Antibacterial Activity of Locally Prepared Herbal Cough Extracts against *Klebsiella pneumoniae* and *Streptococcus pneumoniae*

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ABSTRACT

Cough due to *Klebsiella pneumoniae and Streptococcus pneumoniae* is currently managed by conventional antibiotics and herbal extracts in Uganda. However, much as these herbal extracts are extensively used, their antibacterial activity is not known. This study aimed at determining the antibacterial activity of the selected locally prepared herbal cough extracts against two bacterial strains i.e. *Klebsiella pneumoniae* (ATCC 700603), and *Streptococcus pneumoniae* (ATCC 49619). The herbal cough extracts were screened for antibacterial activity using Agar-well diffusion method for determining zone of inhibition, macro broth dilution method for Minimum Inhibitory Concentration (MIC) determination and Streak plate method for Minimum Bactericidal Concentration (MBC).

In vitro evaluation of antibacterial activity of the 5 brands of herbal cough extracts against *K. pneumoniae* and *S. pneumoniae* revealed that all extracts possessed significant antimicrobial effects against all microorganisms tested ($p < 0.05$). However, MM04 (35.6±0.0) mm and MM03 (33.6±1.5) mm had maximum zones of inhibition as compared to other herbal extracts against *K. pneumoniae* and *S. pneumoniae* respectively.

Average MIC results for extracts against *K. pneumoniae* indicated that MM01 had the highest MIC (2.5000 mg/ml) while MM03 had the least MIC (0.0625 mg/ml). Average MIC results for extracts against *S. pneumoniae* showed MM01 had the highest MIC (2.0000 mg/ml) while MM03 3 had the least MIC (0.0438 mg/ml).

Average MBC results for extracts against *K. pneumoniae* indicated that MM01 had the highest MBC (4.000 mg/ml) while MM03 had the least MBC (0.030 mg/ml). Average MBC results for extracts against *S. pneumoniae* showed MM01 had the highest MBC (4.000 mg/ml) while MM03 had the least MBC (0.033mg/ml).

The results obtained in present study were revealed that locally prepared herbal extracts had significant antibacterial activity. Hence they can be used as promising alternatives of antibiotics used against Respiratory Tract Infections due to K. *pneumoniae* and *S. pneumoniae*.

Keywords

Antibacterial activity, herbal cough extracts, *Klebsiella pneumoniae,* Streptococcus *pneumonia,* Minimum Inhibitory Concentration, Minimum Bactericidal Concentration

INTRODUCTION

Background

Klebsiella pneumoniae and Streptococcus pneumoniae are among the common causes of Respiratory Tract Infections (RTIs) globally especially in children and immune compromised individuals [1] . Worldwide, Respiratory Tract Infections (RTIs) are the largest cause of death in children, causing two to four million deaths each year, with the highest incidence in developing countries[2]. RTIs are normally associated with cough[3]. *K. pneumoniae* and *S. pneumoniae* were indicated to be among the leading causes of pneumonia[4]. Commercial antimicrobial drugs are usually used in treatment of RTIs and this has led to increased multiple drug resistance of microorganisms due to their indiscriminate use worldwide[5]. In addition, antibiotics are sometimes associated with adverse effects on the host such as immune suppression, vomiting, nausea, and hypersensitivity[6]. Although the proportion of people living within 5 kilometers of a health care facility in Uganda rose to 72% in 2010 from 49% in 2000 [7], access to facilities is still limited by poor infrastructure, lack of medicine, lack of accommodation at facilities, shortage of medical human resource among other factors that constrain access to quality service delivery in rural areas[8]. As a result, a large portion of rural population resort to the use of herbal extracts which are thought to be safe, cheaper and accessible[9].

