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Antimicrobial Activity of Kalonji Oil and its Comparison with Methanolic and Aqueous Extracts of *Nigella sativa* (Kalonji) Seeds

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Abstract

Kalonji (*Nigella sativa*) is a reputed multipurpose herb, especially in Middle East Asian countries. Its seed oil and extracts have often been reported to possess an antibacterial activity against wide range of bacteria mostly from culture repositories. This study *in vitro* evaluated the antimicrobial activity of methanolic extract (KME), aqueous extract (KAE) and ether extracts (KEE) of Kalonji seeds on bacteria associated with clinical illness in animals. A total of 381 bacteria (belonging to more than 66 species of 30 genera) isolated from clinically sick animals were tested using standard disc diffusion assay for their sensitivity to methanolic extract (KME), aqueous extract (KAE) and ether extracts (KEE) of Kalonji seeds. Kalonji methanolic extract (KME) was significantly more effective antibacterial than KEE and KAE ($p \leq 0.05$). Antibacterial activity of KME had wide-spectrum but it was significantly ($p \leq 0.05$) more effective on Gram-positive bacteria (GPBs) than Gram-negative bacteria (GNBs). Oxidase positive bacteria were significantly more often sensitive to KME ($p \leq 0.05$) than oxidase negative bacteria. Resistance to KME was significantly correlated ($p \leq 0.05$) to multiple drug resistance (MDR), extended spectrum β-lactamase production and Carbapenem resistance of bacteria. The study indicated that Kalonji extract exhibited only a little potential antibacterial activity on *Escherichia coli, Enterobacter* spp., *Proteus* spp., and *Klebsiella pneumoniae*, but it was active against *Staphylococcus* spp., *Streptococcus* spp., *Brucella abortus* and *Pasteurella multocida* strains. Methanolic preparation among the three tested. Antibiotic drug resistance and Kalonji resistance in bacteria go hand in hand. The antibacterial activity of KME is better against GPBs and oxidase positive bacteria than GNBs and oxidase -ve bacteria. This study will help the researchers to use Kalonji seed extracts for the development of better herbal therapeutic preparations for bacterial infection.

Keywords*: *Herbal antimicrobial; Herbal extract; Multiple drug resistance (MDR); Extended spectrum β-lactamase (ESBL); Carbapenem resistance; E. coli; Pasteurella; Brucella

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Introduction

Kalonji seeds have the reputation of a miracle herb capable of clearing all ailments. Earlier studies have claimed useful antimicrobial activity in edible oil extracted from *Nigella sativa* (Kalonji) seeds [1]. Kalonji oil is reported to kill laboratory strains of *Staphylococcus aureus, S. epidermidis, Bacillus subtilis, B. cereus, Streptococcus faecalis, Corynebacterium xerosis, Escherichia coli, Klebsiella pneumoniae, Salmonella enterica* subspecies *enterica* ser Typhi, and *Proteus vulagaris* but was not effective against *Micrococcus luteus, Enterobacter aerogenes* and *Pseudomonas aeruginosa* [1]. The oil is reported as a good antibacterial against methicillin-resistant *S. aureus* (MRSA) and methicillin resistant coagulase negative staphylococci (MRConS) without any detectable cytotoxicity against gingival fibroblasts cells [2]. The neat Kalonji oil is reported to inhibit 73% and 64% of *E. coli* isolated from human and animals, respectively. However, strains with multiple drug resistance (MDR) were often resistant to Kalonji oil too [3]. In Turkey, the oil of Kalonji was tested against 17 reference strains of different pathogenic and non-pathogenic bacteria and it was the most effective against *Aeromonas hydrophila*, while the least on *Yersinia enterocolitica* [4] strains.

