Antibacterial activity of herbal extracts against multi-drug resistant *Escherichia coli* recovered from retail chicken meat

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Abstract: Increasing incidence rate of multiple drug resistance in *Escherichia coli (E. coli)* due to extensive uses of antibiotics is a serious challenge to disease treatment. Contaminated retail chicken meat is one of the major sources of spread of multi drug resistant (MDR) *E. coli*. Current study has been conducted to study the prevalence of MDR *E. coli* in retail chicken meat samples from Lahore city of Pakistan and it was found that 73.86% of *E. coli* isolates have MDR pattern. *In vitro* evaluation of antibacterial activity of crude ethanolic extracts of six herbs against MDR *E. coli* phenotypes has revealed that clove and cinnamon have maximum zones of inhibition as compared to other herbal extracts. Mint and coriander gave the intermediate results while garlic and kalonji showed the least antibacterial activity against the MDR *E. coli* phenotypes using the agar well diffusion technique. Average Minimum Inhibitory Concentrations (MICs) for clove, mint, cinnamon, coriander, kalonji and garlic extracts were 1.15, 1.38, 0.5, 1.99, 2.41, 8.60mg/mL respectively using the broth micro dilution method. The results obtained in present study were revealed that crude ethanol extracts of selected herbs have had significant antibacterial activity. Hence they can be used as promising alternatives of antimicrobials against MDR *E. coli* species and can be used for cooked food preservation.

Keywords: *Escherichia coli*, multidrug resistance, ethanolic extracts, herbs, MICs, broth microdilution method.

INTRODUCTION

Escherichia coli (*E. coli)* is versatile specie consisting of both commensals of the gastrointestinal tracts of vertebrates and pathogenic strains responsible for various intra- and extra intestinal infections (Clermont *et al.*, 2011; Shahzad *et al.*, 2013). It is a consistent inhabitant of human intestinal tract. But in addition it is responsible for both nosocomial and community-associated infections. They commonly cause urinary tract infection, blood-stream infections, pneumonia, post surgical infections and intra-abdominal infections (Lockhart *et al.*, 2007; Pitout and Laupland, 2008). The prevalence of superbugs in large proportion has emerged as a worldwide problem and is a serious challenge to disease treatment with possibility of complete treatment failure. One of them is multiple drug resistant *E. coli*, heavily contaminated the retail chicken meat (Vincent *et al.*, 2010). Chicken meat is the most commonly used food in Pakistan and is one of the major sources of spread of multi-drug resistant (MDR) *E. coli*. Frequent antimicrobial use creates a pool of resistant commensal bacteria, made them candidates for the spread of resistance genes for pathogenic bacteria (Aarestrup *et al.*, 2008). According to World Health Organization (WHO) half of the antimicrobials produced in the world are used in animal sector for the prophylaxis, control, treatment of infections and as growth promoters as well (Mathew *et al.*, 2007). Therefore, there is a chance that resistant bacteria can infect humans via food which leads to cross resistance and multi resistance patterns (Allen *et al.*, 2010). *E. coli* being present most dominantly in the gut of animals makes it ideal for the study of multiple drug resistance which is mainly due to inappropriate use of human antibiotics as growth promoters and for the treatment of infections (McEwen and Fedorka-Cray, 2002).

Nowadays, people suffering from the side effects of antimicrobial resistance, try to find solution in natural products. Medicinal plants can provide a wealth of antimicrobial agents; Herbs are used to treat various infectious diseases worldwide. Plant-derived materials with therapeutic properties known as herbal medicines; are beneficial for human health (Walter *et al.*, 2011). Interestingly, some herbs have antimicrobial activity against bacterial pathogens in addition to their flavoring effects. From the earliest times, herbal spices were added for improving taste. They can also naturally and safely perk up shelf life of food products. Plants that have been used in medicine as antimicrobial agents since ancient times could provide a promising solution for drug resistant species (Ismail *et al.*, 2012).

Many of the spices used in daily life are antimicrobials. The natural products are found to be more effective with least side effects as compared to commercial antibiotics this is why they are used as an alternate remedy for treatment of various infections. Many medicinal plants have antioxidant and antimicrobial properties, which protect the host from cellular oxidation reactions and other pathogens highlighting the importance of search for natural antimicrobial drugs. Most of the food borne

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bacterial pathogens are sensitive to extracts from plants such as garlic, mustard, onion and oregano (Mukhtar and Ghori, 2012). Therefore the present study was conducted to study the antibacterial potential of herbs against multiple drug resistant *E. coli*.