Herbal extracts are widely used as an alternative in the prevention and treatment of most diseases due to their unique approaches, intrinsic qualities, accessibility as well as affordability [10]. Plants synthesize various chemical compounds which are used to perform biological functions and to defend against microbial, insect, herbivores attack [11]. These chemical compounds are phyto-chemicals and can be used effectively to treat human diseases and most of them have beneficial long term effects on human health when consumed[12]. At least 10% of the phytochemicals have been isolated so far. These phytochemicals mediate their effects on the human body through processes similar to that of chemical compounds in conventional medicine [13, 14]. The World Health Organization (WHO) notes that of 119 plant-derived pharmaceutical medicines, about 74 percent are used in modern medicine in ways that correlated directly with their traditional medicinal uses by native cultures[15]. Many of the pharmaceuticals currently available have long history of use of herbal remedies including aspirin, quinine and opium[16]. The WHO also estimates that about 80% of the population living in developing countries use Traditional Medicine (TM), although, the percentage varies from country to country. For instance 90% in Ethiopia, 70% in Rwanda and 60% in Uganda and Tanzania use TM for their Primary Health Care (PHC) [17]. More than 60% of Uganda's population depends on herbal medicine because it is affordable, accessible and culturally familiar. With an estimated one traditional health practitioner for every 200-400 Ugandans (compared to one western medicine trained doctor per 20,000 people), it is deduced that herbal medicine is more widely used compared to conventional medicine [18]. Herbal extracts have long been used to manage a range of common conditions including malaria, digestive and respiratory problems, tooth aches, skin diseases and reproductive health-related complications [19]. However, in a number of cases, their antibacterial activity and effective dosages / concentrations has not been validated. This necessitates investigating the antibacterial activity, Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of locally prepared herbal cough extracts against *K. pneumoniae* and *S. pneumonia.*

MATERIALS AND METHODS

Study area

The herbal extracts were acquired from drug shops and herbal clinics in Mbarara town in Western Uganda and analyzed for antibacterial activity in the micro-biology laboratory at Mbarara Regional Referral Hospital.

Study Design

This was an experimental study aiming at determining the antibacterial activity of selected locally prepared herbal cough extracts against *K. pneumoniae* and *S. pneumoniae* related cough. Antibacterial activity was determined by the measurement of inhibition zones using agar well diffusion test and through determination of Minimum Inhibitory Concentrations (MICs) of the extracts using macro broth dilution method. The Minimum Bactericidal Concentration was determined by streak plate method. Ciprofloxacin was used as a reference commercial drug whereas peptone water was used as a blank (No drug). Five brands of herbal cough extracts were selected and 5 samples of the extract were obtained from each brand. The brands were chosen based on the level at which they are advertised.

Inclusion and exclusion criteria

A concoction was included in the study only if it was herbal. The extracts were selected based on the level at which they are advertised. To get the concoctions, I had to first pretend I was a customer.

Extracts were bought from drug shops and herbal clinics in Mbarara town from January to February 2015. They were quickly transported and stored appropriately.

Herbal syrup was not used if; the bottle was not properly sealed and those whose expiry date were unknown.

Sub culturing of test organisms.

The bacterial strains *Klebsiella pneumoniae* (ATCC 700603) and *Streptococcus pneumoniae* (ATCC 49619) were obtained from the microbiology laboratory of Mbarara Regional Referral

Hospital. *Streptococcus pneumoniae* was stored in a vial containing nutrient broth and glycerol while *Klebsiella pneumoniae* was stored in brain heart infusion media in a bijou bottle. The test organisms were sub cultured on appropriate media i.e. MacConkey and Blood Agar for *Klebsiella pneumonia* and *Streptococcus pneumoniae* respectively and incubated for 24 hours at 37□.

Gram staining was done on the stock culture after inoculation to ensure that there were no other microbes on the incubated plates. Plate reading was done and based on colonial morphology, cultural characteristics the organisms of interest were identified. Gram staining was again done on the colonies to determine whether the organisms are *Klebsiella pneumoniae* and *Streptococcus pneumoniae*. Growth of *K. pneumoniae* was indicated by presence of large pink mucoid colonies on MacConkey agar and that of *S. pneumoniae* appeared as small white colonies which showed alpha hemolysis on blood agar.

To ensure pure colonies of the organism of interest, biochemical tests were done i.e. *K. pneumoniae* was grown on Simmon's Citrate Agar and green colonies were indicative of a positive test result. Voges-Proskauer test was also done on *K. pneumoniae* which was confirmed by a negative test. *S. pneumoniae was* confirmed by optochin sensitivity and in case a catalase test showed negative. After confirmation, the bacterial cultures were maintained on nutrient agar for subsequent tests.

