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TARGETING POULTRY PATHOGENS: INSIGHTS FROM ISOLATION, ANTIBIOGRAM ANALYSIS, AND ESSENTIAL OIL MODULATION OF KLEBSIELLA SPECIES

Ambreen Rafique[1]*, Nida Baig[1], Iqra Rafiq[2], Aneela Mehboob[3], Asma Naim[3]

[1*]Clinical Laboratory Sciences, Dow Institute of Medical Technology, Dow University of Health Sciences, Karachi 75330, Pakistan
[2]Department of Pharmacy, University of Karachi, Karachi 75300, Pakistan
[3]Department of Microbiology, University of Karachi, Karachi 75300, Pakistan

***Corresponding Author:** Ambreen Rafique
(E-mail: ambreen.rafique@duhs.edu.pk)

Abstract
Poultry meat, a staple in global diets, is known to act as a reservoir for contamination of bacteria. The presence of MDR strains in poultry products has raised concerns about the transmission of genes coding for either virulence or resistance, necessitating thorough investigations into alternative antimicrobial agents for effective bacterial control. This study aimed to investigate the antibacterial activity of commercial essential oils against Klebsiella isolates obtained from poultry meat, as well as to evaluate potential additive effects when combined with antibiotics. Briefly, 150 chicken meat samples (raw) were collected and characterized by conventional methods. Antibiogram analysis and antimicrobial activity of essential oils of the identified Klebsiella isolates was determined by Kirby Bayer disc diffusion and agar well diffusion assay respectively.

The antibiotic susceptibility testing revealed varying degrees of resistance among the Klebsiella isolates, highlighting the importance of alternative antimicrobial agents. Clove and cinnamon essential oils exhibited potent antibacterial activity against 100% of the isolates with zone of inhibition (ZOI) ranges from 20-32 and 15-21.6mm. Compared to this, rosemary and peppermint were less effective against the isolated strains given the activity only against 14.2% and 28.5% isolates. Further investigation into the potential additive effects of clove and cinnamon oils in combination with antibiotics revealed promising results. However, an antagonistic interaction was observed when clove oil was combined with gentamicin, suggesting caution in the concurrent use of certain essential oils and antibiotics.

In conclusion, clove and cinnamon essential oils represent promising natural antimicrobial agents with clove oil demonstrating superior efficacy against Klebsiella isolated from poultry meat. The additive effects observed underscore the potential for synergistic antimicrobial strategies combining essential oils with conventional antibiotics. However, careful consideration of potential interactions, such as antagonism, is necessary to ensure the effectiveness of combined therapies and mitigate the emergence of antimicrobial resistance.

Key words: Gram negative, Essential oil, Antibiotic resistance, Poultry meat.

Introduction

Klebsiella is one of the important pathogens responsible to cause various infections such as pneumonia, meningitis, sepsis, wounds and infections of urinary tract (UTIs) etc. Klebsiella is a Gram-negative fecal coliform bacterium in the family Enterobacteriaceae. It could be isolated by Berry's (1933) and was named after Edin Klebs, a late 19 Century (Baugher and Jaykus 2015, Gato, Rosalowska et al. 2020). It was responsible for several fatal pneumonia (Jong, Hsiue et al. 1995, Decré, Verdet et al. 2011, Gu, Dong et al. 2018) and a variety of clinical syndromes in humans due to consumption of milk (Kleinman, Bahna et al. 1991). Among the Gram-negative bacteria, Klebsiella pneumonia (KP) belongs to the major causative agents of bacteremia with a high mortality rate. Klebsiella species are more related to 100% of immunosuppressed individuals (Al-Rabea, Burwen et al. 1998) causing respiratory, intestinal, meningeal infections due to heat labile and heat stable enterotoxins responsible for the virulence of Klebsiella species (Adinortey 2014).