MATERIALS AND METHODS

Samples

A total of one hundred (n=100), presumptive *E. coli* isolates recovered from retail chicken meat samples from various locations of Lahore city were obtained from University Diagnostic Laboratory (UDL), University of Veterinary & Animal Sciences (UVAS) Lahore.

Biochemical Identification of E. coli

All presumptive isolates were processed for the biochemical identification of *E. coli*, so glycerol preserved isolates of presumptive *E. coli* were revived in Tryptose soya broth (TSB). Subsequently, TSB cultures were inoculated onto MacConkey's agar plates and incubated at 37^{o}C for 24 hours. Biochemical identification of isolates was performed on the basis of IMViC (Indole, Methyl red (MR), Voges Prouskauer (VP) and Citrate utilization test) and sugar (xylose, inositol, maltose, trehalose, lactose, arabinose, glucose, sorbitol and mannitol) fermentation tests following the protocols of (Shank, 1975).

Molecular characterization of E. coli by PCR

Biochemically identified *E. coli* isolates were further confirmed on molecular basis using PCR. The DNA from colonies identified by biochemical tests were subjected to PCR, targeting universal stress protein (*usp*A) gene specific to *E. coli* for confirmation (Chen and Griffiths, 1998).

Bacterial DNA extraction

E. coli DNA was extracted by boiling method (Anonymas, 2008). For extraction of DNA, *E. coli* isolates were inoculated in TSB and incubated at 37^{o}C for 24 hours. After overnight incubation, the broth culture was centrifuged into 1.5mL microfuge tube at 14000g for 30 minutes. Supernatant was carefully removed and cell pellet was suspended in 25µl PCR-grade water. This microfuge tube having suspended cell pellet was boiled for 10 minutes in water bath and then placed on ice for 5 minutes before centrifugation at 14000g for 5 minutes. Cells were settled down and supernatant containing DNA was collected.

PCR of E. coli (uspA gene)

Extracted DNA was amplified using Universal Stress Protein (*usp*A) primers set (Chen and Griffiths, 1998) for confirmation of generic *E. coli.* PCR was performed by using Taq PCR master mix of Fermentas with 5µl of template DNA and 0.5µM of each primer as per manufacturer's instructions. Thermo cycler (EscoSwift™ Mini Thermal Cycler) was programmed according to conditions as mentioned in (table 1).

Multi-drug resistance testing

After confirmation of *E. coli* the isolates were processed for antimicrobial susceptibility testing using Kirby-Bauer disk diffusion method as per Clinical Laboratory Standards Institute (CLSI, 2011) criteria. *E. coli* was inoculated on MacConkey's agar and incubated for 24 hours at 37^{o} C. After incubation, colonies of bacteria were suspended in sterile normal saline (0.85% NaCl) and cell density was adjusted to 0.5 McFarland turbidity standards. Suspension of bacteria was swabbed onto Mueller Hinton agar plate with the help of cotton swab. Disks of Nalidixic acid (30µg), Ampicillin (10µg), Trimethoprim-Sulfamethoxazole (25µg), Cefuroxime (30µg), Chloramphenicol (30µg), Colistin sulphate (10µg), Bacitracin (10µg), Ciprofloxacin (5µg), Norfloxacin (10µg), Tobramycin (10µg), Cefotaxime (30µg), Streptomycin (10µg), Tetracycline (30µg) and Gentamicin (10µg) were applied and incubated at 37^{o} C for 24 hours. Following incubation, zones of inhibition were measured in mm with the help of vernier caliper and recorded for quantitative analysis. Isolates with multi-drug resistance pattern (resistant to three or more than three antibiotics of different classes) were selected for further processing.

Antibacterial activity of herbal extracts

Collection of plant material

Different parts of the selected herbs such as leaves of mint and coriander, cloves of garlic, seeds of kalonji were collected from grocery shop. Clove and cinnamon were taken as such to check the antibacterial activity against the MDR *E. coli* isolates.