Preparation of 0.5 McFarland standard of test organism

Inoculum of test bacterial strains was prepared by transferring a loop full of colonies from media plates into peptone water in a bijou bottle. The absorbance was read at 530 nm and adjusted with peptone water to match that of 0.5 McFarland standards.

Anti-bacterial testing

The anti-bacterial activity of the herbal concoctions was evaluated using Agar well diffusion assay for inhibition zone diameter determination and macro broth dilution for MIC determination and streak plate method for MBC.

Determination of zone of inhibition.

Agar well diffusion method previously described by Jumuna *et al*. (2004) was used to determine the in vitro antibacterial activity of the selected herbal extracts MM01, MM02, MM03, MM04,

MM05 against two bacterial strains i.e. *K. pneumoniae* (ATCC 700603), and *S. pneumoniae* (ATCC 49619).

The media plates were prepared by pouring 15 ml of molten media into sterile plates. Set plates were incubated overnight to ensure sterility before use. The prepared Innocula were uniformly surface spread on sterile Mueller Hinton agar plates (MHA Difco, France) and Chocolate Agar for *K. pneumoniae* and *S. pneumoniae* respectively and allowed to dry for 5 minutes. Wells of 8 mm diameter were dug on the agar plates using a standard sterile cork borer and were aseptically filled with 200 µl of the test herbal extract, positive control (ciprofloxacin) and negative control (peptone water) while avoiding splashes and over filling the wells.

Plates were left for 1 hr. in the refrigerator to allow a period of pre- incubation diffusion in order to minimize the effects of variation in time between the applications of different solutions. Plates were later incubated at 37°C for 24hrs in aerobic and anaerobic conditions for *K. pneumoniae* and *S. pneumoniae* respectively after which the diameter of the inhibition zone was measured using a transparent ruler and values <8 mm were considered as not sensitive against micro-organism. The diameters of zone of inhibition produced by the extract agents were compared with that produced by the commercial control antibiotic

Determination of Minimum inhibitory concentration (MIC)

MIC is defined as the lowest concentration able to inhibit any visible growth of microorganisms in the tubes. The MIC was determined by two-fold serial dilution of the extracts in brain heart infusion broth. To each test tube labeled ½, ¼, 1/8, 1/16, 1/32, 1/64 was added 2ml of brain heart infusion broth. Serial dilutions were carried out by adding 1 ml of the extract into test tube labelled (1/2) and from test tube 1, 1 ml was transferred to test tube 2. The same procedure was repeated for all consecutive tubes but 1 ml (final ml) from test tube 1/64 was discarded. To each test tube was added 100 µl of 0.5 McFarland suspension of organisms depending on the label on the tube i.e. *K. pneumoniae* was put in a tube labeled *K. pneumoniae* and *S. pneumoniae* was put in a tube labeled *S. pneumoniae*. Ciprofloxacin and peptone water were used as reference drugs and blanks respectively.

The set up was incubated at 37□ for 24 hours after which the tubes were evaluated for presence of growth which was indicated by turbidity or absence of growth which was indicated by clear tubes. No growth showed bacteriostatic effect. Since the tubes contain a drug, media (food for microorganisms) and organisms, in case the organisms did not grow this implied that the drug concentration was effective. If there was growth, then this implied that the concentration / dilution of the drug was not effective against the micro-organism.

Determination of Minimum Bactericidal Concentration (MBC)

The MBC test determines the lowest concentration at which an antimicrobial agent kills a particular microorganism. Determination of MBC was done after the MIC test was completed where by dilutions showing no visible growth for MIC were further sub cultured onto fresh drug free nutrient agar i.e. Mueller Hinton Aga (MHA) and Chocolate Agar (CA) plates for *K. pneumoniae* and *S. pneumoniae* respectively using streak plate method. The inoculated media plates were later incubated at 37°C for 24 hrs. Concentrations of the extracts that showed no growth on the culture plates were considered as bactericidal concentrations. The least concentration of the extract that showed no growth on the plates after sub culturing was considered as MBC. It was noted that most of the antimicrobial properties in different extracts shows 2 fold higher MBC than the corresponding MIC.