Essential oils, generally regarded as safe, are complex mixtures of volatile organic compounds, environmentally friendly, and non-toxic. Their antimicrobial properties are mainly a result of the effects of EOs and their constituents like eugenol, phenol, cinnamaldehyde (Spisni, Petrocelli et al. 2020, Al-Harrasi, Bhatia et al. 2022). Evidence of increasing multidrug resistance (MDR) strains at higher rate is seen by means of increased morbidity and mortality. Essential oils possess important volatile compounds with diverse bioactivities including antimicrobial potential. Due to these properties, EOs have been used in drugs, food, and cosmetics. The antimicrobial properties of EOs are mostly related to the individual susceptibility of bacteria. Therefore, research in the future should thus explore the mechanism of individual essential oil components, along with an initiation in systematically investigating the synergy mechanisms among different components (Exner, Bhattacharya et al. 2017).

The plant extracts predominately essential oils have demonstrated antibacterial properties against a number of virulent bacterial strains in various in vitro experiments. These plants have held economic significance for centuries, serving as a food source and therapeutic agents. Cinnamon (CinO) possesses antimicrobial effect against the growth of K. pneumoniae (Mahrous, El-Balkemy et al. 2023). The antibacterial, antifungal, anticancer, antiviral, insecticidal and antioxidant properties of essential oils have been studied in literature (Alves-Silva, dos Santos et al. 2013). Additionally, EOs are known to have a pivotal role in food preservation aromatherapy due to the presence of biologically active compounds (Irshad, Subhani et al. 2020). Therefore, the research aimed to study Klebsiella strains isolated from chicken farms by observing the antibiotic resistance pattern and the effect of some commercial essential oils on the isolated strains.

Material and Method

Isolation of Klebsiella from Raw Chicken Samples

A total 150 fresh raw chicken meat sample randomly obtained from different retail shop located at Karachi, Pakistan were taken in the sampling boxes containing ice packs to maintain the low temperature for sample preservation till the samples carried to laboratory. Total 100µL of each (original, 10^{-1} and 10^{-2} dilutions) were poured on separate MacConkey agar plates and spread by using sterile spreader. Plates were then incubated at room temperature. After 24-48hrs colonies were monitored for their size, pigment production and physical characteristics.

Biochemical Identification and Microscopic Examination

Gram staining of all isolates was performed by heat fixing the bacterial smear on glass slide followed by the gram staining reagents for appropriate timings. In parallel, biochemical tests were performed to confirm the isolates belong to Klebsiella spp.

Disc Diffusion Method

The antimicrobial susceptibility testing was performed against Klebsiella strains (n=7). Antibiotic disc: S; Streptomycin, CHL Chloramphenicol, LEV; Levofloxacin, FOX; Cefoxitin, CN; Gentamicin, CIP; Ciprofloxacin, TE; Tetracycline was used. Briefly, a loopful of each test strain from nutrient agar (NA) were inoculated into 5 mL of Mueller Hinton broth (MHB) and were incubated at the optimum time and temperature. After the incubation, the turbidity in the test tubes was adjusted to McFarland Standard which corresponds to approximately 10^8 cells/mL. 0.1 mL was pipetted out and spread on Mueller Hinton agar (MHA) plates and 6 mm sterile discs were placed on the lawns. The plates were incubated at 37°C over-nightly. The values of ZOI (mm) represent the mean and SD of the triplicate values of each test.

Minimal Inhibitory Concentration (MIC)

MIC assay was performed by the method as described by (Weerakkody et al., 2010). Four commercial essential oils were used namely cinnamon, rosemary, peppermint and clove. Different concentrations of EO (1000, 500, 250, 125, 62.5, 15.625μg/mL) were prepared in a solvent control (10% DMSO and Tween 80 1% v/v) for better diffusion. 1000μL aliquots of sterile MHB were dispensed in eppendorf tubes containing various concentrations of EO and 10μL bacterial suspension. Then tubes were incubated overnight at 37°C. The following day, growth in the form of turbidity was checked and compared with the results of positive control which was also run concurrently.

