

ANTIMICROBIAL ACTIVITY OF WARBUGIA UGANDENSIS AGAINST GRAM-NEGATIVE MULTI-DRUG RESISTANT BACTERIA**M. M. Njire¹, N. L. M. Budambula² and J. Kiiru³**^{1,2}*Department of Botany, Jomo Kenyatta University of Agriculture and Technology, Nairobi Kenya*³*Centre for Microbiology Research, Kenya Medical Research Institute, Nairobi, Kenya*
Email: momunjir@gmail.com**Abstract**

The rise in antibiotic resistance has resulted in decreasing numbers of effective antimicrobial agents available to treat infections caused by multi-drug resistant (MDR) bacteria. This has necessitated a search for new antimicrobial agents. Herbal remedies may offer alternative treatment options especially because they elicit little or no transferable resistance if used in optimal concentrations. This study evaluated the antimicrobial properties of *W. ugandensis* against eight multi drug resistant (MDR) Gram-negative bacterial isolates. The herbal extracts were obtained using methanol as an organic solvent and water as an inorganic solvent. Determination of the Minimum Inhibitory Concentrations (MICs) and the sub-lethal concentrations of the effective extracts was done using broth inoculation method followed by colony count. The test isolates were habituated in sub-lethal extract concentrations (SLC) for 72 h to investigate effect on their sensitivity to conventional antibiotics. Methanol extracts from the root and stem-bark of *W. ugandensis* were active against the test strains and their inhibitory effect was significantly different ($p < 0.05$) from that of other extracts. We determined that the extracts had an inhibitory rather than a lytic (cidal) mode of action. The extracts from this plant had an effective MIC of 42 µg/ml and exhibited an inhibitory mode of action and did not elicit resistance to conventional antibiotics. Methanol extracts from the root and bark of this plant may provide potential sources for further development of alternative antimicrobial agents for the treatment of MDR infections.

Key words: multi-drug resistant (MDR) bacteria, minimum inhibitory concentrations (MICs), sub-lethal concentration (SLC)

1.0 Introduction

Antimicrobial agents have substantially reduced the threat posed by infectious diseases over a period of time since their discovery in the 1940s (Lewis and Ausubel, 2006). However, the escalation of MDR in bacteria in recent years has seriously jeopardized these gains. Increased usage of antimicrobial agents to treat bacterial infections has led to the emergence of MDR strains (Rodriguez *et al.*, 2006). Such strains are resistant to first line of treatments and also the more expensive second and third-line antibiotics. The high cost of such replacement drugs is prohibitive. Furthermore, conventional antibiotics lose their effectivity soon after a certain period of clinical use. This scenario has necessitated a search for new antimicrobial substances from other sources especially plants, which produce diverse chemical compounds with different biological activities (Lewis and Ausubel, 2006).

This study evaluated the *in vitro* antibacterial properties of extracts from *Warbugia ugandensis* against eight Gram-negative clinical isolates. *W. ugandensis* (Canellaceae), commonly known as the East African green wood, is a spreading evergreen tree 4.5-30 m tall with a smooth bark. It is found in many parts of Africa and it has many medicinal uses. It is widely used by Traditional Health Practitioners in Eastern and Southern Africa for treatment of bacterial diseases and conditions associated with HIV/AIDS infections (Kiringe, 2006). The roots, bark and leaves of *W. ugandensis* are used by East African communities as a remedy for bacterial infections besides being used for constipation, stomach ache, coughs, muscle pains, weak joints and general body pains. A decoction of leaves or bark is taken as a cure for malaria (Kokwaro, 1976). Among the Maasai in Kenya, the bark is used for treatment of sexually transmitted diseases, stomachache, throat and chest infections, loss of appetite, malaria, diarrhoea, cough and internal wounds/ulcers (Kiringe, 2006). In Ethiopia the stem bark of *W. ugandensis* is used for the treatment of tuberculosis (Wube *et al.*, 2005). In Arusha and Manyara regions of northern Tanzania the barks and roots of *W. ugandensis* are used for treatment of HIV-related opportunistic infections, including persistent malaria, diarrhoea and flue (Kayombo *et al.*, 2007). Records indicate that all parts of *W. ugandensis* are edible and that leaves, bark, young shoots and fruits are used in curries while roots are used for soup (Kokwaro, 1976; Mbuya *et al.*, 1994), suggesting that the plant is safe for human use. The most commonly prescribed parts of the plant by Traditional Health Practitioners are usually barks and roots, which are harvested in the wild through de-barking and up-rooting the plant, respectively.