Data management and analysis

Results (data) from this study were recorded in my daily record book then transfer was made to Microsoft Excel. Results were analyzed using graph pad and comparisons between groups (test samples, positive and negative controls) were done using one-way ANOVA. The analysis of variance compared variances in antibacterial activity of the standard drug against each herbal extract as well as the differences in terms of action between each extracts. Presentation of data was done using graphs and tables.

RESULTS

Antibacterial activity of the Extracts against *K. pneumoniae* and *S. pneumoniae*

All the herbal extracts had antibacterial activity against both *K. pneumoniae* and *S. pneumoniae* ($p<0.05$). The results showed that MM03 had the highest antibacterial activity against both *K. pneumoniae* and *S. pneumoniae* - (*Figures 1 and 2*).

Antibacterial activity of the Extracts against *K. pneumoniae*

The 5 different brands of herbal extracts were examined for their antibacterial activity against *K. pneumoniae* and the results showed that all the extracts had a significant antibacterial activity against *K. pneumoniae* ($p < 0.05$). From figure 1, it was shown that among the 5 extracts, MM04 (35.6±0.0) had the highest antibacterial activity against *K. pneumoniae*. This was followed by MM03 (29.4±1.3), MM02 (23.8±0.8), MM05 (23.2±1.2) and MM01 (13.4±0.9) in that order. The standard drug had the best action against *K. pneumoniae* compared to all the 5 extracts, however the concentration of active ingredients of the herbal extracts was not calculated / known unlike the standard drug whose active ingredient concentration was known. The antibacterial activity of the standard drug was significantly higher statistically compared to the herbal extracts at <0.0001, <0.0001, 0.0011, 0.0067 and 0.002; for MM01, MM02, MM03, MM04 and MM05, respectively.

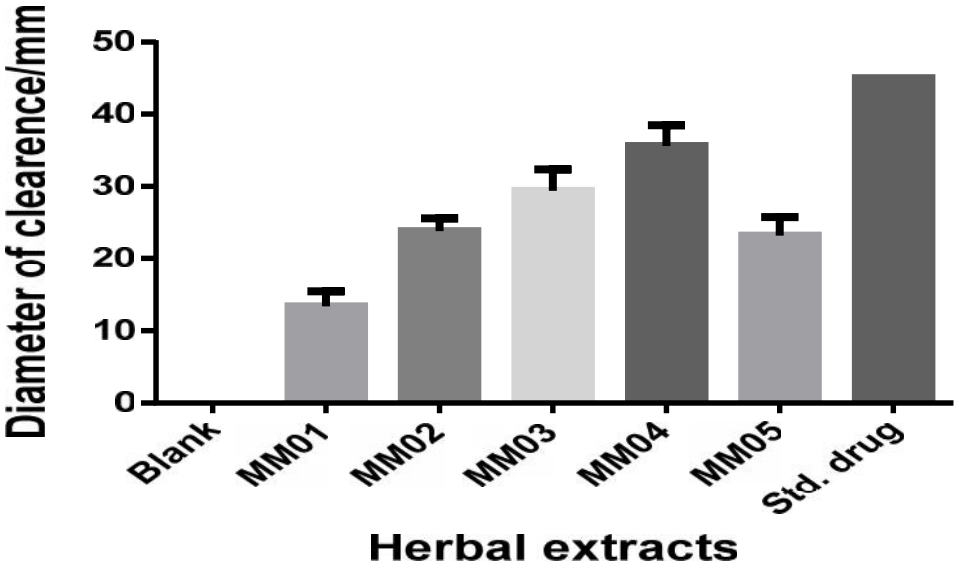

Figure 1: The average Antibacterial activity of the extracts against *K. pneumoniae*

Antimicrobial activity of the Extracts against *S. pneumonia*

The 5 different brands of herbal extracts were examined for their antibacterial activity against *S. pneumoniae* and the results showed that all the extracts had a significant antibacterial activity p <0.05). From figure 2, it was shown that among the 5 extracts, MM03 (33.6±1.5) had the highest antibacterial activity against *S. pneumoniae.* This was followed by MM05 (28.2±1.3), MM04 (25.8±1.6), MM02 (20.6±1.1) and MM01 (14.8±1.3) in that order. The Standard drug had the best action against *S. pneumoniae* compared to all the 5 extracts; however the concentration of active ingredients of the herbal extracts was not calculated/ known unlike the standard drug whose active ingredient concentration was known. The antibacterial activity of the standard drug was significantly higher statistically compared to the herbal extracts at <0.0001, <0.0001, 0.0013, 0.0004 and 0.003; for MM01, MM02, MM03, MM04 and MM05, respectively.

