strains. The activity indices of all plant extracts are tabulated in table 4. It was found that the activity of each plant differed significantly with the type of solvent used for the extraction. Results of the overall activity index of plants indicated that *U. leptocladon* has the highest spectrum (40.7%) of antibacterial activity followed by *K. begoniifolia* (38.8%). Ethyl acetate extract of *U. leptocladon* showed the highest rank of activity index (88.8%) followed by chloroform (77.7%) and ethanolic extracts (66.6%). The methanolic extract showed the lowest rank of activity (11.1%) and extracts obtained from petroleum ether and water showed no activity at all. In the case

Table 4 Antibacterial activity index of plant extracts (%).

Plant species	PE	CHL	EA	MTH	ETH	DW	OAI
<i>A. pirottae</i>	11.1	66.6	55.5	11.1	44.4	0	35.1
<i>G. schweinfurthii</i>	11.1	33.3	66.6	22.2	44.4	0	27.7
<i>K. begoniifolia</i>	22.2	44.4	66.6	22.2	66.6	0	38.8
<i>U. leptocladon</i>	0	77.7	88.8	11.1	66.6	0	40.7

Activity index was expressed as relative antimicrobial activity of respective solvent extract of the plants species against nine ATCC bacteria. Overall activity was expressed as relative antimicrobial activity of all solvent extracts of respective plant species against nine ATCC bacteria. *Zone of Inhibition ≥ 10 mm was considered active. PE- petroleum ether, CHL- chloroform, EA- ethyl acetate, MTH- methanol, ETH- ethanol, DW- distilled water, **OAI- overall activity index of respective plant species.

of *K. begoniifolia*, ethyl acetate and ethanolic extracts showed the highest activity index (66.6%) (Table 4). The mean difference of each extract was significant at the 0.05 level. On comparing the results of activity indices of all organic extracts, it was found that ethyl acetate and ethanol were the best solvents for extracting antibacterial ingredients. Furthermore, no inhibitory effects were shown by negative controls.

Antibacterial activity of *A. pirottae* against ATCC bacterial strains

The overall activity index of *A. pirottae* was 35.1%. Chloroform is the best solvent for extracting antibacterial metabolites. It efficiently inhibited the growth of 66.6% of ATCC strains. The crude chloroform extract of *A. pirottae* produced an inhibitory zone in the range of 12 ± 1.15 mm to 40 ± 1.15 mm against Gram-negative bacteria and 10 ± 0.58 mm to 11 ± 0.58 mm against Gram-positive bacteria. The inhibitory activity was excellent, particularly against Gram-negative bacteria. Chloroform extract showed the highest activity against *E. coli* which produced an inhibitory zone to the extent of 40 ± 1.15 mm and was higher than the reference standard, ciprofloxacin (38 ± 1.45). Moderate levels of activity were showed against *P. mirabilis* (17 ± 0.33 mm), *S. typhimurium* (17 ± 1.15 mm), and *P. aeruginosa* (16 ± 0.58 mm). However, only lower levels of activity are exhibited against Gram-positive bacteria, *S. aureus* (11 ± 0.58 mm), and *E. faecalis* (10 ± 0.58 mm). The second most active was ethyl acetate extract. It produced a zone of inhibition ranging between 11 ± 0.33 mm to 22 ± 0.58 mm against Gram-negative bacteria and also it showed the highest inhibitory activity against *E. coli* (22 ± 0.58 mm). Petroleum ether and methanolic extracts inhibited the growth of *E. coli* in moderate and lower levels respectively (Table 5).

Antibacterial activity of *G. schweinfurthii* against ATCC bacterial strains

The overall activity index of *G. schweinfurthii* was 27.7%. Ethyl acetate was found to be the best solvent to extract antibacterial metabolites from *G. schweinfurthii*. It produced an activity index of 66.6% against the ATCC strains. The highest and moderate levels of activity were exhibited against Gram-negative bacteria, *P. mirabilis* (21 ± 0.67 mm) and *S. typhimurium* (18 ± 1.00 mm). However, only lower levels of activity are shown against *E. coli* (11 ± 0.58 mm), *P. aeruginosa* (13 ± 1.45 mm), *K. pneumoniae* (14 ± 0.67 mm), and *H. influenzae* (12 ± 1.53 mm). The second most active solvent is ethanol. Ethanolic extract showed moderate antibacterial activity to *S. typhimurium* (19 ± 1.45 mm). Chloroform extract showed the highest activity

against *P. aeruginosa* (21 ± 0.67 mm), whereas the petroleum ether extract showed antibacterial activity against only *P. mirabilis*, that too in the lower range (14 ± 1.45 mm). It was noted that no activity was produced solvents against Gram-positive bacteria, irrespective of the solvent used for extraction (Table 5).

