Journal of Institute of Science and Technology, 27(2), 49-59 (2022)
ISSN: 2467-9062 (print), e-ISSN: 2467-9240
https://doi.org/10.3126/jist.v27i2.37483

Research Article

ANTIBACTERIAL ACTIVITY OF LEMON JUICE ON MULTI-DRUG RESISTANT *Klebsiella* SPECIES HARBORING *bla*$_{OXA-48}$ GENE

Babita Malla[1], Santosh Khanal[1,2], Sudeep K.C.[1], Binod Kumar Yadav[3], Dev Raj Joshi[1*]
[1]Central Department of Microbiology, Tribhuvan University, Kathmandu, Nepal
[2]Department of Microbiology, Tri-Chandra Multiple Campus, Tribhuvan University, Kathmandu, Nepal
[3]Shahid Gangalal National Heart Center, Kathmandu, Nepal
**Correspondence: dev.joshi@cdmi.tu.edu.np*
*(**Received:** June 04, 2021; **Final Revision:** October 13, 2022; **Accepted:** November 26, 2022)*

ABSTRACT

Klebsiella species cause a wide range of infections, and infections caused by multi-drug resistant species have significantly higher rates of morbidity and mortality. Extended spectrum β-lactamase ESBL prodan emergingd carbapenem resistant *K. pneumoniae* is an emerging problem in hospital settings worldwise. However, the distribution of *bla*$_{OXA-48}$ in *Klebsiella pneumoniae* has rarely been described from Nepal. This study was carried out to evaluate *in vitro* antibacterial activity of lemon juice against clinical isolates of *Klebsiella* having *bla*$_{OXA-48}$ gene. A total of 667 clinical specimens were processed for the identification of *Klebsiella* species by morphological, cultural and biochemical tests. Methods of antimicrobial susceptibility testing and confirmation of extended spectrum beta lactamase (ESBL) were adopted from the guidelines of the Clinical Laboratory Standard Institute (CLSI, 2018), and *bla*$_{OXA-48}$ gene was detected by polymerase chain reaction. The antibacterial activity of lemon juice was assayed using agar well diffusion, and the bactericidal effect of lemon juice was determined by the broth dilution method. Out of 204 culture positive isolates, 29.5% were *Klebsiella* species of which 16.7% were ESBL producers. Among 19 isolates positive to Modified Hodge test, 68.4% were detected with the plasmid mediated *bla*$_{OXA-48}$ gene. The MIC of lemon juice ranged from 12.5-50% (v/v), and the bacteriostatic activity was observed after 6-8 hours of exposure. The tested varieties of lemon juice showed potential inhibitory activity against *Klebsiella* species indicating that lemon juice has the potential to be used as an antibacterial agent.

Key words: Bactericidal effect, *bla*$_{OXA-48}$, *Klebsiella* species, lemon juice, multi-drug resistance

INTRODUCTION

Klebsiella are Gram-negative problematic pathogens belonging to the family Enterobacteriaceae that inhabit water environments, for example- water, sewage, soil and gut of mammals (Podschun and Ullmann, 1998). *K. pneumoniae* and *K. oxytoca* are two important bacterial pathogens related to community and hospital acquired infections (Zhang *et al.*, 2011; Kohlenberg *et al.*, 2012). *K. pneumoniae* causes severe infections at most of body sites, but the highest incidence is found in the urinary and respiratory tracts as it also causes healthcare-associated infections. It has recently emerged as drug resistant pathogen with possible cause for outbreaks in healthcare setting (Zaman *et al.*, 2014; Harada *et al.*, 2016). World Health Organization (WHO) has listed carbapenem resistant *K. pneumoniae* as one of the antibiotic resistant bacteria posing the greatest threat to human health (WHO, 2017).

One of the foremost prevalent mechanisms of antimicrobial resistance is the production of beta lactamase enzymes like ESBL (Nikolić *et al.*, 2018). The prevalence of bacterial isolates expressing ESBL varies across different geographical regions (Khanfar *et al.*, 2009) and a trend of increased prevalence of ESBLs among *K. pneumoniae* has been globally observed (Rupp and Fey, 2003). Frequency of ESBL production is comparatively higher in *Klebsiella*, for instance a study conducted in Nigeria demonstrated that 38% *K. pneumoniae* were ESBL producer as compared to 31% *E. coli* (Akanbi *et al.*, 2013). In Nepal, 21-38.5% of *K. pneumoniae* were found ESBL producer (Chaudhary *et al.*, 2015; Nepal *et al.*, 2017). The high magnitude of ESBLs may need attributed to lack of antibiotic surveillance, antibiotic misuse and weak infection control measures (Teklu *et al.*, 2019).