Phytochemical investigations of this plant indicate the presence of drimane-type sesquiterpenoids as the main active components (Brooks and Draffan, 1969; Wube *et al.* 2005). Known sesquiterpenoids from *W. ugandensis* include ugandensolide, ugandesidial, warburgin and warburgiadione commonly detected from the heartwood, while muzigadiolide, deacetylugandensolide, cinnamolide, mukaadial, ugandensidial, muzigadial and waburganal are isolated from the stem-bark. Flavonol

glycosides and monoterpenes have also been detected from the leaves of this plant (Manguro *et al.* 2005). It is therefore possible that any of these active compounds could be responsible for the antibacterial activities reported in the current study.

2.0 Materials and Methods

2.1 Test Strains

The bacterial strains were clinical isolates donated from a project by Kiiru *et al.* (KEMRI SSC No. 1177) whose resistance phenotypes were known.

Table 1. Resistance phenotypes of test strains

ID	Species	Antibiogram
E ₁	<i>E. coli</i>	AMP, SMX, AUG, NA, C, NOR, S, ATM,CRO
E ₂	<i>E. coli</i>	NA, C, NOR, SMX, AUG, S, ATM, CXM, CRO
P ₁	<i>P. aeruginosa</i>	ATM, CXM, CRO, FOX, NOR, AMP, AUG, MN
P ₂	<i>P. aeruginosa</i>	NA, N, TM, SMX, AUG, S, ATM, CXM,AMP
K ₁	<i>K. pneumoniae</i>	NOR, SMX, AUG, S, ATM, AMP, TM, S, N
K ₂	<i>K. pneumoniae</i>	NA, N, TM, SMX, AUG, S, ATM, CRO,AMP
S ₁	<i>S. typhi</i>	NOR, SMX, AUG, S, ATM, AMP, CFM, S, N, C
S ₂	<i>S. typhi</i>	CXM, SMX, AUG, S, ATM, AMP, CRO, S, N, C

An isolate was regarded as multi-resistant if it showed resistance against five or more of the antimicrobials listed. NOR, Norfloxacin; SMX, Sulfamethoxazole; NA, Nalidixic acid; TM, Trimethoprim; C, Chloramphenical; N, Neomycin; S, Streptomycin; MN, Minocycline; CXM, Cefuroxime; CRO, Ceftriaxone; ATM, Aztreonam; FOX, Cefoxitin; AUG, Augmentin; AMP, Ampicilin.

Identity of isolates was confirmed by sub-culturing on MacConkey agar (Oxoid, Louis, Mo, USA) and where necessary, the API 20E strips were used (BioMerieux, France). The isolates studied included Gram-negative bacilli (*Escherichia coli*, E₁ & E₂; *Klebsiella pneumoniae*, K₁ & K₂; *Pseudomonas aeruginosa*, P₁ & P₂; and *Salmonella typhi*, S₁ & S₂).

2.2 Plant Materials

Samples of leaves, stem-barks and roots of *W. ugandensis* were obtained from Jomo Kenyatta University of Agricultural and Technology (JKUAT) botanical garden from plants identified by a qualified botanist. The samples were separately air dried until a constant weight was attained after which they were chopped and ground to fine powder using a motor grinder. The herbal extracts were obtained through organic (methanol) and inorganic (water) solvents extraction.