Antibacterial activity of *K. begoniifolia* against ATCC bacterial strains

The overall activity index of *K. begoniifolia* was 38.8%. It was found that ethyl acetate and ethanol were the best solvents for extraction, producing an activity index of 66.6% in both cases. Ethyl acetate extract showed the maximum inhibition against *E. coli* (20 ± 1.15 mm), *P. mirabilis* (21 ± 0.33 mm), and *S. typhimurium* (19 ± 0.58 mm). However, only lower levels of activity are detected against *H. influenzae* (10 ± 0.33 mm), *S. aureus* (10 ± 1.00 mm), and *K. pneumoniae* (10 ± 0.58 mm). Ethanolic extract showed only moderate levels of activity against *S. typhimurium* (15 ± 0.58 mm) and *E. faecalis* (17 ± 0.58). However, methanolic extract showed only a comparatively lower range of activity (10 ± 0.33 to 13 ± 0.67). Based on the results of primary screening, the ethanolic extract of *K. begoniifolia* was selected for further detailed screening of antibacterial activity using MDR bacterial isolates (Table 5).

Antibacterial activity of *U. leptocladon* against ATCC bacterial strains

The overall activity index of *U. leptocladon* was 40.7%. Notably, ethyl acetate extract of this plant efficiently inhibited the growth of all tested ATCC strains except *H. influenzae*. The crude extract of ethyl acetate produced the highest zone of inhibition ranging between 20 ± 1.15 mm to 40 ± 1.45 mm against Gram-negative bacteria and 21 ± 0.58 mm to 28 ± 2.03 mm against Gram-positive bacteria. Among the Gram-negative bacteria, *E. coli* is the most sensitive pathogen which exhibited an inhibitory zone of 40 ± 1.45 mm, and this value is higher than the reference standard, ciprofloxacin (38 ± 1.45). In the case of Gram-positive counterpart, *S. aureus* is the most sensitive which exhibited an inhibitory zone of 28 ± 2.03 mm and is higher than ciprofloxacin (21 ± 0.67 mm). Besides, inhibitory zones produced by the ethyl acetate extract against *K. pneumoniae* (20 ± 1.15 mm), *S. flexneri* (28 ± 1.20 mm), and *E. faecalis* (21 ± 0.58 mm) are also higher than that corresponding to ciprofloxacin. Chloroform extract was the second most active, which also showed high levels of activity against *E. coli* (30 ± 1.86 mm) and *P. mirabilis* (22 ± 0.88 mm), whereas only a lower to moderate range of activity was exhibited by ethanolic extract (12 ± 1.15 mm to 18 ± 1.15 mm). Methanolic extract of *U. leptocladon* inhibited only one bacterium, *S. ty-*

Table 5 Inhibition zones of different plant extracts in primary antibacterial screening against ATCC strains.