Another study reported the antimicrobial activity of aqueous extract of *N. sativa* seeds inhibiting *S. aureus* at 300 mg/mL concentration but not to *E. coli* and *Enterobacter* strains [5]. *In vivo* studies in mice also indicated that not only essential oil but methanolic and chloroform extract of Kalonji seeds offered dose dependent but up to 100% protection to mice infected with a lethal dose of *E. coli* and *S. aureus* [6]. In a study on 99 clinical isolates of MRSA and ATCC strain 25923 of *S. aureus* ethanolic extract inhibited the growth of all strains with a MIC range of 0.2 mg/mL-0.5 mg/mL [7]. In a recent study, use of ethanol and n-hexane extract of Kalonji seeds wide spectrum antibacterial activity is reported against Gram-positive (GPBs) as well as Gram-negative bacteria (GNBs) including *B. cereus, B. subtilis, E. coli, S. epidermidis, K. pneumoniae, P. aeruginosa, Salmonella enterica* ser Typhmurium and *E. aerogenese.* However, the extract was more effective against GPBs (MIC 1 mg/mL, MBC 4mg/mL) than GNBs [8]. In another study, ethanolic extract of Kalonji seeds inhibited *Salmonella enterica ser Typhi* at concentration exceeding 45% in the medium [9]. During a comparative study on GPBs and GNBs, both aqueous and methanolic extract of black seed exhibited a greater inhibition on GPBs (*Streptococcus pyogenes*) compared to GNBs (*P. aeruginosa, K. pneumoniae* and *Proteus vulgaris*) and aqueous extract being slightly better than methanolic extract [10]. The Kalonji seed oil, tested against both clinical and laboratory strains of GPBs and GNBs, is reported as an effective antimicrobial inhibiting not only MRSA, MRConS but *Pseudomonas aeruginosa* too. However, it was not much active against many of the GNBs including *Acinetobacter baumannii, Citrobacter freundii, K. pneumoniae, Proteus vulgaris, P. mirabilis* and *Vibrio cholera* [11].

In general, GPBs have been reported to be more sensitive to essential oil and alcoholic extract of Kalonji [12,13]. Besides antimicrobial and growth promoter activity reported in Kalonji over past few decades [14], *Nigella sativa* seeds (black seed or black cumin, Kala-jeera, seeds of blessing) and oil have been used for other medicinal purposes for centuries in many parts of the world. Kalonji seeds are reported useful in treatment of respiratory, gastrointestinal, kidney, liver, skin, circulatory and immune system ailments [15-20]. In the Middle East Islamic states, Kalonji is one of the most reputed herbs and included in the medicine of the Prophet Mohammed [21].

Considering the wide variation in antimicrobial activity of different types of oils and extracts of Kalonji, the study aimed to test the antimicrobial quality of ether extract, aqueous extract and methanolic extracts of locally available Kalonji seeds (at one of the biggest grocery chains in India). The main objective of the study was to evaluate Kalonji extracts against clinically important bacteria so that its true clinical utility can be stipulated.

Materials and Methods

The study was conducted in the year 2016 at Division of Epidemiology, IVRI, Izatnagar, India. The entire chemicals used in the study were of analytical grade and purchased from SD Fine Chemicals unless specified. Glassware used in the study were all Borosil (India) made with chemically inert glass. All bacteriological media used in the study was purchased from BD, Diffco, USA.

Kalonji seed extracts and discs

Kalonji seeds were purchased from a Big Bazar at Bareilly, Uttar Pradesh, India. Seeds were grounded in a mechanical grinder (Remi, Mixer and Grinder, India). The grounded Kalonji seeds were divided into three equal parts in hermetic sterile jars and a sufficient amount of solvent (triple glass distilled water or methanol or the diethyl ether) was added to the level so that every grain of seed powder remain submerged in the solvent. Jars were sealed with a lid and kept for 24 h at 25°C with a minute of shaking at every two hours. The mixture was strained through fine muslin cloth (Raymonds, India) and collected liquid was centrifuged (Remi, RC 30, India) at 25°C for 10 min at 3000 rpm to remove any particulate matter. The extracts were marked as Kalonji methanolic extract (KME), Kalonji aqueous extract (KAE) and Kalonji ether extract (KEE), respectively after extraction using methanol, distilled water, and ether. The dry matter was estimated and the concentrations of extracts were adjusted to 250 mg of Kalonji extract in each mL. Six mm pre-sterilized discs (cut from Whatman paper No. 3) were loaded with 20 mL of the required extract to make the 5mg extract pre disc [22,23]. Discs having 5 mg of the extract were prepared taking reference from earlier studies reporting a range of MIC between 0.2 mg/mL to 4 mg/mL of Kalonji oil and extracts [7,8] for most of the susceptible bacteria. The discs were stored at 4°C-6°C until the end of the study.