Essential Oil Activity by Agar Well Diffusion Method

To evaluate the antimicrobial activity of essential oils, agar well diffusion method was performed. Briefly, the standardized bacterial suspension was streaked uniformly on prepared sterile MHA plates, then a sterile cork borer (6mm diameter) was used to make the equally sized wells. The labeled wells were filled with 20μL of essential oil with their appropriate MICs and 20μL of 5% DMSO was used as a negative control.

Determination of the Additive Effect of Antibiotics and Clove Essential Oil

To evaluate the combined impact of both EO and standard antibiotics on tested organisms, disc diffusion method was employed as described by (Langeveld, Veldhuizen et al. 2014). 10μL of EO was pipetted out and soaked on antibiotics disc, then plates were allowed to dry for proper diffusion of oils and then plates were incubated at 37°C overnight. Following day, the ZOI was observed in each and results were compared between alone and combined interaction of EO and antibiotics.

Results

The lactose fermenting colonies were subjected to IMViC and SIM test, the isolates exhibiting IMViC pattern --++, non-motile, H_2S negative characteristics and gram-negative rods under the microscope confirmed the organism as Klebsiella. 7 (4.6%) out of samples were positive for Klebsiella strains.

The antibiotics susceptibility trend of the Klebsiella isolates was determined by employing the Kirby-Bauer disc diffusion method using a standard protocol (CLSI, 2008). **Table 1** showed the ZOI of antibiotics according to CLSI standard. Results indicated that antibiotic tetracycline demonstrated the highest inhibition activity with a mean ZOI of 19.6mm followed by levofloxacin and ciprofloxacin measuring mean ZOI of 17.6mm and 17.1mm respectively. In contrast, two antibiotics (cefoxitin and chloramphenicol) exhibited 100% resistance against the strains (**Table 2**). In addition to this, comparative analysis of the isolated strains revealed strain A51K as the most sensitive (mean ZOI 12.42 mm) and A30K as the most resistant strain (mean ZOI 7.72 mm). More than half of the strains (57.1%) were susceptible to tetracycline followed by streptomycin (28.5%) (**Table 3**).
MIC of the essential oils by broth dilution method revealed a variation in the competency of essential oil when tested with different isolates (**Table 4**). The activity of cinnamon oil and clove oil was higher, repressing the growth of all the strains. In the case of clove oil, 28.57% of strains were inhibited at 125μg/mL of concentration. Whereas the inhibition pattern of cinnamon oil was different as mostly 250 and 500μg/mL MIC was evaluated. In contrast to this, essential oils rosemary and peppermint showed the least or no inhibition effect at higher as even 1000 μg/mL concentration in either of the

cases. The least MIC of clove oil was 125µg/mL and for cinnamon oil was 250µg/mL. Whereas in the case of rosemary and peppermint the least MIC was 500µg/mL. Clove oil followed by cinnamon oil showed the most potent among the four tested EOs as clearly observed in **Table 4**.
The combined impact of both clove and standard antibiotics on tested organisms was performed and it was demonstrated by the obtained results (**Figure 1**), the combinations of clove EO with seven tested antibiotics produced synergistic and antagonistic impact. CO with six out of seven tested antibiotics showed synergistic effect against most of the Klebsiella isolates, however an antibiotic gentamicin and levofloxacin showed the antagonistic effect. The ZOI of clove oil in combination with ciprofloxacin was observed to be high (p=0.001) compared to the ciprofloxacin alone. Similarly, the combination of clove oil and cefoxitin significantly enhanced the ZOI as indicated by the p value of <0.0001. Conversely no significant change in the inhibition zones were observed (p=0.2199) when clove oil was combined with tetracycline (**Figure 1**). One interesting finding is that the ZOI of clove oil in combination with gentamicin was observed to be low compared to the clove oil alone (ZOI ranges from 20-32nm) whereas when with gentamicin combined, the value fell up to ZOI 11-14 nm. The same findings i.e. the reduction in ZOI values were observed in the case of levofloxacin except for two strains where the ZOI was increased to 5.1% and 5.9%. Similarly, there was no significant statistical difference was observed between gentamicin and levofloxacin alone and combination (p>0.05 in each of the case). In addition to this, an increase in percent enhancement of inhibition (more than 100%) was observed in some cases of tetracycline, streptomycin and predominately ciprofloxacin (**Table 5**). An arbitrary window of ZOI range of 20-32nm was set to clearly visualize the effect of adding clove oil in antibiotics. The figure clearly illustrates the remarkable enhancement of ZOIs in cases of cefoxitin and tetracycline whereas substantial reduction in ZOI in case of gentamicin and levofloxacin (**Figure 2**).