Preparation of plant extracts

Ethanolic extraction of selected plants was done. The collected plant parts were washed, dried and grinded into fine powder. Powder was weighed 10 g, soaked with 90 mL of 80% ethanol (1:10 w/v) and placed overnight at room temperature with shaking (Optima Tokyo, Japan) at 150 rpm for extraction of active ingredients. Extracts were then filtered through whatman No.1 filter paper and filtrate was dried at 40°C. Stock solution of each extract was prepared in Dimethyl sulfoxide (DMSO) (Obeidat, 2011; Uma *et al.*, 2009).

Agar-Well Diffusion test for herbal plant extracts

The antimicrobial activity of ethanolic plant extracts against MDR *E. coli* isolates was determined by using the agar well diffusion assay. Petri plates with 20mL Muller Hinton Agar were prepared, seeded with 24 hours Inoculum suspension of 1×10^{8} (CFU/mL) of MDR *E. coli* strains and wells of 6mm in diameter were cut in them using sterile borer. 50µl of plant extracts with

concentration of 10, 20 and 30mg/ well were poured in wells and 50 µl of DMSO was used in the same manner as negative control followed by incubation at 37°C for 18 hours. The antibacterial activity was determined by measuring the zone of inhibition in mm around the wells (Hassan *et al.*, 2009).

MIC Calculation of herbal plant extracts

Plant Materials with significant antimicrobial activity against MDR *E. coli* were processed for the minimum inhibitory concentration (MIC) by standardized broth microdilution method using serially diluted plant extracts, with the final inoculums of 5×10^5 colony forming units (CFU) /mL according to the (CLSI, 2011) guidelines. The ethanol extracts were diluted 2 fold to get series of concentrations in sterile phenol red lactose broth up to the 11^{th} well while 12th well was kept as growth control. Then, 100 µl of standardized inoculum suspension of 5×10^5 colony forming units (CFU)/mL was inoculated within 15 minutes in all wells of 96 well plate (Kartel) containing serially diluted herbal extracts. Subsequently, plate was incubated at 37°C for 18 hours. MIC was determined as the lowest concentration or highest dilution of each herb extract that did not give any visible bacterial growth in the micro dilution wells (Hassan *et al.*, 2009).

STATISTICAL ANALYSIS

The data were collected in MS Excel 2007 and was analyzed statistically by Fisher's exact test and Analysis Of Variance (ANOVA) using Statistical Package for Social Science (SPSS) 18.0 software.

RESULTS

In the present study, prevalence of *E. coli* from retail chicken meat samples was 88% in Lahore, Pakistan. All the positive isolates produced smooth, glossy, translucent and typical pink colored colonies on the MacConkey's agar due to fermentation of lactose. They showed positive reaction for Indole and Methyl Red tests. They also gave negative reaction for Voges Prouskauer and Citrate utilization tests. Out of 88 biochemically tested *E. coli* isolates, 80 (90.9%) isolates were found positive through PCR by amplifying *usp*A gene of *E. coli* (fig. 1).

E. coli isolates were surprisingly found resistant to Ampicillin (95.4%) but showed least resistance (3.07%) to Colistin sulphate. Resistance among other groups was also found such as Trimethoprim-sulfamethoxazole (86.2%), Nalidixic acid (81.5%), Streptomycin (64.6%), Tetracycline (60%), Norfloxacin (37%), Ciprofloxacin (30.8%), Chloramphenicol (33.8%), Gentamycin (23.07%), Cefotaxime (26.2%) and 16.9% in Tobramycin (fig. 2). Crude ethanolic extracts of clove, mint, cinnamon, coriander, kalonji and garlic used in current study have depicted antibacterial activity against MDR *E. coli* phenotypes recovered from retail chicken meat.

DISCUSSION

Increasing rates of antimicrobial resistance among meat-borne pathogens and commensal microorganisms poses a serious public health risk (Sheikh *et al.*, 2012). Contaminated retail meat is one of the major sources of food-borne illnesses. According to Food and Agriculture Organization (FAO) survey, chicken meat consumption in Pakistan is increasing day by day, as it is a cheap protein source (Ali *et al.*, 2010). Frequent use of antibiotics in the chicken as growth promoters; globally increase the prevalence of MDR *E. coli* and its high prevalence in retailed chicken meat is not much surprising because *E. coli* is one of the microbial floras of gastrointestinal tract of warm blooded animals and poultry birds (Akond *et al.*, 2009).