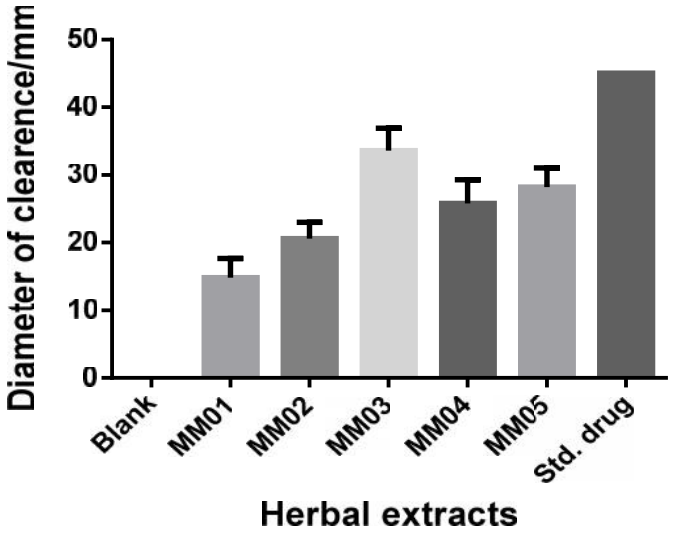

Figure 2: The average Antibacterial activity of the extracts against *S. pneumoniae*

MIC results for the test herbal extracts

Since all the selected herbal extracts had antibacterial activity against both *K. pneumoniae* and *S. pneumoniae* further tests were carried out to determine the least concentration of the herbal extracts that inhibits bacterial growth (MIC). From the study, it was shown that MM03 had the lowest MIC against both *K. pneumoniae* and *S. pneumoniae,* followed by MM02, MM04, MM05

and MM01, respectively in that order. Ciprofloxacin had the lowest MIC compared to the test drugs (*Table 1*). Ciprofloxacin had the lowest MIC compared to all the test drugs**.**

Table 1: Average MICs of the test drugs against the assessed organisms.

	Test Drugs	**Test organisms**	
		K. pneumoniae	***S. pneumoniae***
Average MICs (mg/ml)	MM01	2.5000	2.0000
	MM02	0.1000	0.0813
	MM03	0.0625	0.0438
	MM04	0.3871	0.2815
	MM05	1.9031	0.8880
	Standard drug	0.0200	0.0150

MBC results for the test drugs

We further carried out streak plate method to determine the Minimum Bactericidal Concentration (MBC) to determine the least concentration of the herbal extracts that kills bacteria

From the study results, MM03 had the lowest MBC against both *K. pneumoniae* and *S. pneumoniae* followed by MM02, MM04, MM05 and MM01, respectively in that order. Ciprofloxacin had the lowest MBC compared to the test drugs (*Table 2***)**. Ciprofloxacin had the lowest MBC compared to the test drugs.

Table 2. Average MBCs of the test drugs against the assessed organisms

	Test Drugs	**Test organisms**	
		K. pneumoniae	***S. pneumoniae***
Average MBCs	MM01	4.000	4.000
	MM02	0.200	0.088

(mg/ml)	MM03	0.030	0.033
	MM04	0.563	0.704
	MM05	3.806	1.775
	Standard drug	0.0200	0.0150

DISCUSSION

K. pneumoniae and *S. pneumoniae* still remain the leading causes of Respiratory Tract Infections worldwide. Recent studies have documented that the organisms cause severe RTIs in many individuals particularly children and immunosuppressed individuals [20]. The infections are usually managed using antibiotics, as well as locally prepared herbal extracts, even though the dosages, concentrations and antibacterial activities of the herbal extracts are still not well understood. This particular study was therefore carried out to investigate the antibacterial activity of locally prepared herbal extracts. Specifically; the extracts were assessed against *S. pneumoniae* and *K. pneumoniae,* the most occurring bacteria associated with cough and RTIs.