High burdens of carbapenemase producing *K. pneumoniae* (KPC) have been reported from different countries including China, Brazil and Colombia (Munoz-Price *et al.*, 2013). In Ethiopia, Melese *et al.* (2017) reported 12.2% prevalence of carbapenemase producing Enterobacteriaceae with 10.5% that of *K. pneumoniae.* Although increasing prevalence has been reported, data regarding carbapenemase mediated resistance are scarce in low income healthcare facilities (Ibrahim *et al.*, 2017). Likewise, in Nepal also there is very limited data regarding its occurrence. KPC caused infections are related to increased cost, length of hospital stay also frequent treatment failures and death (CDC, 2009). OXA is an enzyme called oxacillinase and is classified to class D group of β-lactamases (Doi and Paterson, 2015). A few of OXA β-lactamases additionally have the potential to degrade carbapenems (Nordmann *et al.*, 2012). *bla*$_{OXA-48}$ producing *Klebsiella* species are extensively identified in both nosocomial and

community-acquired strains (Chung, 2016) and often also coproduces ESBL (Tzouvelekis *et al.*, 2012).

As there is limited antibiotic therapy against multidrug resistance in particular carbapenemase producers, alternative new sources of antimicrobials should be searched. Medicinal plants from decades are used as major source of alternative medicines to the usual drugs (Al-Mariri and Safi, 2014). Citrus is cultivated for dietary intake, as well as for herbal medicines for long time age (Oikeh *et al.*, 2016; Abdulaziz *et al.*, 2017). In Venezuela, it was claimed that the cholera epidemic was largely resolved by massive consumption of lemon fruit crush (Pamplona-Roger, 1999; Okeke *et al.*, 2015). Citrus plants have an excellent potential for producing new drugs (Nascimento *et al.*, 2000), as they contain bioactive compounds like phenolics, flavonoids, vitamins and essential oils having a variety of protective health benefits including antioxidative, anti-inflammatory, antitumor and antimicrobial activities (Aherne *et al.*, 2007; Aruoma *et al.*, 2012; Al-Mariri and Safi, 2014) and have no any side effects (Henderson *et al.*, 2017).

Treatment failure due to multi-drug resistant *Klebsiella* species is significantly higher in many countries and is a global concern. Considering the same, occurrence and distribution is not comprehensively understood in Nepal so there is a need to determine the prevalence of $bla_{\text{OXA-48}}$ harboring *Klebsiella* species from different clinical specimens. In addition, an alternative natural antimicrobial agent like lemon juice which could be helpful to treat infections caused by *Klebsiella* species, is not well investigated yet in the country. This study provides *in vitro* experimental evidence that lemon juice may be important alternative medicine to treat such infections.

MATERIALS AND METHODS

Study Setting and Ethical Clearance

A hospital based cross-sectional study was conducted at Shahid Gangalal National Heart Center, Bansbari, Kathmandu, a tertiary referral center for heart patients in Nepal and at Central Department of Microbiology, Kirtipur, Kathmandu from May to October 2018. The study protocol was approved by the ethical review board of Nepal Health Research Council (NHRC) (Registration number: 351/2019), Kathmandu.

Isolation and Identification of *Klebsiella* Species

A total of 667 clinical samples (urine, sputum, blood, stool, body fluids, endotracheal tips and secretions were collected and inoculated into MacConkey agar and Blood agar. The identification of the *Klebsiella* species at genera level was in accordance to Bergey's Manual of Systemic Bacteriology, primarily based on colonial morphology, Gram staining reactions and biochemical tests (Cheesbrough, 2006).

Antibiotic Susceptibility Testing

The antibiotic susceptibility testing of the isolates was done by modified Kirby-Bauer disk diffusion method as recommended by CLSI (2018). Amoxyclav (30 μg), ceftriaxone (30 μg), ceftazidime (30 μg), imipenem (10 μg), meropenem (10 μg), amikacin (30 μg), gentamicin (10 μg), norfloxacin (10 μg), ciprofloxacin (5 μg), nalidixic acid (30 μg), nitrofurantoin (300 μg) and cotrimoxazole (25 μg) were used and interpreted on the basis of CLSI (2018). *Escherichia coli* ATCC 25922 was used as reference strain for antibiotic susceptibility teating.

Test for ESBL Producing *Klebsiella* species Isolates

Screening tests

The ESBL producers were screened by using 30 μg each of ceftazidime (CAZ) and cefotaxime (CTX) discs. The screening for the ESBL producer (the zone of inhibition ≤22 mm for CAZ and /or ≤25 mm for CTR) was considered as ESBL producer as recommended by CLSI guideline (2018).

Confirmation test for ESBL producer

ESBL confirmation was done by CAZ (30 μg) and CTX (30 μg) disc alone and in combination with clavulanate (10 μg). If the diameter of zone of inhibition is ≥5 mm in combination discs, i.e., clavulanate/ ceftazidime or clavulanate/ cefotaxime than that of individual disc, it indicates the presence of ESBL in the test organism as recommended by the CLSI (2018)

Test for Carbapenemase Producing *Klebsiella* species Isolates

All isolates resistant to meropenem were subjected for confirmation of carbapenemase production. Phenotypic confirmatory test for carbapenemase producers was carried out by Modified Hodge test as described by CLSI (2018).