Bacterial strains

A total of 381 bacterial strains (69 GPBs and 312 GNBs) belonging to more than 66 species of 30 genera (TABLE 1) isolated from clinical samples of sick animals and available in General Epidemiology Laboratory of the Institute. All the strains were revived and confirmed for identity and purity using standard growth, biochemical and staining characteristics [24,25]. Revived strains were stored at 4°C-6°C on nutrient agar slants until tested.

Antimicrobial sensitivity assay

The sensitivity of test strains to Kalonji extracts and common antimicrobials was determined by disc diffusion assay on Muller Hinton agar (MHA) plates (for non-fastidious bacteria) or on brain heart infusion agar (BHIA) plates (fastidious bacteria as *Streptococcus, Brucella* and *Pasteurella* strains). The results were interpreted for the sensitivity of strains to different antimicrobials on the basis of the diameter of the zone of growth inhibition as per CLSI [22]. Any visible zone against Kalonji extract discs was counted as positive for antimicrobial activity and zone of inhibition was recorded in mm as has been reported earlier [3-5,7]. An all therapeutic drug-sensitive reference strain (*Streptococcus equi* ssp. *equi*, MTCC 3522) available in the laboratory was used as the control. Detection of extended spectrum β-lactamase was decided using

Cefotaxime/Cefotaxime+Clavulanic acid E strips (ESBL CT/CTL, Biomeux, France) by E-test as described by the supplier following standard procedure [26]. To determine the Carbapenem resistance of the test strain 10 µg Imipenem and Meropenem (BD, Diffco, USA) discs were used as per CLSI standards [26]. The strain resistant to 3 or more antimicrobials classes were classified as multiple drug resistant (MDR). Besides, all strains were tested for sensitivity using disc diffusion assay [26] to ampicillin (10 µg), tetracycline (30 µg), gentamicin (30 µg), nitrofurantoin (300 µg), co-Trimoxazole (25 µg), ciprofloxacin (10 µg), chloramphenicol (25 µg), ceftazidime (30 µg), amoxicillin (30 µg) and tigecycline (15 µg). Additionally, Gram-negative bacteria (GNBs) were also tested against colistin (10 µg), moxalactam (30 µg) and aztreonam (30 µg) and Gram-positive bacteria (GPBs) against erythromycin (15 µg), vancomycin (30 µg), clindamycin (10 µg) and cefoxitin (10 µg) for determining MDR potential.

Statistical analysis

To compare the sensitivity of different classes of bacteria to various Kalonji extracts and antimicrobials, a correlation coefficient (*r*) was calculated using the diameter of the zone of growth inhibition measured against the specified discs. To determine significance (at a probability of 95% or more; $p \leq 0.05$) of association between MDR, ESBL, Carbapenem resistance and resistance to methanolic extracts of Kalonji among bacteria of different types, the χ^2 test was performed using Microsoft Excel 2007 tools.

Results

Of the 381 strains of bacteria belonging to more than 66 species of 30 genera only 16.5% strains were sensitive to the methanolic extract of Kalonji seeds (KME) (TABLE 1).