Table 1. Antibiotic ZOI According to CLSI Standards

Antibiotic	Disc Content	Zone Diameter Breakpoints according to CLSI guidelines		
		S	I	R
Cefoxitin	30µg	≥18	15-17	≤14
Levofloxacin	5µg	≥21	17-20	≤16
Gentamicin	10µg	≥15	13-14	≤12
Tetracycline	30µg	≥15	12-14	≤11
Ciprofloxacin	5µg	≥26	22-25	≤21
Chloramphenicol	30µg	≥18	13-17	≤12
Streptomycin	10µg	≥15	12-14	≤11

R= resistant, I= Intermediate, S= Sensitive

Table 2. Antibiogram Profiles of Isolated Strains of Klebsiella.

Strains	Antibiotic discs						
	FOX	LEV	CN	TE	CIP	CHL	S
A30K	0	0	10.6±0.47	12.6±0.47	14.6±0.47	0	16.3±0.47
A43K	0	23.6±0.47	12±0.816	13.3±0.47	21.3±0.47	0	0
A46K	0	14.6±0.47	11.6±0.47	11±0.81	18±0.47	0	0
A47K	0	17.6±0.47	11.6±1.24	19.6±0.94	12.3±0.47	0	0
A50K	0	14.3±0.47	13.3±0.47	23±0.81	12.3±0.47	0	0
A51K	0	17.6±0.47	10.6±0.47	24.6±0.47	16.6±0.47	0	17.6±0.47
A58K	0	19.6±1.24	14.3±0.94	22.3±1.69	24.6±0.47	0	0
Mean ± SD ZOIs (mm)	0	17.6±0.47	11.6±1.24	19.6±0.94	24.6±0.47	0	16.3±0.47

* FOX = Cefoxitin, LEV= Levofloxacin, CN = Gentamicin, TE = Tetracycline, CIP = Ciprofloxacin, CHL = Chloramphenicol, S = Streptomycin

Table 3. Antimicrobial Susceptibility Pattern of Klebsiella Isolates

Antibiotics discs	**FOX**	**LEV**	**CN**	**TE**	**CIP**	**CHL**	**S**
Sensitive	0	1 (14.2%)	1 (14.2%)	4 (57.1%)	0	0	2 (28.5%)
Intermediate	0	3 (42.8%)	1 (14.2%)	2(28.5%)	1 (14.2%)	0	0
Resistant	7 (100%)	3 (42.8%)	5(71.4%)	1 (14.2%)	6 (85.7%)	7 (100%)	5(71.4%)

* FOX = Cefoxitin, LEV= Levofloxacin, CN = Gentamicin, TE = Tetracycline, CIP = Ciprofloxacin, CHL = Chloramphenicol, S = Streptomycin

