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In-vitro antibacterial activity of herbal aqueous extract against multi-drug resistant *Klebsiella* sp. isolated from human clinical samples

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ABSTRACT
The studies were carried out to evaluate antibacterial activity of 35 aqueous herbal extracts against a total of 20 clinical *Klebshiella* sp. isolates. The maximum antibacterial activity was found as 90% in crude extracts of *Syzygium aromaticum* (leaf) and *Citrus limon* L. (fruit) followed by 85% in *Spondias pinnata* (leaf). Sensitivity of these isolates was also evaluated for eight commercial antibiotic discs following disc diffusion assay where most of the isolates found to develop resistance against multiple commercial antibiotics. 85% of isolates exhibited resistant to chloramphenicol and erythromycin and 80% were resiatant to sulfamethoxazole and cephradine. The isolates showed their resistance between 55-60 % to the other four antibiotic discs, *viz*; gentamycin, streptomycin, ciprofloxacin and azithromycin. Among 35 herbal extracts tested, 19 herbal extrats were found to possess antimicrobial activity in all multi-drug resistant isolates. Therefore these herbal extracts could be used in future direction as alternative therapeutic agents for the treatment of human diseases caused by *Klebsiella* sp.

***Key Words*:** *Klebshiella* sp., herbal extract, multidrug resistant, antibacterial activity.

INTRODUCTION

Among the human pathogenic bacteria *Klebsiella* sp. are very notable. They are gram-negative, non-motile, encapsulated, lactose fermenting, facultative anaerobic and rod shaped bacterium found in the normal flora of the mouth, skin, and intestines (Ryan and Ray, 2004). Species of *Klebsiella* are ubiquitous, found naturally in the soil, water and vegetables. In humans, they cause pneumonia (inflammatory illness of the lungs), urinary tract infections (UTI), septicemia, soft tissue infections and abdominal infections (Podschun and Ullmann, 1998). They are opportunistic pathogen and under certain conditions may cause serious infections (Wei *et al.*, 2008). Antibiotics are often used against diseases caused by *Klebsiella* sp. But these pathogens are becoming increasingly antibiotic-resistant, so that many are now labeled as "Multidrug-resistant (MDR) *Klebshiella* sp. (Yan *et al.*, 2001; Ktari *et al.*, 2006). Thus it is an important task for the researcher to find out alternative medicine. Antimicrobials of plant origin have enormous therapeutic potential. Advantages of herbal drugs include true improvement of disease condition. Even in some medical practice herbal treatments are being used. So that Plants based antimicrobials represent a vast untapped source for medicines and further exploration of plant antimicrobials needs to occur. Herbs can be very effective in programs for resolving urinary tract infections and typhoid fever (Tambekar, 2010). Thus, herbal treatment would promise a greater viable solution for effective treatment of diseases caused by bacteria (Khan *et al.*, 2007; Rahman and Hossain, 2010). The efficacy of herbal extract over the multidrug resistant (MDR) *Klebshiella* sp. isolates has been investigated in the present study.

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MATERIALS AND METHODS

Collection of Bacterial Isolates

Human clinical isolates *Klebsiella* sp. were collected from Popular Diagnostic Center, Sylhet branch and Sylhet Osmani Medical College and aseptically transferred to the USDA Project Laboratory of the

Table 1: List of medicinal plants used to evaluate antibacterial activity.