Plant	Solvents	EC	PA	KP	ST	SF	PM	HI	SA	EF
<i>A. pirottae</i>	PE	19±1.00	5	5	5	5	5	5	5	5
	CHL	40±1.15	16±0.58	12±1.15	17±1.15	5	17±0.33	0	11±0.58	10±0.58
	EA	22±0.58	14±0.58	11±0.33	17±1.00	5	16±0.58	0	0	0
	MTH	14±0.67	5	5	5	5	5	5	5	5
	ETH	12±0.33	11±0.00	9±0.00	14±1.15	5	12±0.33	5	5	5
<i>G. schweinfurthii</i>	PE	5	5	5	5	5	14±1.45	5	5	5
	CHL	9±0.00	21±0.67	15±1.76	13±1.67	5	5	5	5	5
	EA	11±0.58	13±1.45	14±0.67	18±1.00	5	21±0.67	12±1.53	5	5
	MTH	9±0.33	11±0.88	5	12±0.33	5	9±0.00	5	5	5
	ETH	5	14±0.58	10±0.33	19±1.45	5	12±0.67	5	5	5
<i>K. begoniifolia</i>	PE	5	5	5	20±1.45	5	5	10±0.00	5	5
	CHL	34±1.00	12±0.58	12±1.53	5	5	30±0.67	5	5	5
	EA	20±1.15	9±0.33	10±0.58	19±0.58	5	21±0.33	10±0.33	10±1.00	5
	MTH	8±0.00	9±0.67	NA	10±0.33	5	5	5	5	13±0.67
	ETH	12±1.15	9±0.58	9±0.33	15±0.58	10±0.33	10±1.15	5	10±1.20	17±0.58
<i>U. leptocladon</i>	CHL	30±1.86	11±1.53	10±0.88	10±1.15	17±1.00	22±0.88	5	10±0.58	5
	EA	40±1.45	28±0.58	20±1.15	30±0.00	28±1.20	32±1.45	8±0.33	28±2.03	21±0.58
	MTH	9±0.67	5	9±0.33	13±1.20	9±0.33	8±0.33	5	5	5
	ETH	18±1.15	5	12±1.15	15±1.20	12±0.88	15±1.33	5	14±2.00	5
Ciprofloxacin (5µg)		38±1.45	31±0.88	18±1.53	37±1.50	25±5.73	39±2.33	17±0.88	21±0.67	18±0.44
The value of inhibition zone (in mm) is expressed by Mean and SEM (standard deviation of the mean), (n=3). CHL- chloroform, EA- ethyl acetate, EC- <i>E. coli</i>, EF- <i>E. faecalis</i>, ETH- ethanol, HI- <i>H. influenzae</i>, MTH- methanol, ATCC strains: PA- <i>P. aeruginosa</i>, PE- petroleum ether, KP- <i>K. pneumoniae</i>, ST- <i>S. typhimurium</i>, SF- <i>S. flexneri</i>, PM- <i>P. mirabilis</i>, SA- <i>S. aureus</i>.										

typhimurium and at the same time, petroleum ether and water extracts were found to be inactive (Table 5). Based on the broadest and highest spectrum of activity, ethyl acetate extract of *U. leptocladon* was further chosen for a comprehensive screening.

Antibacterial screening against MDR clinical isolates
Based on the results of the overall activity indices with ATCC strains, ethyl acetate extract of *U. leptocladon* and ethanolic extract of *K. begoniifolia* were further subjected to a comprehensive antibacterial screening test against six MDR bacterial isolates sourced from different clinical samples.

Antibacterial activity of *K. begoniifolia*

It was found that the antibacterial activity of the ethanolic extract of *K. begoniifolia* was moderate against the MDR clinical isolates. It only inhibited the growth of two clinical isolates. Inhibitory activity was in the range of 14.7±0.7 mm to 17.3±0.7 mm respectively against *S. aureus* and *E. coli* (Table 6).

Antibacterial activity of *U. leptocladon*

The antibacterial activity of ethyl acetate extract of *U. leptocladon* was prominent against MDR isolates. The highest inhibitory zones of 23.7±0.7 mm and 20.7±0.7 mm were showed against two clinical isolates of *S. au-*

Table 6 Inhibition zones exhibited against MDR bacterial isolates.

Plant species	Solvent	PA	SA-1	SA-2	EC	KP
<i>U. leptocladon</i>	EA	15.7±0.3	20.7±0.7	23.7±0.7	20.0 ±1.7	16.3±0.7
<i>K. begoniifolia</i>	ETH	0±.00	14.7±0.7	0±.00	17.3±0.9	0±.00

EA- ethyl acetate, ETH- ethanol, EC- *E. coli*, KP- *K. pneumoniae*, PA- *P. aeruginosa*, SA-1- *S. aureus* (ear discharge), SA-2- *S. aureus* (wound).

Table 7 MIC and MBC of ethyl acetate extract of *U. leptocladon* against ATCC strains.