TABLE 1**. Antimicrobial activity of methanolic extract of *Nigella sativa* (Kalonji) seeds on bacterial strains isolated from clinical samples from sick animals 2015-2016.**

Genus	Species, number of strains	Total strains tested	Sensitive to Kalonji methanolic extract (5 mg discs)	ESBL producers	MDR strains	Carbapenem resistant
Acinetobacter	*A. aclcoaceticus* 2, *A. lowffii* 2	4	0	2	3	3
Aerococcus	*Aerococcus* spp.	1	0	1	1	0
Aeromonas	*A. bestriarum* 4, *A. eucranophila* 1, *A. media* 4, A. *popoffii* 4, *A. salmonicida* 1, *A trota* 4	18	3 (*A. eucranophila, A. media, A. trota*)	12	11	7
Aggregatibacter	A. actinomycetemcomitans	1	1	1	0	0
Alcaligenes	*A. faecalis*	7	3	3	4	1
Arsenophonus	*A. nasoniae*	2	2	1	0	1
Bacillus	*Bacillus* spp.	5	2	2	2	0
Brucella	*B. abortus*	1	0	0	0	0
Citrobacter	*C. freundii*	3	0	3	0	0
Edwardsiella	*E. tarada* 12, *E. hoshiniae* 1	13	0	4	2	1
Enterobacter	*E. aerogenese* 2, *E.*	46	0	23	24	11

	agglomerans 41, *E. gregoviae* 1, *E. intermedius* 1, *E. nimipressaralis* 1					
Erwinia	*E. chrysanthemi*	3	0	2	2	0
Escherichia	*E. coli 126, E. frgusonii 4, E. vulneris 1*	131	4 (*E. coli*)	87	102	36
Geobacillus	*G. stearіothermophilus*	3	3	1	1	0
Hafnia	*H. alvei*	3	0	3	3	0
Klebsiella	*K. pneumoniae*	23	0	8	13	8
Kluyvera	*K. cryocrescens*	3	0	0	0	0
Micrococcus	*Micrococcus* spp.	2	2	2	0	0
Moraxella	*M. osloensis* 8, *M. phenylpyruvica* 3	11	3 (*M. osloensis*)	4	5	0
Obesumbacterium	*O. proteus*	1	0	1	1	0
Pasterurella	*P. multocida*type B	1	1	1	1	0
Pragia	*P. fontium*	1	0	0	0	0
Proteus	*P. mirabillis* 11, *P. penneri* 6	17	0	8	15	13
Providencia	*P. alkalifaciens*	2	0	0	1	0
Pseudomonas	*P. aeruginosa*	4	0	2	4	2
Raoultella	*R. terrigena*	5	0	4	5	3
Salmonella	*S. enterica*ssp. *enterica*	8	0	4	2	1
Staphylococcus	*S. arlettae* 1, *S. aureus* 7, *S. auricularis* 1, *S. capitis* ssp. *capitis* 2, *S. capitis* ssp. *Urealyticus* 1, *S. caseolyticus* 2, *S. chromogenese* 2, *S. delphini* 1, *S. epidermidis* 2, *S. equorum* 1, *S. felis* 1, *S. haemolyticus* 6, *S. hyicus 1, S. intermedius* 8, *S. lentus* 4, *S. sciuri* 1	41	29 (*S. aureus* 4, *S. auricularis* 1, *S. capitis ssp. capitis* 2, *S. caseolyticus* 1, *S. chromgenes* 2, *S. epidermidis* 2, *S. haemolyticus* 5, *S. hyicus* 1, *S. intermedius* 7, *S. lentus* 3, *S. sciuri* 1)	26	21	3
Streptococcus	*S. canis* 1, *S. equi*ssp. *Equi* 1, *S. equi*ssp. *Equisimilis* 2, *S. iniae* 3, *S. milleri* 5, *S. pneumoniae* 1, *S. pyogenes* 3, *S. suis* 1	17	10 (*S. equi* ssp. *equisimilis* 2, *S. iniae* 1, *S. milleri* 4, *S. pneumoniae* 1, *S. pyogenes* 2)	7	11	3
Xenorhabdus	*X. bovienii*	4	0	2	3	0

Total	381	63 (16.5%)	214 (56.2%)	237 (62.2%)	93 (24.4%)

The KME was the most potent Kalonji extract (p= 0.06) inhibiting the growth of a maximum number of bacterial isolates while KAE and KEE could inhibit the growth of only 5.13% of the isolates tested for their sensitivity using 5 mg discs (TABLE 2).