2.3 Water Extraction

Warm water extraction was done to simulate the traditional decoction method of preparing herbal preparations. Sixty (60) g of the ground powder was soaked in 300 ml sterile distilled and deionised water at 50°C for 1 hr, placed in an orbital shaker at 100 rpm for 24 h at 25°C. The resulting elute was membrane-filtered (0.45 µm) and lyophilized into granules (LyoBeta range, Telstar, UK). Stock solutions were prepared by dissolving 0.4 g of the granules in 20 ml of deionised sterile distilled water while another set of a similar amount of preparations was dissolved in TBE buffer (pH 8) as a control, and stored at -20°C.

2.4 Organic Extraction

Organic extraction was done using methanol. Sixty (60) g of the ground powder was soaked for 72 hr in a beaker containing 300 ml of methanol (Analytical Grade, 99.9%, Sigma-Aldrich, UK) and placed in an orbital shaker (Basic Model, Cole-Parmer, UK) at 100 rpm for 72 h at 25°C. The resulting extracts were filtered on a Whatman paper number 1 (General Lab Supply, USA) then membrane-filtered for sterility. The extracts were concentrated using a vacuum rota-vapour (Basic Model, Buchi, UK) at 50°C and 100 mbar. In order to evaporate the methanol used as the extraction solvent, the concentrates were oven-dried at 25°C until a constant weight was attained. Stock solutions of the methanol-free paste were prepared by dissolving 0.4 g in 20 ml of deionised sterile distilled water. Another preparation was dissolved in Tris-borate-EDTA (TBE) buffer at pH 8 as an alternative diluent. The preparations were then stored at -20°C until further use.

2.5 Testing for Antibacterial Effects

Preliminary screening of the plant extracts for antibacterial effect was done using *E. coli* J53 as the test organism. This strain has a defined genetic background and is susceptible to all conventional antibiotics except Sodium azide. A confluent lawn of the bacteria was prepared from a 0.5 MacFarland equivalent (approximately 10⁶ CFU/ml) and placed in an incubator to dry after which 0.5 µl of the stock test extracts were point inoculated using a micropipette (Eppendorf, Hamburg, Germany). Other two sets of preparations were made in a similar way and used for testing the efficacy at each of the following concentrations: 5 µg/ml, 15 µg/ml, 25 µg/ml, 35 µg/ml, 45 µg/ml, 55 µg/ml and 65 µg/ml. Extract-free sterile distilled water and TBE buffer (the diluents used for making the stock solutions of the test extracts), were inoculated in another set of these plates and used as negative controls. The plates were allowed to stand for at least 1 hr at room temperature for the extracts to diffuse at the point of inoculation before incubation at 37°C for 8 h. Observation of a clear zone on bacterial lawn at the point of inoculation of the extract was interpreted as evidence of inhibition of bacterial growth. The diameter of these zones were measured and recorded to the nearest size mm.

2.6 Determination of MICs and SLCs of the extracts against clinical MDR Isolates

Duplicate tubes containing 2 ml Muller-Hinton broth were prepared. To one set of these tubes, separate concentrations of the extract in the range of 5 µg/ml to 65 µg/ml were added to separate tubes before a uniform inoculum of 10^6 CFU/ml of the test isolates was added to each tube. Negative controls were set in a similar way but using extract-free distilled water and TBE buffer. The preparations were incubated for 8 h at 37°C with continuous shaking at 100 rpm in an orbital shaker. An inoculum of 1 µl of the preparations was streaked onto MacConkey plates, incubated for 8 hr and the colonies counts determined. The tube containing the lowest concentration at which no colonies were observed was identified and calculations done to determine the appropriate concentration of the herbal extract added. This concentration was identified as the MIC. The tube containing the preparation at which the last visible growth was observed was identified as the highest amount of extract that does not inhibit bacterial growth. This concentration was therefore identified as the highest Sub-Lethal Concentration (SLC) of the extract. This sub-lethal concentration was used as the reference concentration in the habituation experiment.