In the present study, 73.86% of total *E. coli* isolates have MDR (three or more than three antimicrobials belonging to different categories) pattern. The high prevalence rate of multiple drug resistant (MDR) *E. coli* in current study indicated the irrational uses of antimicrobials in poultry farming. Almost 80% of administration of these antimicrobials is unnecessary. This misuse of antimicrobials in food animals has adverse effects on human health and contributing in the development of resistance in microorganisms (Ogunleye *et al.*, 2008). Ciprofloxacin is used in GIT infections of poultry in Pakistan, which may be responsible for the development of resistance. High resistance (73.17%) to trimethoprim-Sulphamethoxazole was also found in *E. coli* recovered from avian origin by (Zhang *et al.*, 2012). In the present study, least resistance (3.07%) to Colistin sulphate was found. Similarly, in a previous conducted study *E. coli* recovered from chicken from slaughterhouses in Tunis were found 4% resistant to Colistin sulphate (Soufi *et al.*, 2009).

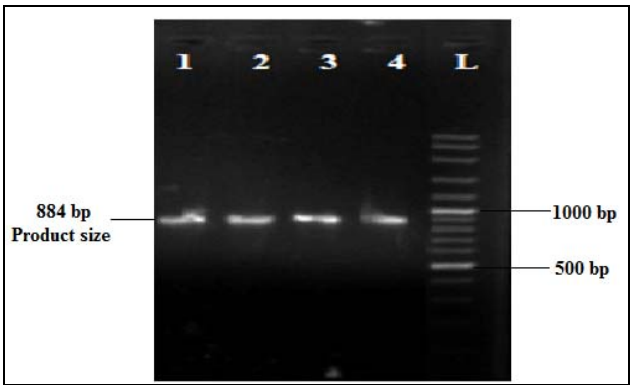

Fig. 1: PCR results for *usp* A gene amplification of *E. coli* isolates

The antibacterial activity of herbal plants is most likely due to adsorption of polyphenols to bacterial membranes with disruption of membrane and subsequent leakage of cellular contents (Negi, 2012). It has been revealed from previous studies that garlic, cinnamon, cloves, onion, sage and other spices can inhibit the growth of both Gram negative and Gram positive food borne or food spoiling bacteria (Ismail *et al.,* 2012). *Syzhygium aromaticum* (Clove) have been extensively studied for its antimicrobial potential against wide range of microorganisms. In this study, crude ethanolic extract of clove gave maximum zones of inhibition of 14.76 mm, 16.67 mm and 18.79 mm at average with 10, 20 and 30 mg/well concentration respectively (table 2). The average MIC of clove was 1.15mg/mL against the MDR *E. coli* isolates using broth microdilution technique. One of the previous conducted study revealed the growth inhibition zones of 14mm diameter of crude ethanol extract of clove against *E. coli* and its MIC was found to be 100 mg/mL (Gupta *et al.,* 2012). In another study, the antibacterial activity of Clove was tested against *E. coli* and the methanolic extract of clove exhibited MIC value of 2.31 mg/mL for *E. coli* (Pandey and Singh, 2011). Previous studies proved that *Cinnamomum zeylanicum* (Cinnamon) is the most effective herb against growth of the microorganisms *in vitro*. Similarly, in current study the crude ethanolic extract of cinnamon showed maximum growth inhibition zones against MDR *E. coli* isolates with average diameters of zone of inhibition of 15.44, 16.98 and 18.89 mm at 10, 20 and 30 mg/well concentration by agar well diffusion assay (table 2). The MIC of crude ethanolic cinnamon extract was 0.49mg/mL on an average. Previous conducted study on use of ethanolic extracts of cinnamon against *E. coli* (whether or not it was MDR) showed presence of sensitive *E. coli* against cinnamon. The zone of inhibition of 7 mm diameter and MIC value (64µg/mL) of cinnamon was found against *E. coli* by (Usha *et al*., 2012). But another previous conducted study depicted that *E. coli* was resistant to cinnamon extract (Shafique *et al.,* 2010).