TABLE 2**. Percent bacterial strains showing resistance to different *Nigella sativa* (Kalonji) seeds extracts and different types antimicrobial resistance detected.**

Types of bacteria tested	Number of strains tested	Kalonji aqueous extract (KAE) 5 mg	Kalonji ether extract (KEE) 5 mg	Kalonji methanolic extract (KME) 5 mg	ESBL producers	MDR strains	Carbapenem resistant
Gram +ve	69	85.71	85.71	33.33	56.52	52.17	8.70
Gram -ve	312	96.88	96.88	94.55	56.09	64.42	27.88
Oxidase +ve	53	100.00	92.31	66.04	52.83	52.83	18.87
Oxidase -ve	328	92.31	96.15	86.28	56.71	63.72	25.30
Gram +ve and Oxidase +ve	10	100.00	100.00	30.00	50.00	30.00	0.00
Gram +ve and Oxidase -ve	59	80.00	80.00	33.90	57.63	55.93	10.17
Gram -ve and Oxidase +ve	43	100.00	90.91	74.42	53.49	58.14	23.26
Gram -ve and Oxidase -ve	269	95.24	100.00	97.77	56.51	65.43	28.62
Total	381	94.87	94.87	83.46	56.17	62.20	24.41

The resistance among GNBs to KME was more pronounced than ESBL production, multiple drug resistance and Carbapenem drug resistance (TABLE 3) among bacteria isolated from sick animals. However, GPBs were often more often sensitive to KME than to their ability to produce ESBL being multiple-drug-resistant and Carbapenem drug resistance ($p \leq 0.05$).

TABLE 3. **Comparison of bacteria for their resistance (χ^2 statistics) to methanolic extract of Kalonji (KMER), aqueous extract of Kalonji (KAER), ether extract of Kalonji (KEER), multiple drug resistance (MDR), extended spectrum β-lactamase activity (ESBL) and Carbapenem drug resistance (CR).**

Type of bacteria	Traits compared	*KAE/KEER*	*ESBL*	MDR	CR
G +ve	KME	0.007[b]	0.006[b]	0.025[b]	0.004[a]
	KAE/KEE	1	0.134	0.089	1.01E-07[a]
	ESBL	-	1	0.608	2.07E-09[a]
	MDR	-	-	1	2.86E-08[a]
G -ve	KME	0.4272	7.83E-29[a]	1.17E-20[a]	1.79E-65[a]
	KAE/KEE	1	7.36E-06[a]	0.0002[a]	4.90E-15[a]
	ESBL	-	1	0.0334[b]	9.48E-13[a]
	MDR	-	-	1	5.46E-20[a]
Oxidase +ve	KME	0.383	4.90E-17[a]	2.52E-11[a]	1.05E-55[a]
	KAE/KEE	1	0.0004[a]	0.003[a]	8E-13[a]
	ESBL	-	1	0.066553	2.93E-16[a]
	MDR	-	-	1	4.22E-23[a]
Oxidase -ve	KME	0.383	4.90E-17[a]	2.52E-11[a]	1.05E-55[a]
	KAE/KEE	1	0.0004[a]	0.003[a]	8.00E-13[a]
	ESBL	-	1	0.0666	2.93E-16[a]
	MDR	-	-	1	4.22E-23[a]
Total	KME	0.06	2.27E-27[a]	4.20E-11[a]	4.17E-60[a]
	KAE/KEE	1	2.67E-06[a]	0.00005[a]	1.24E-19[a]
	ESBL	-	1	0.090	4.00E-19[a]
	MDR	-	-	1	6.43E-26[a]

[a] Trait in 2nd column was significantly more common than trait compared in the row; [b] Trait in 2nd column was significantly less common than the trait compared in the row. E indicates the position of decimal as 2.45E-5=0.0000245.

The GPBs were more often sensitive to Kalonji extracts than GNBs (TABLE 3). Only 5.4% GNBs were inhibited by KME while two third numbers of GPBs could not grow in presence of KME discs. Similarly, oxidase positive bacteria were more often sensitive to KME than oxidase negative bacteria (TABLE 3) and the difference among oxidase positive and negative strains was more apparent (TABLE 3).