Detection of $bla_{\text{OXA-48}}$ Gene

The plasmid DNA of bacterial isolates (n=26) was extracted using alkaline hydrolysis method (Sambrook and Russell 2001). The extracted plasmids were suspended in TE buffer (pH=7.5,), labeled well and stored at -20 °C. For gene amplification, 3 μL plasmid DNA, 21 μL of master mix (2X) and nuclease free water (1:1) and 0.5 μL each of reverse and forward primers were added to make a final mixture volume of 25 μL. The primer pair used for the detection of the $bla_{\text{OXA-48}}$ gene were $bla_{\text{OXA-48}}$ F (5'-GCTTGATCGCCCTCGATT-3') and $bla_{\text{OXA-48}}$ R (5'-GATTTGCTCCGTGGCCGAAA-3') and a band of 281 bp was considered positive (Dallenne *et al.*, 2010). The thermal cycling process (30 cycles) consisted of initial denaturation at 95°C for 15 minutes, denaturation of 94°C for 30 seconds, annealing at 57°C for 90 seconds, with extension at 72°C for 90 seconds and final extension at 72°C for 10 minutes. The amplified products along with 100 bp DNA ladder were subjected to gel electrophoresis with 2% gel stained with 0.5 μg/ml ethidium bromide at 100 V for 1 hour.

Extraction of Lemon Juice and Sterility Testing

The *Citrus limon* (Variety A) and *Citrus limon* (Variety B) (on the basis of their location only, unknown phylogenetic history), were respectively collected from

Kavre and Dhading districts of Nepal. The lemons were rinsed thoroughly with distilled water and 75% ethanol and cut into halves. The lemons were squeezed, and the extracts were filtered through Whatman filter paper (pore size 20 μm). The extract was considered as 100% and diluted with sterile distilled water to different concentrations (75%, 50% and 25%). The lemon juice extract, citric acid (pH 2.2 - 2.5) was then stored at 4°C in sterile airtight glass bottle until subsequent use. Assurance of sterility test of lemon juice was done if no microbial growth was seen in nutrient agar plate after incubation at 37°C for 24 hours (Wasihun and Kasa, 2016).

Antimicrobial Assay of Lemon Juice

The antibacterial assay was carried out using agar well diffusion method. The various concentrations (100%, 75%, 50% and 25%) of lemon juice (100 μl) and distilled water (100μl) as negative control were poured into 6 mm wells on Mueller Hinton agar plate. The plates were incubated at 37°C for overnight and the zone of inhibition was noted (Aibinu *et al.*, 2007).

Determination of MIC and MBC

The minimum inhibitory concentration (MIC) was determined using broth tube dilution methods (Wasihun and Kasa, 2016).The bacterial population size (10^5-10^6 CFU/ml) was in the suspension was maintained equivalent to the turbidity of 0.5 McFarland Standard. The two-fold serial dilution of lemon juice was carried out and the process was continued until the dilution of 1:128 was reached. Each dilution was inoculated with 0.1 mL aliquot of test organism except negative control. The tubes were incubated at 37°C for 24 hours. The MIC end point was determined as the lowest concentration of extract that did not permit any visible growth. The sub-culture from tubes showing no visible signs of growth was done on nutrient agar plate and incubated at 37°C for 24 hours. The least concentration in which there was no bacterial growth was considered as MBC (CLSI, 2018).

Antimicrobial Efficacy Testing

The antimicrobial efficacy of the lemon juice was determined by plotting time kill curve with viable cell count (CFU/ml)against time of incubation (Yadav *et al.*, 2015). MBC concentrations of lemon juice were used initial dose and the incubated bacterial suspensions were sampled out at 0, 2, 4, 6, 8 and 24 hours respectively to count viable cells.

RESULTS

Bacterial growth pattern

A total of 204 isolates were obtained from 667 clinical specimens including urine (309), blood (240), sputum (42), pericardial fluid (34), Endotracheal (ET) secretions (23), ET tubes (4), ET suction (3), Central venous pressure (CVP) tips (4), Stool (2) and other body fluids (6). Gram negative bacteria were predominant (75.5%) than Gram positive bacteria (24.5%). Among Gram negative bacteria, *E. coli* (31.4%) was common followed by *Klebsiella* species (29.5%) while among Gram positive bacteria *Staphylococcus aureus* (19.6%) was most frequent (Table 1).