Table 4. Minimum Inhibitory Concentration Determined by Broth Dilution Method

Strains	Clove		Cinnamon		Rosemary		Peppermint	
	MIC	ZD	MIC	ZD	MIC	ZD	MIC	ZD
A30K	500	22.6±0.47	250	21.3±0.47	1000	0	1000	0
A43K	500	20.3±0.47	250	21.6±0.47	1000	0	1000	0
A46K	250	25.3±0.47	500	15.6±0.47	1000	0	1000	0
A47K	125	23.6±0.47	250	21±0.81	1000	0	1000	0
A50K	250	23.6±0.47	500	17.6±1.24	1000	0	1000	11.3±0.47
A51K	500	27±0.81	500	15±0	500	12.3±0.47	500	10.6±0.47
A58K	125	32.3±0.47	500	17±0.81	1000	0	1000	0
Mean ZOIs		24.95±0.47		18.4±0.81		1.75±0.81		3.12

Table 5. Antibacterial Activity of Essential Oil of Clove Oil and its Combined Effects with Standard Antibiotics

Strains	**Clove oil**	**Essential Oils and standard antibiotic discs (Percent Enhancement)**						
		FOX	**LEV**	**CN**	**TE**	**CIP**	**CHL**	**S**
A30K	22.6±0.47	18.3±0.47	13±0.81	13±0.81 (22%)	22.3±0.47 (76%)	31±0.81 (112%)	18.3±0.47	14.3±0.47
A43K	20.3±0.47	16.6±0.47	25±0.81 (5.93%)	12.6±0.47 (5%)	18±0.81 (35%)	27.6±0.47 (29%)	16.6±0.47	18.3±0.94
A46K	25.3±0.47	21.3±0.47	13.3±0.94 (-8.9%)	13.6±0.47 (17.2%)	22.3±0.47 (102%)	32.6±0.47 (77%)	21.3±0.47	22.3±0.47
A47K	23.6±0.47	25.6±0.47	15±0.81 (-1.4%)	12.6±0.47 (8.6%)	33.6±0.47 (71%)	28.6±0.47 (132%)	25.6±0.47	25.6±0.47
A50K	23.6±0.47	36.6±0.47	13±0.81 (-9%)	12.6±1.24 (-5.2%)	34.6±0.47 (50.4%)	26.3±0.47 (113%)	36.6±0.47	24.3±0.94
A51K	27±0.81	18.6±0.47	19.6±1.24 (11.3%)	11.6±1.24 (9.4%)	32.6±0.47 (32.5%)	25.3±0.47 (52%)	18.6±0.47	42.6±0.47 (142%)
A58K	32.3±0.47	18.6±0.47	20.6±1.24 (5.1%)	13.3±1.24 (-6.9%)	20.3±0.47 (-8%)	26.6±0.47 (8%)	18.6±0.47	21±0.81

* FOX = Cefoxitin, LEV= Levofloxacin, CN = Gentamicin, TE = Tetracycline, CIP = Ciprofloxacin, CHL = Chloramphenicol, S = Streptomycin