2.7 Habituation Tests

This was done according to McMahon *et al.* (2007) with slight modifications. Two sets of duplicate tubes containing 2 ml MH broth were prepared. A sub-lethal concentration of the extract (30 µg/ml) was added and inoculated with approximately 10^6 CFU/ml bacterial cells of the clinical isolates or controls ATCC *E. coli* strains 25922. Negative controls were prepared using similar sets of bacterial preparations but instead of the extracts, deionised sterile distilled water or TBE buffer (pH 8) were used. All preparations were incubated for 72 hr at 37°C with constant shaking at 100 rpm after which colony counts were determined on MacConkey agar.

2.8 Effect of pH on Antimicrobial Potential of the Extracts

Two sets of the extract concentrations in the range of 5 µg/ml to 65 µg/ml were prepared by dissolving the extract in TBE buffer of pH 3, 8 and 11. The extracts were separately added to duplicate tubes containing 2 ml MH broth and inoculated with 10^6 CFU/ml of the test isolates. The extract suspended in TBE buffer of pH 8 (the pH of the buffer used as diluent in standard experiments) was used as the positive control. All preparations were incubated for 8 h at 37°C, after which colony counts were determined on MacConkey agar.

3.0 Results

The antibacterial activity of crude extracts from *W. ugandensis* against *E. coli* ATCC J53 strain was assessed qualitatively by measuring and calculating means of three sets of the inhibition zone diameters and quantitatively by determining minimal inhibitory concentrations against the clinical MDR isolates. In the pre-screening assay, the extracts from *W. ugandensis* inhibited the growth of *E. coli* ATCC J53. However, methanol extracts had a high antimicrobial effect than the water extracts with inhibition zones of 30 mm, 28 mm and 16 mm for the root, stem-bark and leaf extracts respectively at 100 µg/ml concentration. Inhibition zones for the water extracts were 20 mm, 18 mm and 12 mm for the root, stem-bark and leaf extracts respectively at equivalent concentration of 100 µg/ml. For both methanol and water extracts, the antibacterial activity of the root and stem-bark extracts was higher than of the leaf extracts. However, the inhibition characteristics of root and stem-bark water extracts were not significantly different (t-test, $p > 0.05$).

In determining efficacy of the extracts against the clinical MDR isolates, methanol extracts from the root and stem-bark of the plant had inhibitory effect while methanol extracts from the leaf and all water extracts were non-inhibitory

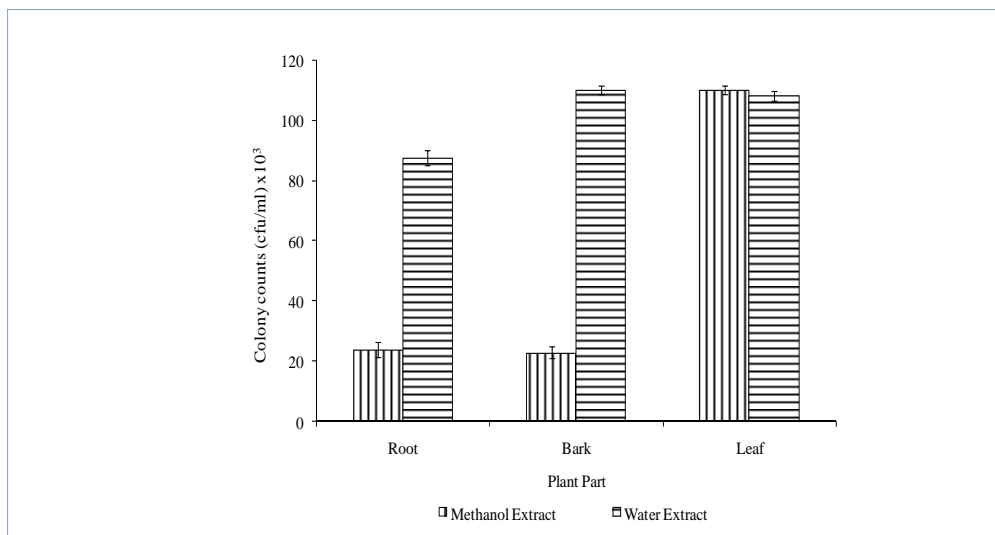


Figure 1: Differences in the effectiveness of methanol and water extracts of *W. ugandensis* against a clinical *S. typhi* isolate, S_1 .