Table 1. Frequency of bacterial isolates

Organisms isolated	Frequency (%)
Gram negative bacteria	**154 (75.5)**
E. coli	64 (31.4)
Klebsiella spp.	60(29.4)
Pseudomonas aeruginosa	16 (7.8)
Acinetobacter spp.	12 (5.9)
Citrobacter freundii	1 (0.5)
Proteus mirabilis	1 (0.5)
Gram positive bacteria	**50 (24.5)**
Staphylococcus aureus	40 (19.6)
Enterococcus spp.	9 (4.4)
CONS	1 (0.5)
Total	**204 (100)**

Distribution of *Klebsiella* species among clinical specimens

Among 60 *Klebsiella* isolates, 56 were *K. pneumoniae* and the rest *K. oxytoca*. The isolates were more predominant in urine samples (65%) followed by blood (11.7%). There was a significant statistical association ($p<0.05$, at 95% confidence interval) between clinical specimens and infections caused by *Klebsiella* species (Table 2).

Table 2. Distribution of *Klebsiella* species among different clinical specimens

Clinical Samples	*Klebsiella* species N (%)	*p*-value*
Urine	39 (65.0)	
Blood	7 (11.7)	
Sputum	8 (13.3)	
ET secretion	1 (1.7)	
ET tube	1 (1.7)	0.046
ET suction	1 (1.7)	
Pericardial fluid	1 (1.7)	
Stool	1 (1.7)	
CVP tips	1 (1.7)	
Total	**60 (100.0)**	

** Chi-square test*

Antibiotic susceptibility pattern of *Klebsiella* species isolates

The antibiotic susceptibility pattern of *Klebsiella* species isolates towards 12 different antibiotics shows that 60% of the isolates were sensitive to imipenem followed by meropenem (56.7%) and cotrimoxazole (45%). Most of the isolates were resistant towards amoxyclav (80%), amikacin (78.3%) and ciprofloxacin (78.3%). The bacterial isolates had high degree of resistance to quinolones (80%), fluroquinolones (75-78.3%), aminoglycosides (75-78.3%) and β-lactams (73.3-80%) whereas least resistant towards carbapenem (40-43.3%) (Fig. 1).

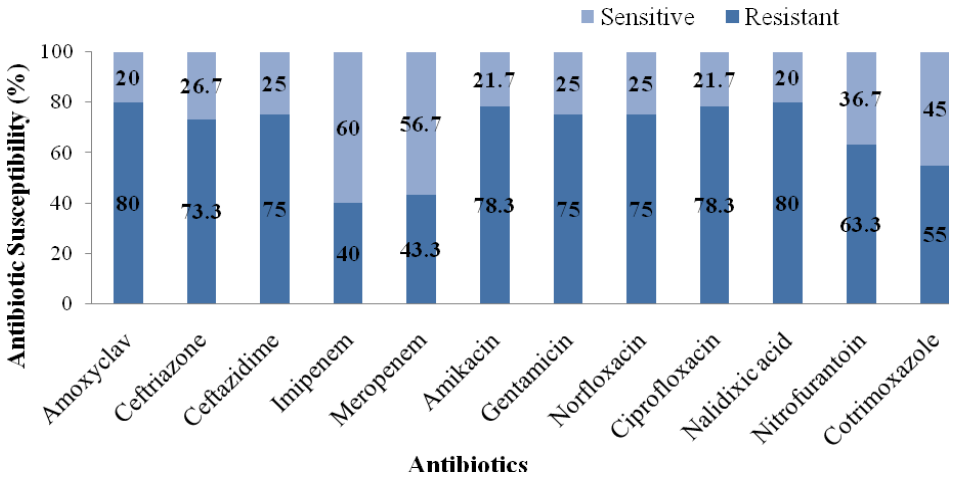

Figure 1. Antibiotic susceptibility pattern of *Klebsiella* species isolates

Distribution of MDR isolates of *Klebsiella* species

In this study, 80% of *Klebsiella* species isolates were MDR (Fig. 2). Among them 82.1% were *K. pneumoniae* and 50% were *K. oxytoca*.

ESBL producers among MDR *Klebsiella* species

Among MDR isolates (n=48), 7 isolates were confirmed as the ESBL producers (Table 3). The association of distribution of ESBL isolates among MDR *Klebsiella* species was statistically insignificant (Table 3).

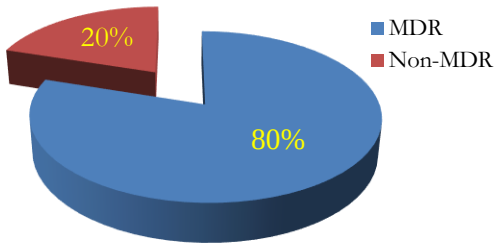

Figure 2. Frequencies MDR isolates of *Klebsiella* species

Table 3. ESBL producers among MDR *Klebsiella* species

Isolates	MDR	Non-MDR	Total	*p-value**
ESBL	7(100%)	0(0.0%)	7(100%)	0.159
Non-ESBL	41(77.4%)	12(22.6%)	53(100%)	
Total	48(80.0%)	12(20%)	60(100%)	

** Chi-square test*

Distribution of bla_{OXA-48} gene among *Klebseilla* isolates

Among 26 examined isolates (19 MHT positive and 7 MHT negative isolates), the carbapenemase genes bla_{OXA-48} was detected in only 13 isolates of *K. pneumoniae* Fig. 3).