In this study all the herbal cough extracts used against *K. pneumoniae* and *S. pneumoniae* exhibited a certain level of antibacterial activity. This implies that all the herbs used in this study could be having some active ingredients that inhibit growth of the bacteria and/or kill the organisms. This is in agreement with early studies that elucidate that herbal extracts have anti-bacterial activity against both *K. pneumoniae* and *S. pneumoniae* [21]. The antibacterial activity of herbal cough extracts was probably due to active substances that may include mircene, neral, geranial, and other unidentified compounds[22]. However, among all the extracts used, MM03 showed the highest antibacterial activity against both *K. pneumoniae* and *S. pneumoniae* followed by MM04. This is probably because they contain the other ingredients against both *K. pneumoniae* and *S. pneumoniae* or were simply well prepared with a high concentration of the active ingredient. This could also indicate that such extracts could be developed further into novel antibiotics. This is in agreement with the studies conducted previously that explain that herbal extracts have antibacterial activity against *K. pneumoniae* [23]. The herbs could also act by destroying the biofilm, a slime layer that is formed by bacteria to expose that organism to the action of the active ingredients. *K. pneumoniae* are known to protect themselves against routine

antibiotics by making shields of slime called biofilm [24]. Of the 5 extracts, MM03 and MM05 showed the highest antibacterial activity against *S. pneumoniae*. This means that the herbal cough extracts could have more active ingredients that inhibit growth of the bacteria and/or kill *S. pneumoniae*. This is in agreement with early studies that reveal that herbal extracts have anti-bacterial activity against *S. pneumoniae* [25]. As hypothesized in the results for herbal extracts against *K. pneumoniae*, the herbal extracts could as well contain active ingredients *S. pneumoniae* especially phytochemicals that have an anti-bacterial activity as reported by [26].

Ciprofloxacin is a pure compound routinely used in the treatments against *K. pneumoniae and S. pneumoniae* infections especially RTIs. It is a fluoroquinolone that acts by inhibiting the action of DNA gyrase hence interfering with the process of DNA replication henceforth causing death of the organism [26]. Ciprofloxacin also had the best action against both *K. pneumoniae* and *S. pneumoniae* compared to all the 5 extracts because it's a pure compound unlike herbal extracts that are crude. Since in this study active ingredients were not determined, the mode of action of the herbal drugs could not be explained.

MM03 had the lowest MIC and MBC against both *K. pneumoniae and S. pneumoniae,* followed by MM02, MM04, MM05 and MM01 in that order. Ciprofloxacin had the lowest MIC compared to the test drugs. The MIC and MBC results are in agreement with the Agar well diffusion results which also show that MM03 had the best antibacterial activity against both *K. pneumoniae* and *S. pneumoniae.* A low MIC indicates that the herbal drug is needed in small concentrations to inhibit the growth of the organism [27], hence having a high efficacy. A low MBC means that the herbal drug is needed in less concentration to kill the organism[28].

CONCLUSION

Generally, all herbal extracts used in this study had antibacterial activity against *K. pneumoniae* and *S. pneumoniae*. From the results, it was shown that MM03 and MM04 had the best antibacterial activity against *K. pneumoniae* and therefore can be promising medicine against these bacterial infections. The MIC and MBC values of all extracts were relatively low meaning that the herbal extracts at low concentrations are able to inhibit and /or kill both *K. pneumoniae* and *S. pneumoniae.*

RECOMMENDATIONS

Herbal cough extracts should be used as an alternative medication against *K. pneumoniae* and S. *pneumoniae* since they were effective against the organisms under study and had low values for both MIC and MBC.

Future studies should aim at identifying the active ingredients with a view to determining their dosage and possible side effects.

After identifying the active ingredients in the herbal extracts, they should be purified and concentrated to improve antibacterial activity.

Toxicity studies should also be carried out to determine the suitability for use of these herbal extracts as antimicrobial agents, as well as their side effects.

ETHICAL APPROVAL

Permission to carry out the study was sought from the department of pharmacy, clinical and comparative medicine Makerere University. For laboratory investigations, standard strains of bacteria were used.

CONFLICT OF INTEREST

Authors declare that there is no exisiting conflict of interest.

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