Test organisms	MIC (µg/ml)	MBC (µg/ml)	MBC/MIC
<i>E. coli</i>	7.8	15.62	2
<i>P. mirabilis</i>	31.25	62.5	2
<i>K. pneumoniae</i>	15.62	62.5	4
<i>P. aeruginosa</i>	31.25	125	4
<i>S. typhimurium</i>	31.25	62.5	2
<i>S. flexineri</i>	125	500	4
<i>E. faecalis</i>	250	500	2
<i>S. aureus</i>	125	500	4

reus from the wound and ear discharge respectively. It displayed the highest activity against *E. coli* ie., to an extent of 20.0 ± 1.7 mm. A moderate range of activity only was shown against *K. pneumoniae* (16.3±0.7 mm) followed by *P. aeruginosa* (15.7±0.3 mm) (Table 6).

Determination of MIC of *U. leptocladon* extract against ATCC strains

The MIC was determined by tube dilution assay using ethyl acetate extract of *U. leptocladon*. The determined MIC and MBC values of *U. leptocladon* against the ATCC strains ranged between 7.8µg/ml - 250µg/ml and 15.62 µg/ml - 500 µg/ml respectively. The least MIC and MBC values were produced against the Gram-negative bacteria, *E. coli* (7.8 µg/ml & 15.62 µg/ml). In the case of Gram-positive bacteria, *S. aureus*, the least MIC and MBC values were 125 µg/ml and 500 µg/ml respectively (Table 7).

Determination of MIC of *U. leptocladon* extract against MDR clinical isolates

The determined MIC value against MDR clinical isolates ranged between 62.5 µg/ml – 250 µg/ml. The

least MIC value of 62.5µg/ml with a corresponding MBC value of 250 µg/ml was recorded against the Gram-negative bacteria, *K. pneumoniae*. However, the MIC and MBC values of *P. aeruginosa* were slightly higher (125µg/ml and 500 µg/ml). Concerning the Gram-positive bacteria, the lowest MIC and MBC values of 125 µg/ml and 500 µg/ml respectively were displayed against both strains of *S. aureus* (Table 8).

Discussion

Overall activity

This is the first study that demonstrates the *in vitro* antibacterial potentials of *A. pirottae*, *G. schweinfurthii*, *K. begoniifolia* and *U. leptocladon* plant species, which are widely in use in the traditional medicines of south Omo Zone. The selection of plants based on ethno medicinal information can probably yield good floral candidates with much higher potential for antimicrobial and medicinal property rather than a random selection.²⁰ Hence, based on the ethnobotanical information collected from the Omo valley, these four

Table 8MIC and MBC values of ethyl acetate extract of *U. leptocladon* against MDR clinical isolates

Test organisms	MIC (µg/ml)	MBC (µg/ml)	MBC/MIC
<i>E. coli</i>	250	1000	4
<i>K. pneumoniae</i>	62.5	250	4
<i>P. aeruginosa</i>	125	500	4
<i>S. aureus</i> [1]	125	500	4
<i>S. aureus</i> [2]	125	500	4

plants were chosen. It was found that antibacterial activity significantly varied among the plant species, and also in the case of each plant, it fluctuated depending on the type of solvent used for the extraction, and the genus of bacteria tested. Alterations in antimicrobial activities may be attributed to differences in the time of harvest,²¹ the developmental stage of plants,²² the method of extraction employed, virulence factors of bacteria tested, and even the specific plant parts used.^{18,23}