Sl. No.	Local name	English Name	Botanical name	Parts of plant used
01.	Amloki	Amla	*Phyllanthus emblica* L.	Fruit
02.	Arjun	Arjuna	*Terminalia arjuna*	Leaf
03.	Amra	Hog plum	*Spondias pinnata*	Leaf
04.	Lobongo	Clove	*Syzygium aromaticum*	Fruit
05.	Lebu	Lemon	*Citrus limon* L.	Fruit
06.	Daruchini	Cinnamon	*Cinnamomus Zeylanicum*	Bark
07.	Eucalyptus	Eucalyptus	*Eucaplytus globules*	Leaf
08.	Kalijira	Black cumin	*Nigella sativa* L.	Seed
09.	Rosun	Garlic	*Allium sativum*	Bulb
10.	Arjun	Arjuna	*Terminalia arjuna*	Bark
11.	Jolpai	Olive	*Olea europaea* L.	Leaf
12.	Peyaj	Onion	*Allium cepa*	Bulb
13.	Dumur	Common fig	*Ficus carica*	Leaf
14.	Paan	Betel leaf	*Piper betle* L.	Leaf
15.	Jarul	Banaba Plant	*Lagerstroemia speciosa*	Leaf
16.	Chalta	Chalta	*Dillenia indica* L.	Leaf
17.	Mehedi	Henna	*Lawsonia inermis* L.	Leaf
18.	Ada	Ginger	*Zingiber officinale*	Rhizome
19.	Lojjaboti	Mimosa	*Mimosa pudica*	Leaf
20.	Tejpata	Indian bay leaf	*Cinnamomum tamala*	Leaf
21.	Bel	Bael	*Aegle marmelos*	Leaf
22.	Lebu	Lemon	*Citrus limon* L.	Leaf
23.	Pepe	Papaya	*Carica papaya*	Leaf
24.	Helencha	Spinach	*Atternatherna phloxeroides*	Leaf
25.	Chatni	Palm-leaf Marshmallow	*Althaea cannabina*	Leaf
26.	Allamanda	Allamanda	*Allamanda schottii*	Leaf
27.	Thankuni	Asiatic Pennywort	*Centella asiatica*	Leaf
28.	Dhonia	Coriander	*Coriandrum sativum* L.	Leaf
29.	Casava	Casava	*Manihot esculenta*	Leaf
30.	Tulsi	Tulsi /HolyBasil	*Ocimum sanctum*	Leaf
31.	Horitoki	Black Myrobalan	*Terminalia Chebula*	Leaf
32.	Joba	China rose	*Hibiscus rosa-sinensis* L.	Leaf
33.	Tetul	Tamarind	*Tamarindus indica* L	Leaf
34.	Cha	Tea	*Camellia sinensis*	Leaf
35.	Bhringraj	False Daisy	*Eclipta alba* (L.) Hassk.	Leaf

Department of Genetic Engineering and Biotechnology, Shahjalal University of Science and Technology for further studies. Twenty clinical isolates of presumptive *Klebsiella* sp. were collected during March, 2011. Presumptive *Klebsiella* sp. isolates were obtained from urine samples of patiences who claimed fever, pneumonia and urinary tract infection (UTI). All presumptive *Klebsiella* sp. isolates were collected in chromogenic media plates, as blue-purple mucoid colony. After collection of all the isolates, were labeled, sub cultured and stored at -20°C for further use.

Identification of *Klebsiella* sp.

A series of morphological, physiological and biochemical tests were performed to identify the suspected *Klebsiella* sp isolates. The test included Gram staining, motility, oxidase activity, catalase production, acid production in glucose, oxidation-fermentation (OF) test (glucose, lactose and sucrose fermentation), Voges-Proskauer Test (VP) and Hydrogen Sulfide Production. All tests were conducted according to The Bergey's Manual of Determinative Bacteriology (Bergey and John, 1994).

Antibiotic sensitivity of the isolates

The antibiotic sensitivity test was performed following the disc diffusion technique as described by Rahman and Hossain 2010. The nutrient broth culture containing desirable bacterial culture was incubated at 37°C for 24-48 hours in a shaking incubator. After obtaining suitable growth of bacterial culture by observation of turbidity, 50μl of broth culture were dropped in the nutrient agar plate. Culture was spread by sterile "L" shaped glass rod for the preparation of spread plate culture to set the antibiotic discs. The anti-microbial discs were dispensed onto the surface of the inoculated agar plate. Eight commercially prepared antibiotics discs *viz*., gentamicin (10μg/disc), ciprofloxacin (20μg/disc), streptomycin (10μg/disc), chloramphenicol (30μg/disc), azithromycin (25μg/disc) sulphamethoxazole (25μg/disc), erythromycin (15μg/disc), and cephradine (30μg/disc) manufactured by Oxoid Ltd. and gentamycin (10μg/disc) manufactured by Becton Disc kinson & company were placed on the surface of the medium with sterile forceps and pressed gently to ensure good contact with the surface of the medium. The plates were inverted and placed in an incubator at 37°C within 15 minutes after the discs were applied. After 16 to 18 hours of incubation, each plate was examined for the determination of the zone of inhibition of the antibiotics. The anti-microbial activities of discs were determined by measuring the zone of inhibition expressed in millimeter.

Antibacterial activity of herbal extract to Klebsiella sp.

A total of 35 herbal extracts prepared from 33 plants were used in this study to screen their antibacterial activity to the *Klebsiella* sp **(Table 1).** Most of the herbs were collected from different parts of Sylhet district. The fresh parts of plants such as young leaves, bark, bulb, root, flower, rhizome or petiole were collected and washed several times with distilled water. The plant parts were cut into small pieces and paste was made by using mortar-pestle. Approximately 10μl of individual herb extract was inoculated onto spread plate culture of the *Klebshilla* sp. isolates. The plate was then allowed to incubate at 37°C for overnight. After 12-24 h of incubation, the herb extract was noted for zone of inhibition for each *Klebsiella* sp. isolates. The diameter of the herb extracts and the diameter of the zone of inhibitions were measured by measuring scale in millimeter (mm). The ratio between the diameters was calculated.