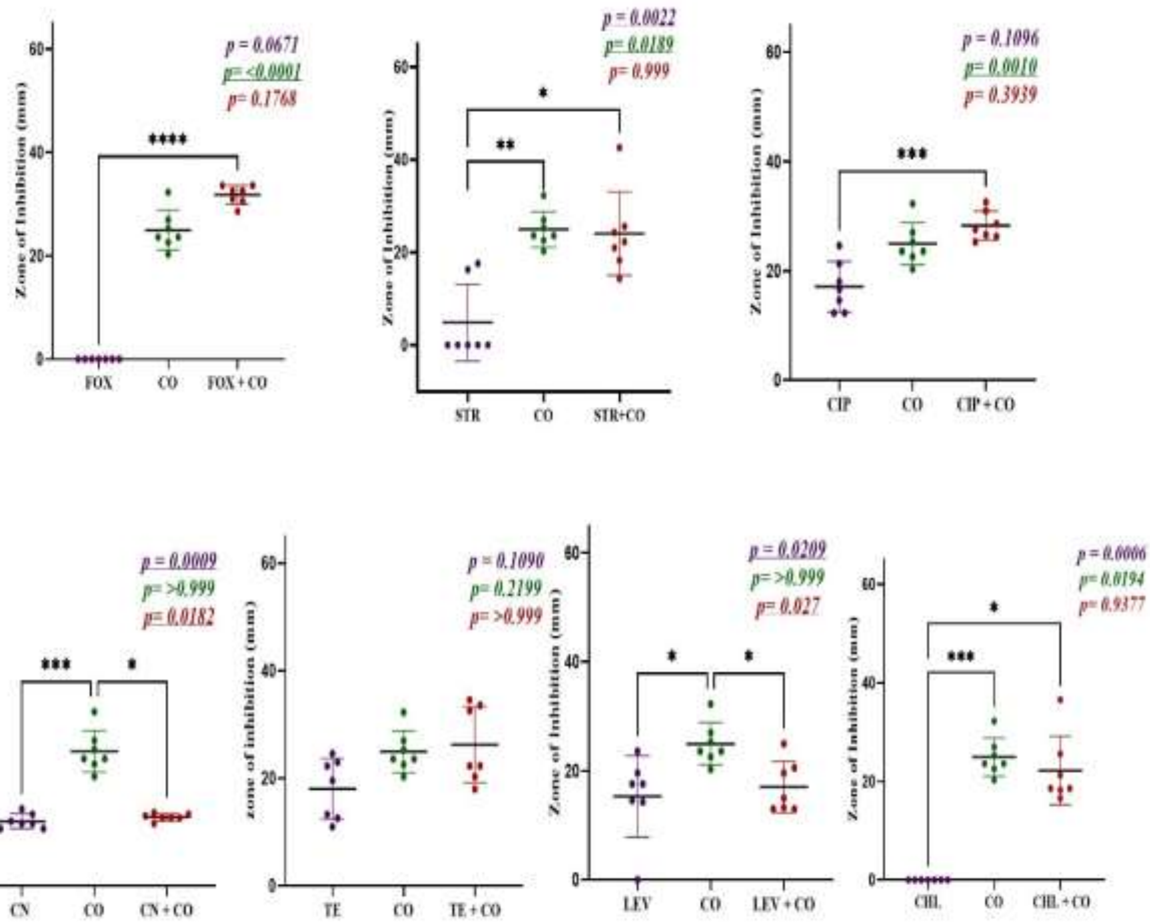

Figure 1: Antimicrobial Activity of Antibiotics, Clove Essential Oil and Their Combination Against Klebsiella Strains; STR; Streptomycin, CHL Chloramphenicol, LEV; Levofloxacin, FOX; Cefoxitin, CN; Gentamicin, CIP; Ciprofloxacin, TE; Tetracycline. Klebsiella strains with antibiotics alone, clove oil at its appropriate MIC and combination of clove oil and antibiotic via drop dilution method were incubated for 24 h and 37 ºC. Data represents mean values of triplicate measurements of zone diameters ±SD. ANOVA ($p < 0.05$) was performed, followed by Kruskal-Wallis Test. While asterisk (*) represent significant differences between the effect of antibiotic and Clove oil. The statistical support values of difference between antibiotic and CO, antibiotic + CO and antibiotic and CO and antibiotic + CO are shown in an inset with purple, orange and green colors, respectively. One asterisk (*) indicates p value smaller than 0.05 ($p<0.05$). Two asterisks (**) indicate p value smaller than 0.01 ($p<0.01$). Three asterisks (***) indicate p value smaller than 0.001 ($p<0.001$).