For GNBs, erythromycin was the least effective antibiotic inhibiting only 18.2% isolates still it was more effective than KME, effective only on 5.4% isolates. For GPBs colistin was the least effective antibiotic inhibiting only 27.5% strains followed by tetracycline (63.8%) while KME could inhibit the growth of 66.7% strains. For GPBs the most effective antimicrobials in the study were tigecycline (100%) and imipenem (97.2%) followed by nitrofurantoin and chloramphenicol (92.8%), meropenem (91.3%), moxalactam and cefoxitin (87%), cefepime (82.6%) and ceftazidime (81.2%). However,

carbapenem was not the best antimicrobials for GNBs as it failed to inhibit>22% strains, the best antibiotics for GNBs was tigecycline inhibiting 93.6% strains followed by ceftazidime (89.7%), chloramphenicol (88.1%), cefepime (84.6%), moxalactam (84.3%) and gentamicin (82.1%). None of the other antimicrobials could inhibit more than 80% of the strains tested.

TABLE 4**. Comparison of bacteria of different genera for their sensitivity (χ^2 statistics) to methanolic extact of Kalonji (KMES), multiple drug resistance (MDR), extended spectrum β-lactamase activity (ESBL) and carbapenem drud resistance (CR).**

Bacteria	Compared with	KMES	ESBL	MDR	CR
KMES	KME resistant	NA	0.036[b]	0.119	0.006[a]
Gram +ve	Gram -ve	3.08E-25[a]	0.948	0.0576	0.0008[b]
Oxidase +ve	Oxidase -ve	0.000233[b]	0.598	0.129	0.311
Aeromonas	*Edwardsiella*	0.121	0.0484[a]	0.0109[a]	0.05[a]
	Enterobacter	0.0046[a]	0.228	0.518	0.231
	Escherichia coli	0.011[a]	0.983	0.119	0.317
	Klebsiella	0.042[a]	0.0427	0.767	0.786
	Moraxella	0.494	0.111	0.411	0.0176[a]
	Staphylococcus	0.0001[b]	0.810	0.483	0.0029[a]
	Streptococcus	0.0099[a]	0.130	0.826	0.164
	Proteus	0.0783424	0.241	0.067	0.0247[b]
Edwardsiella	*Enterobacter*	1	0.219	0.0183[b]	0.199
	Escherichia coli	1	0.011[b]	1.61E-06[b]	1.19E-01
	Klebsiella	1	0.806	0.0162[b]	0.071
	Moraxella	0.044[b]	0.772	0.106	0.347
	Staphylococcus	0.000008[b]	0.039[b]	0.0228[b]	0.964
	Streptococcus	0.0007[b]	0.558	0.0069[b]	0.426
	Proteus	1	0.367	0.000066[b]	0.00183[b]

Enterobacter	*Escherichia coli*	1	0.0483[b]	0.000934[b]	0.637
Escherichia coli	*Klebsiella*	1	0.231	0.733	0.341
	Moraxella	0.00027[b]	0.416	0.689	0.0710
	Staphylococcus	2.83E-12[b]	0.208	0.929	0.035[a]
	Streptococcus	1.31E-07[b]	0.534	0.374	0.595
	Proteus	1	0.836	0.009[b]	0.000[b]
	Klebsiella	1	0.004[a]	0.03[a]	0.475
	Moraxella	0.0004[b]	0.046[a]	0.017[a]	0.044[a]
	Staphylococcus	7.65E+22[b]	0.724	0.0009[a]	0.0071[a]
	Streptococcus	1.44E-13[b]	0.042[a]	0.230	0.387
	Proteus	1	0.117	0.323	0.0005[b]
Klebsiella	*Moraxella*	0.0087 [b]	0.928	0.545	0.025[a]
	Staphylococcus	4.92E-08[b]	0.0276[b]	0.783	0.005[a]
	Streptococcus	2.16E-05[b]	0.679	0.601	0.230
	Proteus	1	0.433	0.031[b]	0.009[b]
Moraxella	*Staphylococcus*	0.009[b]	0.106	0.734	0.355
	Streptococcus	0.102	0.799	0.315	0.141
	Proteus	0.023[a]	0.576	0.014[b]	0.00007[b]
Staphylococcus	*Streptococcus*	0.379	0.119	0.347	0.239
	Proteus	9.39E-07[a]	0.249	0.0082[b]	8.16E-08[b]
Streptococcus	*Proteus*	0.002[a]	0.729	0.106	0.0009[b]