Antibacterial activity of lemon juice against *Klebsiella* species

Klebsiella species showing zone of inhibition were considered as lemon juice susceptible isolates. Juice from Variety A and B of *Citrus lemon* demonstrated inhibitory activity against 50/60 (83.3%) and 53/60 (88.3%) of *Klebsilla* species isolates, respectively. Zone of inhibition (mean±SD) due to lemon juice decreased proportionally with the dilution (decreasing concentration) of lemon juice (Table 4).

3.8 MIC of lemon juice against *Klebsiella* species isolates

MIC of lemon juice ranged from 12.5 - 50% v/v for both the varieties. The inhibitory activity of both the varieties of lemon juice against *Klebsiella* species is shown in Table 5. MIC of Variety A lemon juice was determined 12.5% (v/v) against majority of isolates (60.6%) of *Klebsiella* species while that of Variety B was 50% (v/v) against majority of the isolates (69.7%).

In order to test the activity of lemon juice against ESBL producing isolates of *Klebsiella* species, numbers of isolates with lemon juice MIC values were compared. The MIC values of both varieties of lemon juice were not associated statistically with ESBL and non ESBL producing capability of the isolates (p>0.05) as shown in Table 6.

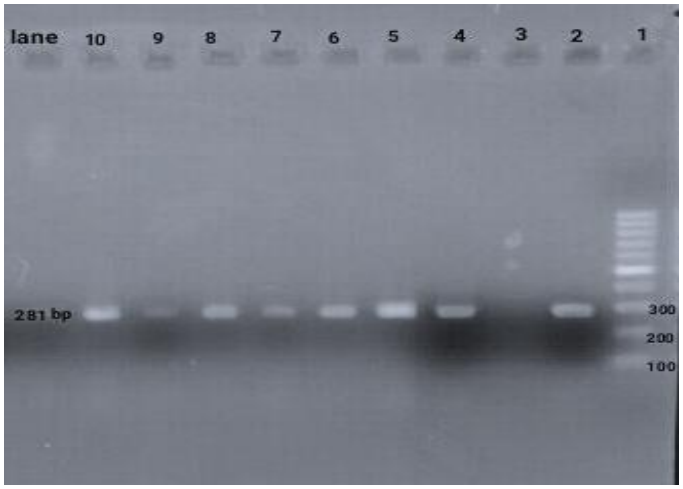

Figure 3. Electrophoretic gel showing PCR bands of 7 isolates of *bla*$_{OXA-48}$ gene. (Lane 1: 100 bp DNA ladder; Lane 2: Positive Control ; Lane 3: Negative Control; Lane 4, 5, 6, 7, 8, 9, 10 test plasmids positive for *bla*$_{OXA-48}$ gene)

Table 4. Antibacterial activity (zone of inhibition) of lemon juice against *Klebsiella* species (n=60)

Lemon juice	Number of isolates	Concentration of lemon juice (%)	Zone of inhibition Mean ±SD (mm)
Variety A	17 14 12 7	100	18.0±1.581
		75	16.6±1.540
		50	14.8±1.467
		25	12.5±1.460
Total	50 (83.3%)		
Variety B	19 11 15 8	100	17.9±2.936
		75	16.6±2.924
		50	14.9±2.614
		25	12.0±2.614
Total	53 (88.3%)		

Table 5. MIC of *Citrus limon* juice on *Klebsiella* species

Variety of Lemon juice	Frequency of isolates with different MIC of lemon juice (v/v)			Total
	12.5%	25%	50%	
	N (%)	N (%)	N (%)	
Variety A	20 (60.6)	9 (27.3)	4 (12.1)	**33 (100)**
Variety B	2 (6.1)	8 (24.2)	23 (69.7)	**33 (100)**

Table 6. Association between MIC of *Citrus lemon* juice and ESBL producing *Klebsiella* species

Varieties of Lemon Juice	Resistance type	MIC of lemon juice (v/v)			Total	*p*-value*
		12.5%	25%	50%		
		N (%)	N (%)	N (%)		
(Variety A)	ESBL	3 (42.9)	4 (57.1)	0	7	0.108
	Non-ESBL	17 (65.4)	5 (19.2)	4 (15.4)	26	
	Total	**20 (60.6)**	**9 (27.3)**	**4 (12.1)**	**33**	

(Variety B)	ESBL	0	1 (14.3)	6 (85.7)	7	0.541
	Non-ESBL	2 (7.7)	7 (26.9)	17 (65.4)	26	
	Total	**2 (6.1)**	**8 (24.2)**	**23 (69.7)**	**33**	

**Chi-square test*

The presence of *bla*$_{OXA-48}$ gene in bacterial genome is indicative of potential antibiotic resistance. Table 7 shows MICs of both varieties of lemon juice against *bla*$_{OXA-48}$ gene harboring *Klebsiella* species isolates. Statistical analysis showed that there is no association (p>0.05) between MIC of lemon juice and presence/absence of *bla*$_{OXA-48}$ gene in bacterial isolates.