The colony counts from the leaf methanol, root water, stem-bark water and leaf water extracts were not significantly different from those of the negative controls ($P > 0.05$), indicating that there was no inhibition. All the clinical Gram-negative MDR isolates were inhibited by the methanol extracts from the root and stem-bark of *W. Ugandensis*, indicating that the extracts under *in vitro* study has broad-spectrum antibacterial activity. There was a gradual decrease in the number of colonies as the concentration of the root and stem-bark extracts obtained using methanol

increased. There were no colonies at concentrations $> 42 \mu\text{g/ml}$ for the stem-bark and root extracts. Therefore, the MIC for *W. ugandensis* root and stem-bark methanol extracts was $42.5 \mu\text{g/ml}$. Consequently, the highest sub-lethal extract concentration for these extracts was $32.5 \mu\text{g/ml}$.

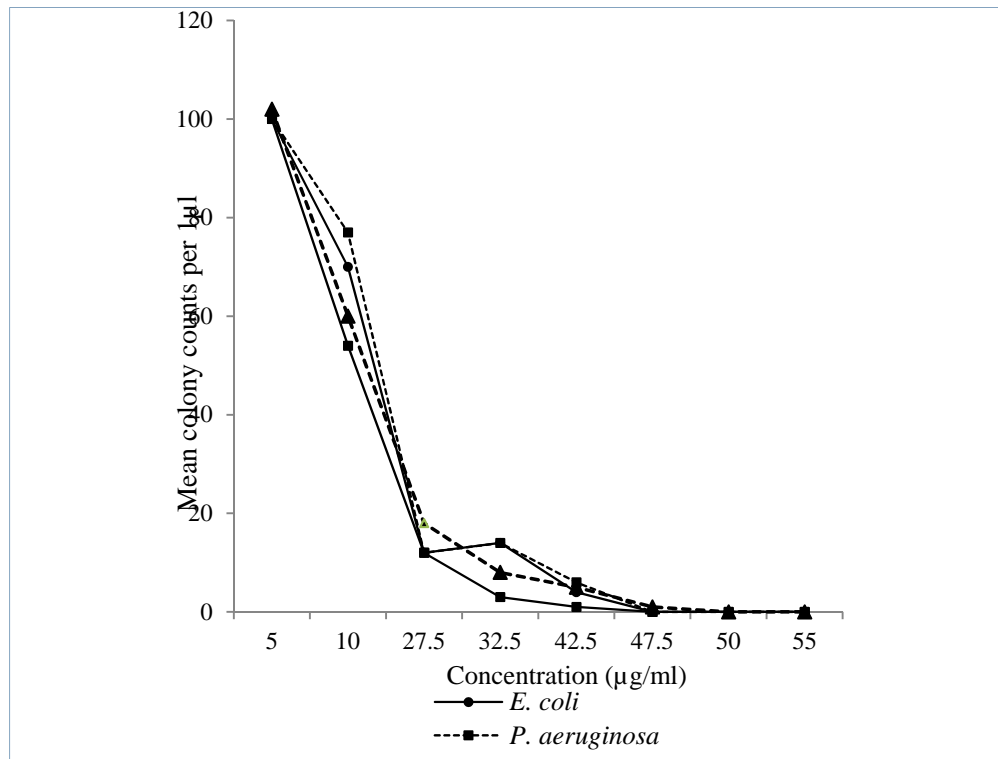


Figure 2: Inhibitory effect of methanolic stem bark extracts from *W. ugandensis* against the MDR clinical isolates. Test isolates represents one strain for each of the five Gram-negative species used. Colony counts were an average of the duplicate set for each species strain and were obtained from $1 \mu\text{l}$ of broth preparation.