[a] bacteria in Ist column were significantly more common carrier of the trait than bacteria compared in the 2nd column; [b] bacteria in Ist column was significantly less common carrier of the trait than bacteria compared in the 2nd column. E indicates the position of decimal as 2.45E-5=0.0000245.

In general, bacteria resistant to KME were more often carbapenem-resistant (TABLE 4) than KME sensitive strains while for ESBL opposite was the observation. Among GPBs, ESBL potential was more among KME resistant (KMER) strains (p=0.001) but MDR had a better relationship with carbapenem-resistance (p=0.01). More of the KMER GNBs were MDR type (p=0.009) than KMES GNBs, and MDR strains were more often (p=4.70 E-11) CR type than non-MDR strains. However, among oxidase positive strains neither ESBL nor MDR was significantly high among KMER strains but Carbapenem-resistance was significantly more common (p=0.05) among KMER strains than KMES strains, and MDR was associated with CR (p=0.001).

Although KEE and KAE inhibited only a few bacteria, their zone of inhibition (ZI) correlated well with each other (r=0.59; p=0.001) and that of KME (r=0.39; p=0.001). The ZI by KME discs could be negatively associated with MDR, ESBL and CR potential of the bacterial strains (r, $\leq$ -0.18; p, 0.05) in the study. The correlation in ZI of KME and of other antimicrobials was always positive but insignificant for gentamicin and ceftazidime ($r \leq 0.12$) and was best correlated with ZIs induced by amoxicillin, vancomycin, and clindamycin ($r \geq 0.54$; $p \leq 0.001$).

In the current study, the strains of genera of bacteria including>10 isolates (*Aeromonas* 18, *Edwardsiella* 13, *Enterobacter* 46, *Escherichia* 131, *Klebsiella* 23, *Moraxella* 11, *Staphylococcus* 41, *Streptococcus* 17, *Proteus* 17) were compared for their sensitivity to KME, production of ESBL and having CR and MDR (TABLE 4). Among all the GNBs, *Aeromonas* were more often sensitive to KME than *Enterobacter*, *Escherichia*, and *Klebsiella* species strains. Among other GNBs, *Moraxella* strains were also more often sensitive to KME than Enterobacteriaceae strains. More of the *Staphylococcus* strains were sensitive to KME than strains of Gram-negative bacteria. Though *Streptococcus* species strains were less often sensitive to KME than staphylococci, the difference was insignificant (TABLE 4).

Among all the GNBs, Carbapenem resistance was more common among the strains of *Proteus* species strains (TABLE 4). Aeromonads more often had CR than *Edwardsiella*, *Moraxella*, and *Staphylococcus* strains. Strains of *E. coli* and *Klebsiella* were more commonly CR types than other bacterial species strains.

Discussion

In the present study, out of 69 GPBs and 312 GNBs isolated from clinical cases from animals 66.7% GPBs and 5.4% GNBs were sensitive to KME. The KME was significantly more antibacterial than KEE and KAE (p=0.06). The observations are in concurrence with earlier observations indicating better antibacterial activity of Kalonji towards GPBS [1,3,4,8,10-13]. Better antibacterial activity in the methanolic extract of Kalonji (KME) might be similar to reported antibacterial activity in the ethanolic extract that too against GPBs [8,13]. Although a few reports earlier indicated similar antibacterial activity in methanolic and aqueous extracts against GPBs or slightly better in aqueous extracts [10] than alcoholic extract. However, in the present study aqueous extract was found be effective only on 14.3% GPBs while methanolic extract was active against 66.7% GPBs. In an earlier study too [22] aqueous extract of Kalonji inhibited only 17% bacterial strains. Similar to observations in the present study, the antibacterial activity of aqueous extract of Kalonji was reported significantly more on *Aeromonas*, *Staphylococcus* and *Streptococcus* species strains [22]. The difference from earlier studies in antibacterial activity of different extracts might be either due to the number of strains tested, origin of the strains tested or due to variation