Table 7. Association between MIC of *Citrus limon* juice and *bla*$_{OXA-48}$ gene harboring *Klebsiella* species

Varieties of Lemon Juice	Isolates	MIC of lemon juice (v/v)			Total	*p*-value*
		12.5%	25%	50%		
		N (%)	N (%)	N (%)		
Variety A	*bla*$_{OXA-48}$	9 (69.2)	4 (30.8)	0	13	0.325
	Non *bla*$_{OXA-48}$	7 (53.8)	4 (30.8)	2 (15.4)	13	
	Total	**16 (61.5)**	**8 (30.8)**	**2 (7.7)**	**26**	
Variety B	*bla*$_{OXA-48}$	2 (15.4)	3 (23.1)	8 (61.5)	13	0.263
	Non*bla*$_{OXA-48}$	0	2 (15.4)	11 (84.6)	13	
	Total	**2 (7.7)**	**5 (19.2)**	**19 (73.1)**	**26**	

**Chi-square test*

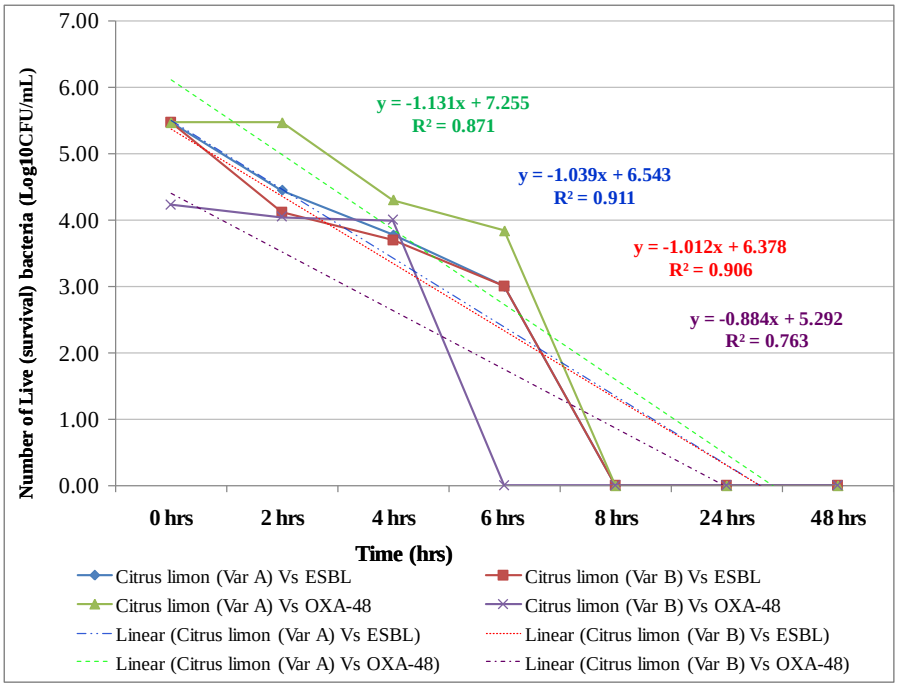

Figure 4. Bactericidal effect of lemon juice against *Klebsiella* isolates

Antimicrobial Efficacy Testing

Time kill curve demonstrates the bactericidal activity of the lemon juice. The time kill curve of both varieties of lemon juice against those *Klebsiella* species isolates which were positive both for ESBL and *bla*$_{OXA-48}$ are shown in the Figure 4. The linear curve was plotted against the $\log_{10}$ CFU/mL of bacteria (survival) vs. time (hours). As shown in the curve, both the types of lemon juice

exhibited inhibitory activity against *Klebsiella* isolates within 6-8 hours. There was a rapid decreamente in bacterial population, predicting about half population reduction within 4 hours of exposure to both the varieties of juices. The complete inhibition of ESBL isolates was observed after 8 hours of exposure to both varieties of *Citrus limon*. While the *bla*$_{OXA-48}$ gene harboring isolates showed inhibitory activities after 6- and 8-hours exposure to Variety B and Variety A, respectively.

DISCUSSION

The incidence of infections with multi-drug resistant *Klebsiella* species is increasing tremendously worldwide. The emergence of antibiotic resistant bacteria is a significant issue as many strains are developing resistance to most available antibiotics. WHO has listed carbapenemase producing *K. pneumoniae* as one of the antibiotic resistant priority pathogens posing greatest threat to human health (WHO, 2017).

In the present study, a total of 204 bacterial isolates were obtained from 667 various clinical specimens. Among total isolates, Gram negative bacteria were predominant constituting 75.5% and Gram positive bacteria constituted 24.5%, which was in accordance with results of previous studies (Bhandari *et al.*, 2016; Sah *et al.*, 2017) suggesting predominance of Gram negative bacteria over Gram positive bacteria. Of all isolates, *E. coli* (31.4%) was the most prevalent bacteria followed by *K. pneumoniae* (27.5%). Corroborating with the present study, several other studies have reported similar pattern of pathogens recovery from clinical specimens in Nepal and elsewhere (Akanbi *et al.*, 2013; Ghimire *et al.*, 2017; Sah *et al.*, 2017).