Secondary metabolites responsible for the antibacterial activities in the assayed plants are not elucidated in this study. However, an extensive literature survey revealed that each of the four genera included in this work has been studied by various authors from different locales. To mention it precisely, a recent comprehensive review had appeared describing the constituents of various species of the genus *Aloe* and attributing the antibacterial activity to the presence of polysaccharides, along with synergistic interaction from components such as emodin, aloin A, aloemodin, saponins, chrysophanol, acemannan and pyrocatechol.^{24,25,26} In the case of *U. leptocladon*, there are some interesting reports regarding its bioactivities.^{27,28} This species is reputed for bioactive metabolites such as uvaretin, diuvaretin, triuvaretin, isotriuvaretin, angoluvarin, and chamuvaretin.^{27,28} Species belonging to the genus *Grewia* is known to contain various bioactive compounds such as triterpenoids, steroids, glycosides, flavones, lignans, phenolics, alkaloids, lactones, anthocyanins, flavones, and organic acids and, *G. asiatica* is one of the most studied species.²⁹ Species belonging to the genus *Kosteletzkya* and its chemical profiling of bioactive constituents are scanty. However, *K. virginica* has been screened for its bioactive metabolites. This species is found to contain β -sitosterol, jatrocinn B, cleomiscosin A, B, C and D, syringaresinol, α -spinasterol and lignoceric acid.^{30,31,32} It is generally acknowledged that antimi-

crobial activities in plants are due to a wide variety of secondary metabolites, such as tannins, terpenoids, alkaloids, lectins, polysaccharides, polypeptides, and flavonoids.³³

Ethanol and ethyl acetate were found to be the best solvents to extract antimicrobials. These findings infer that solvents used for the extraction had a definite effect on the isolation of antimicrobials. The highest activity demonstrated by the crude ethanolic and ethyl acetate extracts might be due to the presence of higher concentrations of the medium polar to polar antibiotic constituents that are fairly soluble in these solvents. Literature shows that antimicrobial metabolites from several other plants are also well soluble in ethanol and ethyl acetate.^{11,17,34} For instance, ethyl acetate extract of *Nicotiana tabacum* prepared from leaves (1 mg/ml) by immersion method efficiently repressed the growth of bacterial uro-pathogens.³⁴ Likewise, another study reported that ethanolic leaf extract obtained by this method having a concentration of 2 mg/ml from *Moringa stenopetala* inhibited the growth of Methicillin-resistant *S. aureus* (MRSA).¹¹ The antimicrobial activity of plants varies with respect to the production of secondary metabolites, which in turn is season dependent.²¹ However, water-based extracts had no antibacterial activity. Similar results were reported by several researchers.³⁵⁻³⁶

Our results revealed that each medicinal plant showed different degrees of inhibition against all the tested microorganisms. It is a known fact that Gram-negative bacteria are prominent pathogens compared to Gram-positive counterparts due to the morphological differences between them.³⁷ Antibacterial activities of four plant extracts included in this study are significant towards Gram-negative bacteria. This observation was by and large similar to a study conducted in other plant species from India which reported that the extract of *Acacia nilotica* inhibited the growth of Gram-negative bacteria such as *K. pneumo-*

niae and *E. coli* and failed to inhibit *S. aureus*.³⁸ This trend has already been reported from Ethiopia in the case of extracts of *A. harlana* and *A. pulcherrima* showing higher activity towards Gram negative compared to Gram-positive bacteria.³⁹⁻⁴⁰ Indeed, the difference in the sensitivity patterns between Gram-negative and Gram-positive bacteria may be correlated to the variation in their cell wall structure and permeability. The cell wall of the latter group consists of 70–100 layers of peptidoglycans.^{18,41} However, further investigation is required to draw a conclusion. Overall results of primary screening revealed that, among the four plants, *U. leptocladon* and *K. begoniifolia* have commendable antibacterial activities. Therefore, these two plants were further selected for a comprehensive screening against MDR bacterial isolates. Besides, the reduced degree of activity exhibited by the extracts of *A. pirottae* and *G. schweinfurthii*, do not intend that these plants are inactive nor do they completely lack antimicrobial compounds. However, it can be assumed that some of the bioactive compounds in the crude extract may be present only in insufficient quantities or they are highly volatile causing nominal activity. Another possibility is that there exist antagonistic effects exerted by several constituents of conflicting chemical nature.⁴²