in active ingredient(s) in Kalonji used in the study [13]. Besides aqueous extract, ether extract equivalent to oil of Kalonji was also effective against 14.3% strains of GPBs and 3.1% strains of GNBs in the study. The observation is in contrast to most of the earlier observations indicating very good antibacterial activity of Kalonji oil inhibiting>64% *E. coli* isolated from clinical cases of human and animal origin. The difference might be due to the fact that in the reported study [3] bacteria were tested against 100% Kalonji oil which does not appears to be a practical approach. In the present study, MIC of selected sensitive and resistant strains (results not shown) indicated that all sensitive strains had MIC<5 mg/mL while resistant strains had MIC of KME>5 mg/mL. In the earlier study on 99 MRSA, MIC of alcoholic extract of Kalonji has been reported to range between 0.2 to 0.5 mg/mL [7]. Though in the present study 70.7% strains of *Staphylococcus* were sensitive to KME, 29.3% strains had MIC>5mg/mL. However, sensitivity among MDR strains of *Staphylococcus* for KME was much more ($p=0.03$) than non-MDR strains of *Staphylococcus* indicating better antimicrobial activity of Kalonji on drug resistant strains as reported earlier [7]. However, the ABST profile of all the bacteria indicated that more MDR or ESBL potential was associated with higher chances of resistance to KME. Among *Staphylococcus aureus* strains, 43% were sensitive to KME which is much lower figure than reported earlier [7,8,13] and it might be due to variation in susceptibility of strains associated with different clinical conditions.

In the study, oxidase positive GNBs including strains of *Aeromonas* and *Moraxella* species were more often sensitive to KME than oxidase negative strains. The observation is in accordance with earlier study [4,22] reporting *Aeromonas* as the most sensitive bacteria to Kalonji oil. However, no earlier study reported testing of Kalonji extract on *Moraxella* and several other bacterial strains in the present study for comparison. In the study *Proteus, Klebsiella* and *Pseudomonas* species strains were among the most resistant strains for KME (TABLE 1) similar to earlier reports on Kalonji extracts [10,13].

The study revealed that Kalonji seeds have a limited clinical utility as therapeutic herb and more studies are needed to reveal its active antimicrobial ingredients and true antimicrobial potential. The observation of the study might be an important milestone in planning for development of an effective therapeutic preparation from Kalonji at least for topical infections. Some of the bacteria causing topical infections as *Staphylococcus aureus* and some oxidase positive (*Aeromonas, Moraxella*, and *Pasteurella*, often associated with wound infections) were quite sensitive to methanolic Kalonji extract. The study concluded that methanolic extract of kalonji seeds was much more potent antibacterial than aqueous and ether extracts of Kalonji. Antibacterial activity of Kalonji though wide-spectrum was more directed towards GPBs and some oxidase positive bacteria including *Aeromonas, Moraxella*, and *Pasteurella* species strains. There was significant negative correlation ($r \leq -0.18$; $p=0.05$) between KME sensitivity and antimicrobial drug resistance among clinical strains of bacteria. The observation indicated that antibiotic drug resistance and Kalonji resistance in bacteria might be going hand in hand. The study has indicated that none of Kalonji extracts revealed potential anti-bacterial activity for clinical utility against enteric bacteria including *Escherichia coli, Enterobacter* sp., *Proteus* spp., and *Klebsiella pneumoniae* strains. However, KME might be a source of an antibacterial component of Kalonji for topical application to inhibit the growth of bacteria causing skin infections. The antibacterial activity of KME against GPBs and oxidase positive bacteria was good but more studies are needed for successfully exploiting the quality for therapeutic purposes.

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Conflict of Interest

None declared.

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