The increased use of antibiotics and persistent exposure of *K. pneumoniae* to a number of antimicrobial agents, facilitate the emergence of multi-drug resistant strains (Heidary *et al.*, 2018). Of the total 60 isolates of *Klebsiella* species, 80% isolates were MDR in present study. A previous study reported that all isolates of *Klebsiella* species were MDR from nosocomial respiratory tract infections (Shrestha *et al.*, 2011). A similar study conducted in Shahid Gangalal National Hearth Centre (Ghimire *et al.*, 2017) on MDR Gram negative bacteria reported 94.4% of *K. pneumoniae* as MDR strains. But the reduced multi-drug resistance in present study might be attributed to the decreased burden possibly due to implementation of National Antimicrobial Resistance Containment Action Plan in Nepal (DoH/MoH, 2016). In fact, lack of surveillance and infection control practices contribute to high drug resistance rate (Gashaw *et al.*, 2018).

In the study, imipenem (60%) was found to be the most effective antibiotic toward *Klebsiella* species followed by meropenem (56.7%) and cotrimoxazole (45%). However, 80% isolates of *Klebsiella* were resistant to amoxicillin-clavulanic acid followed by amikacin (78.3%) and ciprofloxacin (78.3%). A study by Melese *et al.* (2017) in Ethiopia reported the resistance pattern of *Klebsiella* species to amoxicillin-clavulanic acid (89.5%), which is high as compared to the present study while resistance pattern for norfloxacin was low (15.8%). Likewise, Manikandan and Amsath (2013) reported the lowest rate of resistance towards nalidixic acid (50.6%) and norfloxacin (25%) in comparison to the present study. The antibiotic resistance pattern may vary according to geographical location and prevailing factors (Navon-Venezia *et al.*, 2017).

The prevalence of ESBL among *Klebsiella* species (16.7%) reported in this study was comparable to Chander and Shrestha (2013) in tertiary hospital of Nepal who showed 16.55% ESBL producing *K. pneumoniae* in similar clinical samples. However, Sharma *et al.* (2013) and Sah *et al.* (2017) reported 67% and 38.5% isolates to be ESBL positive among Gram negative bacteria, respectively. According to Melese *et al.* (2017), 84.2% *K. pneumoniae* and all isolates of *K. oxytoca* were ESBL positive; this was much higher than the present study. Ibrahim *et al.* (2017) in Nigeria reported the prevalence of ESBL to be 38.4% from the clinical isolates. Similarly, a study conducted by Nepal *et al.* (2017) reported ESBL production to be 34.5% with *K. pneumoniae* (38.5%). Extensive use of broad spectrum antibiotics, prolonged hospitalization, indwelling devices and severe underlying diseases are reported as factors that have led to the spread of ESBLs (Zaki, 2007). This study also revealed that all ESBL producers were MDR but all the MDR isolates were not ESBL producers and the association between ESBL producing isolates among MDR *Klebsiella* species was found to be statistically insignificant (p>0.05) and data published by Nepal *et al.*(2017) and Shakya *et al.*(2017) also indicated similar result pattern.

In this study, PCR amplification of the *bla*$_{OXA-48}$ carbapenemase gene revealed that 68.4% isolates harbored *bla*$_{OXA-48}$ gene. Although the frequency of *bla*$_{OXA-48}$ has not been studied in Nepal, the studies revealed vast range from 0.9% in Iran (Hosseinzadeh *et al.*, 2018) to 78% in Saudi Arabia (Shibl *et al.*, 2013). Similarly, study conducted in Jordan and Taiwan reported that 33.3% and 71.4% isolates of *K. pneumoniae* harbored *bla*$_{OXA-48}$ from clinical specimens (Huang *et al.*, 2014; Aqel *et al.*, 2017). According to this study, high number of *bla*$_{OXA-48}$ isolates were from inpatients in comparison to outpatients indicating that hospital environment may favor spread of carbapenemase. Shibl *et al.* (2013) and Hosseinzadeh *et al.* (2018) reported widespread occurrence of *K. pneumoniae* isolates harboring *bla*$_{OXA-48}$ among inpatients in healthcare facilities. Furthermore, this result also demonstrates that *bla*$_{OXA-48}$ is circulating in the hospital setting and is associated with emerging infections. However, its dissemination in healthcare facilities in Nepal is still to be understood and analyzed. Among different variants, *bla*$_{OXA-48}$ is particularly important because of high conjugation rate of the pOXA-48a and lack of hydrophobic bridge across the active site (Evans and Amyes, 2014; Potron *et al.*, 2014). Other *bla*$_{OXA-48}$ variants were also observed elsewhere (Castanheira *et*

al., 2019) but with resource limitation, it could not be done in the present study despite the high prevalence of gene.