The antibiotic sensitivity profiles of the clinical isolates and ATCC *E. coli* strains 25922 and J53 was not significantly different (t-test, $p > 0.05$) following exposure to a sub-lethal concentration ($30 \mu\text{g/ml}$) of *W. ugandensis* root and stem-bark methanol extracts.

Table 2: Mean inhibition zones of 15 conventional antibiotics against a *S. typhi* isolate, S₂, before and after exposure to sub-lethal concentration of *W. ugandensis* methanol root extract.

	Antibiotics														
	NOR	SMX	NA	TM	C	N	S	MN	CXM	CFM	CRO	ATM	FOX	AUG	AMP
Mean zones of isolate S ₂ before exposure	23	11	13	10	12	15	9	15	12	18	14	14	15	9	8
Mean zones of isolate S ₂ after exposure	22	12	14	9	13	16	10	16	13	17	15	16	16	8	6
t-test (p value)	0.03	0.14	0.27	0.13	0.96	0.21	0.45	0.35	0.56	0.92	0.91	0.61	0.9	0.17	0.16

The choice of pH for the diluents used to dissolve the herbal extracts was found to influence inhibitory characteristics of the extracts. Normal saline (pH 7), distilled water (pH 7) and TBE buffer pH 8 (containing no extract) did not inhibit bacterial growth. However, root and stem-bark methanol extracts dissolved in TBE buffer pH 3 and pH 11 had increased inhibitory characteristics. Bacterial colony counts from extract preparations dissolved in TBE pH 3 and pH 11 were significantly lower than those dissolved in TBE buffer pH 8 ($p < 0.05$). Therefore the optimal pH for use of TBE buffer as a diluent in which no inhibition of bacterial growth was observed was pH 8.

4.0 Discussion

This study reports the antibacterial effect of the root and stem-bark methanol extracts from *W. ugandensis* against both the ATCC *E. coli* strains and clinical MDR isolates. *Warbugia ugandensis* has been used traditionally to manage bacterial infections and previous studies have reported its antibacterial activity. Crude extracts and purified compounds from *W. ugandensis* have been reported to be effective against *Mycobacterium tuberculosis*, *E. coli* and *Vibrio cholerae* (Mbwambo *et al.*, 2009). Wube *et al.* (2005), reported antimycobacterial activity of dichloromethane stem bark extracts against *Mycobacterium aurum*, *Mycobacterium fortuitum*, *Mycobacterium phlei* and *Mycobacterium Smegmatis*. The active constituents showed MIC values ranging from 4 to 128 $\mu\text{g/ml}$ compared to the antibiotic drugs ethambutol (MIC range from 0.5 to 8 $\mu\text{g/ml}$) and isoniazid (MIC range from 1 to 4 $\mu\text{g/ml}$).

These earlier reports corroborates with the findings of this study in which all the clinical isolates were inhibited by methanol extracts from the roots and stem-barks of *W. ugandensis* with an MIC value of 47.5 $\mu\text{g/ml}$. The isolates used were resistant to more than four classes of conventional antibiotics such as aminoglycosides, quinolones, cephalosporins and β -lactams among others, and thus were multi-drug resistant (Koronakis *et al.*, 2000). Therefore, the inhibitory action of the extracts indicates their potential application as alternative broad-spectrum antibacterial remedies. In effect, further characterization, isolation and utilization of the bioactive compounds in the extracts can offer more effective and less expensive treatment options to bacterial diseases.

In contrast to the previous findings that Gram-negative bacteria are hardly susceptible to the plant extracts in doses less than $2 \times 10^5 \mu\text{g/ml}$ (Suffredini *et al.*, 2006), our results showed inhibition at concentrations as low as 42.5 $\mu\text{g/ml}$. The variation of susceptibility between Gram-positive and Gram-negative bacteria could be attributed to their intrinsic properties that are related to the permeability of their cell surface to the extracts. Therefore, these findings further support the idea that *W. ugandensis* may be an important source of compounds with broad-spectrum antimicrobial properties.