Table 2: Biochemical characteristics of Presumptive *Klebshiella* sp.

Biochemical characteristics of isolates	Isolate No. K1-K20
Gram staining	-
Grow Aerobically	+
Grow Anaerobically	+
O-F	F
Motility	-
Lactose fermentation	+
Sucrose fermentation	+
Glucose fermentation	+
Indole test	-
H_2Sproduction	-
MR	-
VP	+

Note: + = Positive, - = Negative, F= Fermentative

RESULTS AND DISCUSSION

The study was performed to investigate the antibacterial properties of some medicinal plant extracts to *Klebshiella* sp. isolates. All of the presumptive isolates that exhibited the colony characteristics similar to *Klebsiella* sp. isolates were sub-cultured and assessed for their morphological, physiological and biochemical characteristics. All bacterial isolates were Gram negative, facultative anaerobes, and negative in Indole test. The isolates were non-motile, lactose fermenter, positive in VP test and negative in MR test and able to ferment glucose and sucrose but did not produce H_2S in TSI. Based on all of the above tests isolates were identified to belong to the genus *Klebshiella* **(Table 2).**

The *Klebshiella* sp. isolates showed variable result in their antibiotic sensitivity pattern against eight commercial antibiotic discs tested. Most of the isolates exhibited resistant to multiple commercial antibiotics and refereed as "Multidrug-resistant organisms" (MDROs). Antibiotics often used against diseases caused by *Klebsiella* sp. But multidrug-resistant (MDR) strains of *Klebsiella* sp. are now encountered frequently and the rates of multiple drug-resistances have increased considerably in recent years. In reviewed study it is available that the frequency of ESBL producing *Klebshiella* sp. were found to be resistant against ciprofloxacin (74.8%) cephalosporin (23%) (Podschun *et al*., 1998; Alipourfard and Yeasmin, 2010). In the present study, 85% of *Klebsiella* sp. isolates were resistant to chloramphenicol and erythromycin followed by 80% to

Table 3: Antimicrobial activity of aquas herbal extract against *Klebshiella* sp. isolates.

Name of herbs	Name of isolates with their sensitivity pattern (K1-K20)																			
	K1	K2	K3	K4	K5	K6	K7	K8	K9	K10	K11	K12	K13	K14	K15	K16	K17	K18	K19	K20
Spondias pinnata	2.90	1.44	2.42	2.21	2.8	-	2.85	2.75	2.95	2.75	2.29	2.29	2.75	2.88	2.45	-	2.60	2.00	2.90	-
Allium sativum	2.55	-	-	2.75	-	-	2.80	1.95	-	2.65	1.88	-	2.00	2.25	2.00	-	2.29	2.77	2.52	2.05
Terminalia arjuna (leaf)	1.44	-	-	2.75	-	1.80	-	1.73	-	1.95	1.45	1.95	1.75	1.36	1.20	1.20	1.55	2.00	1.36	1.70
Citrus limon L. (fruit)	3.00	4.00	4.60	4.20	3.80	4.00	-	4.40	4.00	4.40	3.40	2.90	2.90	3.00	-	3.60	3.60	3.60	3.20	2.40
Tamarindus indica L	-	2.16	2.36	-	2.43	2.25	-	-	-	2.30	2.40	2.33	-	2.25	2.14	2.76	2.45	2.47	2.33	2.20
Phyllanthus emblica L.	2.44	2.25	-	2.44	2.40	-	-	2.90	2.30	2.36	2.44	2.70	-	2.40	2.70	2.20	2.70	2.00	3.22	2.50
Syzygium aromaticum	2.50	2.20	2.38	2.44	-	2.30	2.44	-	2.50	2.30	2.50	2.95	2.40	2.35	2.40	2.70	2.30	2.35	2.50	2.45
Olea europaea L.	-	-	2.50	3.00	2.90	1.40	-	-	1.50	2.25	2.75	-	-	2.90	2.75	2.95	2.45	2.60	1.90	2.85
Terminalia arjuna (bark)	2.50	-	-	2.90	-	-	-	-	-	-	-	1.65	1.40	2.33	-	1.85	1.95	-	-	2.05
Eucaplytus globules	2.44	2.58	-	2.87	2.33	-	-	-	2.33	1.33	2.40	-	-	2.44	2.56	2.44	2.30	2.35	2.45	2.44
Nigella sativa L.	2.50	-	2.44	2.57	2.33	2.59	2.44	1.75	-	2.45	2.51	2.25	-	2.55	-	1.95	-	2.58	1.80	2.73
Cinnamomus Zeylanicum	-	1.45	1.55	1.39	-	1.20	-	1.80	1.35	1.25	-	-	-	-	-	1.33	-	-	1.30	1.45
Allium cepa	-	-	1.55	1.70	-	1.25	-	-	-	1.28	1.36	1.45	1.50	1.20	-	-	1.30	1.45	-	1.40
Cinnamomum tamala	2.42	2.20	2.40	2.44	-	-	-	2.13	-	2.40	2.20	2.15	-	-	-	-	-	-	-	-
Camellia sinensis	3.5	2.25	2.22	2.15	-	2.15	-	-	2.25	-	-	-	-	2.55	1.65	-	2.5	-	-	1.25
Citrus limon L. (leaf)	-	-	1.95	-	-	1.15	-	-	-	1.25	-	-	-	-	-	-	-	1.70	-	-
Polygonum tomentosum	-	1.45	-	1.33	-	1.88	-	-	-	1.33	1.25	-	1.05	1.19	-	1.25	-	-	-	-
Ficus carica	-	-	-	-	-	2.00	-	-	-	-	-	-	1.95	-	-	1.85	-	-	-	-
Lagerstroemia speciosa	1.33	-	-	-	-	-	-	-	-	1.41	-	-	-	-	-	1.19	-	1.41	1.33	-