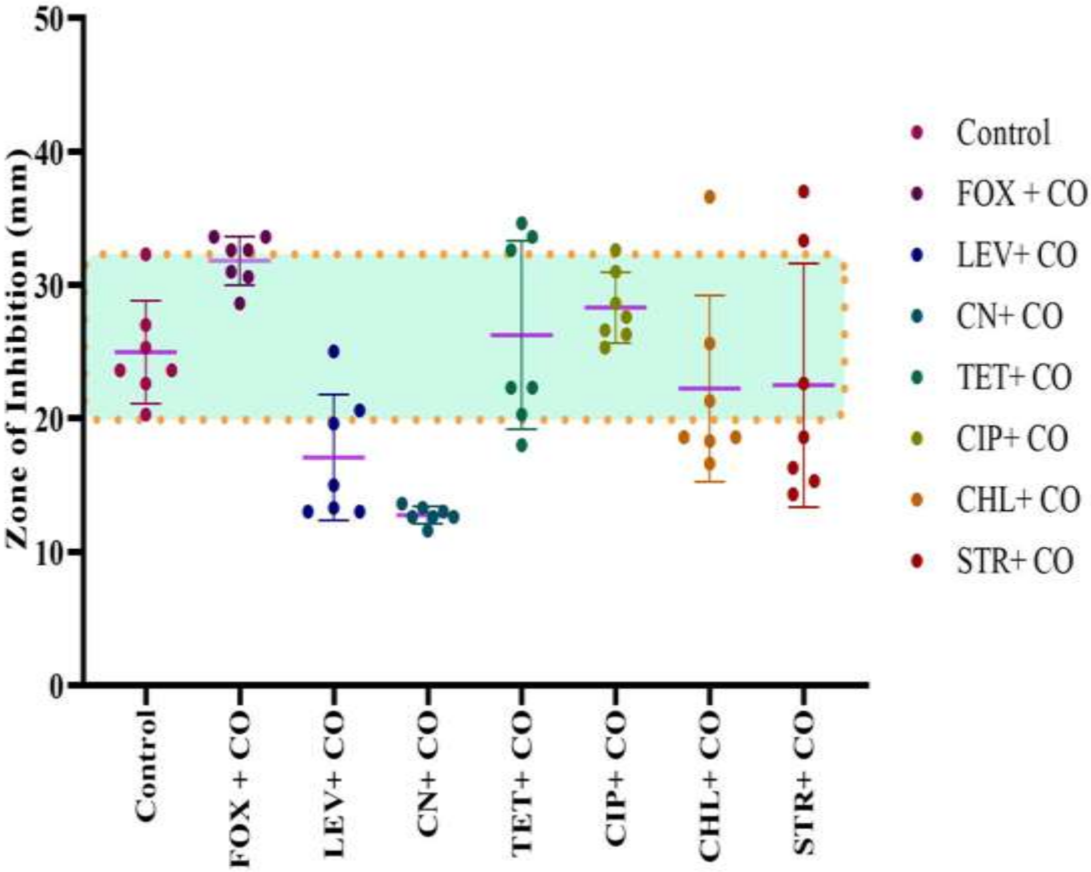

Figure 2: Comparison of Clove Oil Alone and in Combination with Antibiotics: STR; Streptomycin, CHL Chloramphenicol, LEV; Levofloxacin, FOX; Cefoxitin, CN; Gentamicin, CIP; Ciprofloxacin, TE; Tetracycline. The data points reflect the mean of the triplicate values of zone of inhibitions for each experimental group. The blue colored box with a yellow outline is the representation of the window of Clove oil activity.

Discussion

It is noteworthy that the food producing animals are categorized as the major reservoirs for the transmission of food pathogens to humans through the food chain. These pathogens are known to cause life threatening diseases which are having adverse effects both on human health and economy. The unhygienic and unsanitary poultry meat handling production practices are increasing and changing both the virulent and resistance pattern of pathogens such as E. coli, Salmonella, Staphylococcus spp., Klebsiella spp. The food pathogens perform oxidation reactions during slaughtering, processing and storage of poultry products results in undesirable and adverse effects on product quality like decrease in nutritional quality, changes in color, flavor and smell, and the production of toxic compounds and virulence determinants. Amongst the food items which are associated with the diseases, poultry products rank the topmost. In Pakistan, the rate of production in the poultry industry and its market share has increased up to 25% per year as reported by Hussain et al., in the year 2010. On the other hand, the same study revealed that reduced market share for beef and mutton went up to 55% and 20% respectively.