Antibacterial activity of *A. pirottae*

The chloroform extract of *A. pirottae* showed the highest antibacterial activity against Gram-negative ATCC bacteria such as *E. coli*, *P. mirabilis*, *S. typhimurium*, and *P. aeruginosa*. Similar activity was shown against Gram-positive bacteria also. However, chemical profiling of the extract has not been performed. Our results are in line with the studies previously done using two other species of *Aloe*: such as *A. sinana* and *A. harlana* which had displayed significant activities towards *E. coli*, *S. typhimurium* and *S. aureus*.^{39,43} Likewise, a study from the Jimma zone of Ethiopia reported that crude acetone extract (200 mg/ml) of *A. pulcherrima* prepared by percolation method shown a strong *in vitro* antibacterial activity against *E. coli*, *P. aeruginosa*, and *S. aureus*.⁴⁰ It is generally believed that the antibacterial activity of *Aloe* plants is initiated by a major group of compounds, polysaccharides by triggering phagocytic leukocytes destroying bacteria.²⁴

Antibacterial activity of *G. schweinfurthii*

As per the primary screening, ethyl acetate and chloroform were the best solvents to extract antibacterial metabolites from *G. schweinfurthii*. These two extracts showed significant antibacterial activity against *P. aeruginosa* and *P. mirabilis* followed by *S. typhimurium*. In contrast to our study, the methanolic extract of

other species of *Grewia*, ie., *G. ferruginea* inhibited the growth of both ATCC and clinical isolates of *P. aeruginosa*.⁴⁴ Methanolic extract of another species, *G. occidentalis*, from South Africa, showed significant inhibition at a concentration of 4 mg/ml against Gram-positive and Gram-negative bacteria in the agar dilution method.²⁰ A notable result of our study is that *G. schweinfurthii* exhibited antibacterial activity towards *H. influenzae* which is considered as a bacterial pathogen of respiratory tract infections in children and adults. However, Gram-positive bacteria were found to be highly resistant. It is envisaged that some antimicrobial constituents in plants have a specific spectrum of activity (narrow) and therefore could be rendered inactive against certain species of pathogens.⁴⁵

Antibacterial activity of *K. begoniifolia*

In the present study, the second most active plant was *K. begoniifolia* which exhibited efficient activity against Gram-negative bacteria. Ethyl acetate extract of *K. begoniifolia* was found to be very effective. In contrast to our results, petroleum ether, chloroform and methanolic extracts of *K. begoniifolia* from the Jimma zone of Ethiopia showed antibacterial activity in an earlier study.⁴⁶ The discrepancy in the results could be correlated to the differences in concentration, sample processing as well as the habitat of the plant.⁴⁶ In the comprehensive screening, the extract of *K. begoniifolia* significantly inhibited the growth of *S. aureus* isolated from ear discharge. In accordance with our study, similar results were reported by other plant species of the same family (Malvaceae), particularly, *Sida acuta* which efficiently inhibited the clinical isolate, *S. aureus* in comparison to the ATCC strains.⁴⁷ The same authors also demonstrated that the presence of alkaloids is responsible for the antimicrobial activity of *S. acuta*.⁴⁷ Besides, *K. begoniifolia* needs special citation as the ethyl acetate extract of this plant showed activity against *H. influenzae*. Studies pertaining to the antibacterial activity of *K. begoniifolia* are scanty.⁴⁶ Therefore, our results provide a scientific basis for the traditional use of this plant as an antibacterial agent, since it showed a moderate spectrum of inhibitory activity. However, further investigation is essential to elucidate the pharmacological mechanisms and the traditional value and hence the usefulness of this plant.

Antibacterial activity of *U. leptocladon*

The antibacterial activity of *U. leptocladon* was significantly higher when ethyl acetate was used as the solvent for extraction. It efficiently inhibited the growth of 93% of the pathogens tested except *H. influenzae*.