sulfamethoxazole and cephradine. 55-60% of *Klebsiella* sp. isolates were resistance to streptomycin, azithromycin, gentamicin and ciprofloxacin **(Figure 1)**.

The prevalence of resistant *Klebsiella* sp. is significant and deserves more consideration. To overcome all of these constrains now we are taking shelter to our ancestor's medicinal practice. According to former customs we are taking interest about herbs. Reviewed studies stated that enormous work has done to screen the antibacterial activity of herbs against human pathogen (Jahan *et al.*, 2007; Khan *et al.*, 2007; Rahman *et al.*, 2008; Misra *et al.*, 2009). Among the 35 samples tested in the present study, 19 crude herb extracts were found to exibit their antibacterial properties against *Klebshiella* sp. isolates. *viz. Spondias mombin* L., *Allium sativum, Terminalia arjuna* (leaf), *Citrus limon* L. (fruit), *Tamarindus indica* L, *Phyllanthus emblica* L., *Syzygium aromaticum, Olea europaea* L., *Terminalia arjuna* (bark), *Eucaplytus globules, Nigella sativa* L., *Cinnamomus zeylanicum, Allium cepa, Cinnamomum tamala, Camellia sinensis, Citrus limon* L. (fruit), *Polygonum tomentosum, Ficus carica* and *Lagerstroemia speciosa.* The maximum antibacterial activity were found up to 90% in *Citrus limon* L. (fruit) and *Syzygium aromaticum* (leaf) followed by 85% activity were found in *Spondias pinnata* (leaf) **(Table 3).**

In recent years, work is being done with organic solvent to find out which solvent is more efficient. Phytochemical studies are also going on with important medicinal herbs to analyze their pharmacological properties. There is strong need to develop alternate antimicrobial drugs for the treatment of diseases caused by *Klebsiella* sp. Because of huge emergence of multi-drug resistant (MDR) bacteria it is an urgent need to discover new therapeutics that

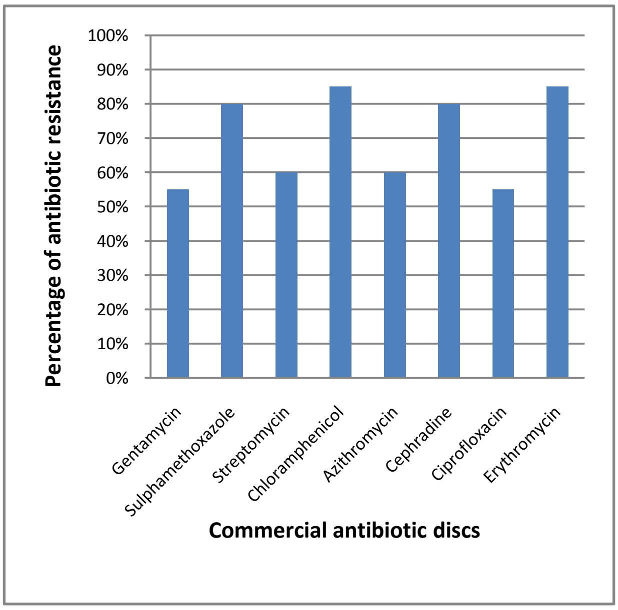

Figure 1: Percentage of Resistance of Klebshiella sp. isolates to commercial antibiotics.

would be effective against MDR strain. This study suggested that herbs with unique chemical compounds that can either inhibit the growth of pathogens or kill them considered as potential candidates for developing new antimicrobial drugs.

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