Klebsiella spp. are found in variable niches such as soil, water, sewage and as opportunistic pathogen in human body (Rocha, Henriques et al. 2022). Isolation of multi-drug resistant (MDR) strains from poultry environment suggest that the chicken meat is the most profound route for gene transmission predominately those express for antibiotic resistance (Smet, Martel et al. 2010). Among the investigated Klebsiella strains, 14.28% were resistant to all the antibiotics tested. Antibiotics cefoxitin and chloramphenicol failed to inhibit any of the strain reported no ZOI. This is in agreement with the previous reports noting the prevalence of MDR isolates of Klebsiella from poultry environment (Landman, Georgescu et al. 2008, Krasucka, Cybulski et al. 2012, Prevention, Control et al. 2015). Eventually this poses a threat to the effectiveness of antibiotics which are used in the treatment of infectious diseases. Therefore, it mainly suggests that there should be a proper control and regulatory system for the use of antibiotics, as the studies involved in One Health issues also take part in reduction the spread of antibiotic resistance bacteria. Tetracycline was observed to be most effective (57.14%) against the isolated strains followed by streptomycin (28.57%). The finding corroborates with the previous research on chicken reported 60% of Klebsiella isolates were sensitive to tetracycline. Additionally, a high prevalence of MDR isolates indicates the frequent indiscriminate use of antibiotics in livestock for the growth promotion and prophylaxis (Laube, Friese et al. 2014). Taking One Health perspective into account, it is imperative that the inappropriate use of antibiotics should be limited both in humans and animals.

Since past several decades, essential oils and extracts have been used for in food preservation, pharmaceuticals, alternative medicine and natural therapies (Djilani, Dicko et al. 2012, Reddy and Applications 2019, Rafique, Baig et al. 2022). It is observed that the tested essential oil can act as a promising antibacterial in the treatment of diseases caused by K. pneumoniae (Derakhshan, Sattari et al. 2010, Meng, Li et al. 2016).

Essential oils are potential sources of novel antimicrobial compounds especially against bacterial pathogens. In-vitro studies in this work showed that the essential oils inhibited bacterial growth, but their effectiveness varied (Giannenas, Sidiropoulou et al. 2020, Rafique, Naim et al. 2024). The antimicrobial activity of many essential oils has been previously reviewed and classified as strong, medium or weak. Several studies have shown that cinnamon, clove and rosemary oils had strong and consistent inhibitory effects against various pathogens.

Clove oil has biological activities, such as antibacterial, antifungal, insecticidal and antioxidant properties, and is used traditionally as a savoring agent and antimicrobial material in food (Nuñez and D'Aquino 2012, Zubair, Altaf et al. 2023).

The results indicated that clove oil was efficient at all concentrations in suppressing the Klebsiella strains. The antibacterial activity has been attributed to the presence of some active constituents in the oil. It's possible that eugenol and phenolic compounds are the major components responsible for clove oil's antibacterial properties (Marchese, Barbieri et al. 2017). The available antibiotics can be combined with curcumin or clove oil to enhance the antibacterial spectrum against antibiotic resistant pathogens.

Conclusion

From this study it can be concluded that despite the treatment interventions of MDR strains with multiple antibiotics, the results of essential oils were way more promising. Clove oil followed by cinnamon oil were observed to have most potential antimicrobial activity against the isolated strains however the other oils in the study gave the lesser effect emphasizing the enhanced use of clove and cinnamon oils.

Although the elevated inhibition percent were observed when the antibiotics were combined with clove oil, nevertheless gentamicin and levofloxacin were idiosyncratically opposite to what was observed in other antibiotics. The finding of the study identified the gaps and gives confidence for further exploration as a means of appropriate representation of MDR at genomic level and the treatment with combination for better therapeutic approaches.

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Authors Contribution

All authors have equal contribution in the study. They have reviewed and agreed to its publication.

Declaration of competing interest

The authors declare no conflict of interest

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