U. leptocladon displayed considerable efficacy, particularly against the ATCC bacteria. Ethyl acetate extract of *U. leptocladon* exhibited significant activity against *E. coli*, *S. flexneri*, *K. pneumoniae*, *S. aureus* and *E. faecalis* and was even higher than the reference, ciprofloxacin. The pronounced inhibitory activity of ethyl acetate extract might be due to the solubility and availability of medium polar antibiotic constituents.³⁴ A recent study showed that other species of *Uvaria* (*U. chamae*) inhibited the growth of *E. coli*, *Klebsiella* sp., *Proteus* sp., *P. aeruginosa*, and *S. aureus*.⁴⁸ In the comprehensive screening, MDR bacterial isolates, particularly the Gram-negative ones were found to be moderately susceptible. A notable result of this study is that the *U. leptocladon* used to treat urinary tract and wound infections in the Omo valley are significantly effective against MDR strains of *E. coli* and *S. aureus*, the most common causative agents of the urinary tract and wound infections. However, the resistance exhibited by the Gram-negative MDR isolates might be due to the permeability barrier provided by the cell wall or to the membrane accumulation mechanism.¹⁸ A literature survey revealed that hitherto no studies had specifically reported the antibacterial activity of *U. leptocladon* against ATCC or MDR clinical bacterial isolates. However, the antimicrobial activity of other species of *Uvaria* (*U. rufa* and *U. chamae*) against *M. tuberculosis*, vancomycin-resistant Enterococcus, and MRSA are already reported.^{49,50} Besides, pharmacological studies also proved that some species of the genus *Uvaria* possess potential antimicrobial activities.^{50,51} The notable antimicrobial activity demonstrated by the *U. leptocladon* pellucidly indicates that this plant is a potential source of antibacterial components with a broad-spectrum of activity. As discussed earlier, this species is unique and well-known for some special metabolites such as dihydrochalcones, uvaretin, and isouvaretin having antimicrobial activity.⁵² Our results revealed wide variations in the MBC/MIC values against ATCC and MDR isolates of bacteria tested. MIC values of *U. leptocladon* against the ATCC strains were less prominent compared to MDR bacterial isolates. In the case of ATCC strains, the lowest MIC value was recorded against *E. coli*. The MBC/MIC ratio was determined to identify whether the active crude extract of *U. leptocladon* was bactericidal or bacteriostatic in nature. The ratio of MBC/MIC against four ATCC strains was ≤ 2 and this value inferred that the antibiotic mechanism of plant extract is bactericidal. Concerning MDR bacterial isolates, the lowest MIC value was recorded against both *E. coli* and *K. pneumoniae*. It could be en-

visaged that the antibiotic mechanism against MDR isolates is bacteriostatic rather than bactericidal because of the ratio of MBC/MIC was ≥ 2 .¹⁸ The variation in MBC/MIC values could be due to fluctuations in factors such as the crude nature of extract, concentrations of active constituents, cytoplasmic permeability associated with different metabolites, and virulence factors of bacteria.¹⁸ The exact mechanism of action of crude extract of *U. leptocladon* was not examined in this study. However, one could support that antibacterial components present in the *U. leptocladon* extract might be able to interrupt the permeability of the cytoplasmic membrane of bacteria, thereby inhibiting their growth. Therefore, the overall results of antibacterial activities provide some scientific credence to the use of *U. leptocladon* in traditional medicines for the treatment of urinary tract and wound infections.

Conclusion

Antibacterial activity of the four medicinal plants varied with species, type of solvents used for extraction, and also the strains of bacteria tested. Under this experimental condition, ethyl acetate and ethanol are effective for extracting antimicrobial components from all four plant species. Two plant species, *A. pirottae* and *G. schweinfurthii* demonstrated moderate activity against ATCC bacterial strains tested. *K. begoniifolia* and *U. leptocladon* showed remarkable antibacterial activity. Among the four plants screened, crude ethyl acetate extract prepared from *U. leptocladon* significantly inhibited the growth of both ATCC and MDR bacterial isolates indicating that metabolites present in this extract are antibacterial with a broad spectrum of activities. Based on the overall findings, it could be inferred that *U. leptocladon* is a promising source of novel antibiotic leads. A detailed study is being underway on the bioassay-guided fractionation and isolation of bioactive constituents from these plants.

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Περίληψη

***In vitro* αντιβακτηριακή δράση τεσσάρων εκχυλισμάτων φυτών χρησιμοποιούμενων στην παραδοσιακή θεραπευτική από την περιοχή του Omo στην νότια Αιθιοπία**