As the present study revealed the occurrence and dissemination of ESBL and carbapenemase producing *Klebsiella* species in the clinical setting, the major objective of this study was to evaluate inhibitory activity of lemon juice against target *Klebsiella* species. Lemon fruit is popular for its culinary and medicinal uses (Henderson *et al.*, 2017). Lemon juice has been found to possess potential antibacterial activity against *K. pneumoniae* (Adeshina, 2014; Kato *et al.*, 2018). From the antibacterial assay, the highest zone of inhibition exhibited by lemon juice was 21 mm and lowest 9 mm, which correlates with the study of Chowdhury *et al.* (2013) in Bangladesh. The results showed that lemon juice exhibited a level of antibacterial activity, which increased with increase in concentration. In agreement to the present study, antimicrobial activity of lemon juice alone and in combination has been investigated (Adeshina, 2014; Jaafar, 2016; Okojie*et al.*, 2017). The antibacterial activity of concentrated or freshly squeezed lemon juice has been reported against *Vibrio* species, *Salmonella* Typhimurium, *P. aeruginosa*, *E. coli* and *S. aureus* (Tomotake *et al.*, 2006; Jayana *et al.*, 2010; Kumar *et al.*, 2012; Okeke *et al.*, 2015; Saeb *et al.*, 2017). Ekawati and Darmanto (2019) reported that bioactive compound and active ingredients in juice enhanced the antibacterial activity as citrus fruits contain phytochemicals while another study suggested that bacteriostatic effect was mainly induced by low pH due to the presence of citric acid (Kato *et al.*, 2018). Possibly both the acidic environment and active ingredients are instrumental in inhibiting the pathogens.

In order to find the dose or concentration for optimal antibacterial activity, MIC of lemon juice was estimated. In this study the MIC was ranged from 12.5% to 50% (v/v) that indicates that even 12.5% (v/v) concentration of *Citrus limon* was sufficient to kill bacterial isolates, however, few isolates were inhibited only at higher concentrations of 25% and 50%. The MIC values for two different varieties of lemon juice showed potential bacteriostatic activities against *Klebsiella* species. Adeshina (2014) also reported the susceptibility of *K. pneumoniae* towards lemon juice at 25% v/v concentration, which was within the range of this study.

In antimicrobial efficacy test, *Klebsiella* isolates on exposure to lemon juice showed rapid decrease in bacterial population with complete killing within 6-8 hours. In this study, when 8 $\log_{10}$ CFU/mL of *Klebsiella* isolates were exposed to 12.5% and 25% concentrations the juice extract showed considerable bactericidal activity. Adeshina (2014) reported that lemon juice completely killed *K. pneumoniae* isolates at 2 hours. The variation in antibacterial activity or MIC may be due to the difference in varieties of lemon used in different studies. However, in this study killing effects of lemon juice of both the varieties was almost similar. Thus, both the varieties of lemon juice were found to exhibit a good and rapid bactericidal activity within 6-8 hours for *Klebsiella* isolates. This indicates the potential of lemon juice to be used as antibacterial agent.

The present study only investigated the *in vitro* inhibitory activity of lemon juice against strains of *Klebsiella* species expressing ESBLs and $bla_{\text{OXA-48}}$ harboring gene. However, *in vivo* tests were not performed. Therefore, further tests are recommended to ensure *in vivo* application of lemon juice as antibacterial agent. However, the present study definitely provides important clues that lemon juice may be used as an alternative therapeutic option.

CONCLUSIONS

Klebsiella isolates were predominant Gram-negative bacteria after *E. coli* causing infections. ESBL producing *Klebsiella* species was found to be 16.7% and $bla_{\text{OXA-48}}$ gene was detected in more than two-third of *K. pneumoniae*. Both the tested varieties of lemon juice showed potential inhibitory activity against multi-drug resistant *Klebsiella* species with MIC 12.5% to 50% (v/v) and the reduction of 8 $\log_{10}$ (10^{-8} CFU) per mL bacterial population within 6-8 hours. However, additional studies are needed that would clarify various other aspects of lemon juice before its development as therapeutic agent.

ACKNOWLEDGEMENTS

Authors sincerely acknowledge Shahid Gangalal National Heart Center, and Central Department of Microbiology, Kathmandu for providing clinical samples and laboratory facilities. This research did not receive any specific grant funding agencies in public, commercial, or not-for profit sectors.

AUTHORS CONTRIBUTION STATEMENT

BM: designed the study, conducted laboratory experiments and drafted manuscript; SK: supported to PCR experiments and analyzed data; SKC: analyzed data and revised the manuscript; BKY: supervised clinical part of research at the hospital; DRJ: supervised overall research and revised the manuscript.

CONFLICT OF INTEREST

The authors do not have any conflict of interest pertinent to this work.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author, upon reasonable request.

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