The lack of inhibition by the leaf extracts may suggest diverse resistance mechanisms such as efflux pumps. Many efflux systems are multidrug transporters capable of expelling a wide spectrum of structurally unrelated drugs and antimicrobials, thus contributing significantly to bacterial multidrug resistance (van Veen and Konings, 1997). Contrasting results could also be attributed to differences in bioassay techniques used and diluents used for extraction and elution. In his study, Manguro *et al.* (2003) used the disc diffusion technique to assay the antimicrobial activity of the plant extracts. Such standard antimicrobial susceptibility testing methods like the agar diffusion and Kirby-Bauer may result to misinterpretation of results especially for extracts with low antimicrobial activity or in cases where the active ingredient(s) may irreversibly bind to the paper discs (Das *et al.*, 2010). To overcome these drawbacks, the antimicrobial activity of the crude plant extracts was detected by performing viable counting following direct inoculation. This method allows for maximum action by the extract on the bacterial cells hence generating more reliable data. Besides the methodological approaches used by various investigators, the contrasting results could also be attributed to the locality of plant species, storage conditions, test strains used, and lack of appropriate positive and negative controls in some of these studies.

The current study reports that exposure of the MDR strains to sub-lethal extract concentration of *W. ugandensis* extracts had no effect on their susceptibility to conventional antibiotics. Therefore, this study indicates the safety of using the plant extracts as herbal remedies in the treatment of bacterial infections as they do not affect susceptibility profiles of bacteria to conventional antibiotic. In addition, this study provides useful data that will shed light on the use of the selected herbal extracts as alternatives to conventional antimicrobials.

The use of potentially toxic solvents for extraction and elution requires that the solvent tolerance of the test strains be tested to establish the optimal concentration and pH values at which the solvents would result to significant reduction in viable cell counts. Good experimental designs ensuring that the extraction solvents such as methanol is fully evaporated should be ensured in order to avoid misleading results. Evaporation conditions should also be controlled so as to minimise the chances of losing the thermal-labile active ingredients. Similarly, the pH of the eluting buffers should be optimized to enhance their ability to dissolve the active ingredients in the plant extracts. However, if the potential toxicity of the eluting solvents is also not checked, the results could be misinterpreted. The higher potency observed in methanol extracts unlike the water extracts may be due to polarity of the solvents and their ability to better elute the active ingredients (Parekh *et al.*, 2005). It is known that highly polar solvents are able to extract phytochemicals efficiently and thus methanol or related organic solvents may be ideal for elution of active ingredients. These differences in polarity determine the solubility of the extracts into the growth media although further characterization of the test extracts

would be necessary to prove this hypothesis. Previous studies have shown that plant extracts in organic solvents like methanol provided consistent antimicrobial activity as compared to those extracted in water (Parekh *et al.*, 2005). Additionally, methanolic extracts from plants consistently provide more antimicrobial activity compared to those extracted in ethanol, or other more polar substances. The higher anti-bacterial activity of methanol extracts is hypothesized to be due to the polarity of the solvent, and to the ability to dissolve or diffuse into the media used in the assays (Cowan, 1999).

5.0 Conclusion

The five clinical isolates under investigation strains were multi-drug resistant. Methanol extracts had better inhibitory effects than water extracts. Methanol extracts from root and stem-bark of *W. ugandensis* exhibited better antimicrobial properties than the leaf extracts. The study established that the MIC of the root and stem-bark extracts of *W. ugandensis* obtained using methanol against the MDR gram-negative bacterial strains was 42.5 µg/ml while the highest sub-lethal concentration was 32.5 µg/ml. Exposure of the clinical MDR Gram-negative isolates and ATCC *E. coli* strains 25922 and J53 to sub-lethal extract concentrations of the effective extracts of *W. ugandensis* do not affect their sensitivity profiles to conventional antibiotics. This further indicates that extracts from these plants may not jeopardize the use of conventional antibiotics.

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