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Ο έλεγχος φυτών που χρησιμοποιούνται στην παραδοσιακή θεραπευτική μπορεί δυνητικά να προσφέρει αξιοποιήσιμες πληροφορίες σχετικά με αντιμικροβιακά δραστικές ουσίες. Η παρούσα μελέτη εκτίμησε την αντιβακτηριακή δράση ακατέργαστων εκχυλισμάτων τεσσάρων θεραπευτικών φυτών, *in vitro*, έναντι πρότυπων στελεχών από το ATCC (American Type Culture Collection) (δύο Gram-θετικά και επτά Gram-αρνητικά βακτήρια) και κλινικών στελεχών πολυανθεκτικών (MDR) βακτηρίων (δύο Gram-θετικά και τρία Gram-αρνητικά βακτήρια), εφαρμόζοντας μέθοδο διάχυσης σε άγαρ. Βάσει εθνοβοτανικών δεδομένων, επιλέχθηκαν τέσσερα φυτά τα οποία συνελέγησαν από διαφορετικές περιοχές του νοτίου Omo. Φύλλα (*Aloe pirottae*, *Kosteletzkya begoniifolia* και *Uvaria leptocladon*) και ρίζες (*Grewia schweinfurthii*) των φυτικών ειδών υπέστησαν εκχύλιση με τη χρήση έξι διαφορετικών οργανικών διαλυτών. Τα φυτά που εμφάνισαν τους μεγαλύτερους δείκτες δραστικότητας μελετήθηκαν ακολούθως και έναντι MDR βακτηριακών στελεχών και υπολογίσθηκαν οι ελάχιστες ανασταλτικές και βακτηριοστατικές συγκεντρώσεις [MIC (minimum inhibitory concentration) και MBC (minimum bactericidal concentration)] για το πλέον δραστικό φυτικό εκχύλισμα. Τα αποτελέσματα του αρχικού ελέγχου κατέδειξαν ότι δύο φυτά (*K. begoniifolia* και *U. leptocladon*) εμφάνισαν υψηλή δραστικότητα έναντι ATCC στελεχών. Το εκχύλισμα οξικού αιθυλεστέρα του *U. leptocladon* εμφάνισε τη μέγιστη ζώνη αναστολής, μεταξύ 20 ± 1.15 mm έως 40 ± 1.45 mm έναντι Gram-αρνητικών βακτηρίων, και 21 ± 0.58 mm έως 28 ± 2.03 mm έναντι Gram-θετικών βακτηρίων. Παρόμοια, εκχυλίσματα του *K. begoniifolia* στον ίδιο διαλύτη παρουσίασαν ζώνη αναστολής εύρους 10 ± 0.33 mm και 20 ± 1.15 mm έναντι Gram-θετικών και Gram-αρνητικών βακτηρίων αντίστοιχα. Τα αποτελέσματα του περαιτέρω ελέγχου κατέδειξαν ότι το εκχύλισμα σε οξικό αιθυλεστέρα του *U. leptocladon* κατέστειλε αποτελεσματικά την ανάπτυξη MDR βακτηριακών στελεχών. Συνολικά, η μελέτη συμπέρανε πως και τα τέσσερα φυτά εμφανίζουν, κυμαινόμενου εύρους, αντιβακτηριακές δραστηριότητες. Το εκχύλισμα οξικού αιθυλεστέρα του *U. leptocladon* εμφάνισε την ευρύτερη και υψηλότερη αντιβακτηριακή δραστικότητα, εύρους 15.7 ± 0.3 έως 23.7 ± 0.7 mm με τη μέθοδο διάχυσης σε άγαρ, ενώ οι τιμές MIC του *U. leptocladon* έναντι Gram-αρνητικών βακτηρίων κυμάνθηκαν μεταξύ 7.8 και 125 µg/ml και οι αντίστοιχες τιμές MBC μεταξύ 15 και 500 µg/ml. Οι αντίστοιχες τιμές MIC και MBC βρέθηκαν στο ελάχιστο, 125 µg/ml και 500 µg/ml αντίστοιχα, για τα Gram-θετικά βακτήρια. Συνολικά τα αποτελέσματα επιβεβαιώνουν το ουσιαστικό της χρήσης του *U. leptocladon* στην παραδοσιακή θεραπευτική ως αντιβακτηριακού παράγοντα.



Λέξεις κλειδιά

αντιμικροβιακή δράση, θεραπευτικά φυτά, νότιο Omo, *U. leptocladon*, *A. pirottae*, *G. schweinfurthii*, *K. begoniifolia*.



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