Mint have strong antimicrobial activity due to polyphenolic compounds, terpenoids, flavonoids, and other volatile compounds (Gulluce *et al.*, 2007). Crude ethanol extract of *Mentha piperita (*Peppermint) produced average zones of inhibition of (13.63, 15.78 and 17.74 mm) diameter against MDR phenotypes of *E. coli* using the same above discussed concentrations (table 2). Average MIC of mint was found 1.38 mg/mL. In another study, It was found that ethanolic extract of mint gave the zone of inhibition of 2 mm against *E. coli* (Irshad *et al.*, 2011).

Crude ethanolic extract of *Coriandrum sativum (*coriander) gave average inhibition zones of 12.25, 14.5 and 16.28 mm and MIC was found to be 1.99mg/mL against MDR *E. coli* phenotypes (table 2). Previous study showed that methanol extract of Coriander has antimicrobial potential and revealed that its extract have significant antibacterial activity against *E. coli*, *Salmonella* sp. and *Shigella* sp. (Uma *et al.,* 2009). In another study, the extract of coriander was studied as effective antibacterial agent against human pathogens. According to this previous study, crude methanol extract produced zone of inhibition of 8 mm against *E. Coli* and the MIC value was found 32µg/mL (Dash *et al.*, 2011).

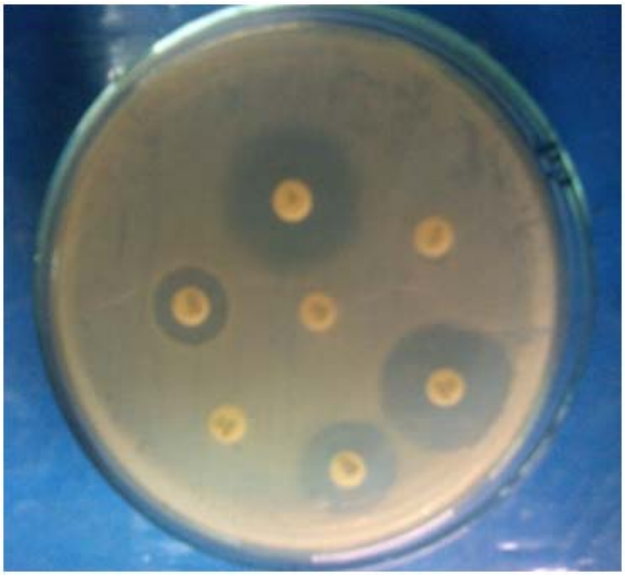

Fig. 2: Multi drug resistance monitoring by disk diffusion assay

One of the previously conducted studies was considered inhibitory zone (>12 mm) to be significant in case of plant extracts (Hannan *et al.*, 2008). In the present study, low antimicrobial activity of *Nigella sativa* (Kalonji) extract was observed with growth inhibition zones of 7.34, 9.67 and 10.88 mm in agar well diffusion test (table 2) and average MIC value of 2.41mg/mL against MDR *E. coli* phenotypes but by Hannan *et al.,* (2008) MIC of *Nigella sativa* was found 0.5mg/mL (500µg/mL). In another study the researchers tested antibacterial activity of ethanolic black seed extract against *E. coli* and according to their results MIC for ethanolic extract was 0.125g/100mL (1250µg/mL) (Shahid *et al.*, 2013). Antibacterial activity of *Allium sativum* (Garlic) has been proved against *E. coli* and many other bacterial pathogens. The selected MDR phenotypes of *E. coli* were found resistant to Garlic extract depicted the average inhibition zones of 5.39, 8.36 and 10.18 mm using the well diffusion method (table 2) and average MIC value of 8.60mg/mL. In contrast a work in recent past proves antibacterial activity of garlic extracts with the zone of inhibition of 20.33±0.47 mm against *E. coli* (Sudhir *et al.*, 2012).

Table 1: Thermo cycler conditions for *usp*A gene PCR

Stages	PCR Conditions	Cycles
Initial Denaturation	95°C for 5 Min	1
Denaturation	94°C for 30sec	30
Annealing	56°C for 30sec	
Extension	72°C for 1 Min	
Final Extension	72°C for 5Min	1

Table 2: Average values of zone of inhibitions formed by crude ethanolic extracts of herbs against various multidrug resistant *E. coli* isolates using well diffusion technique

Concentration (mg) /well	Average zones of inhibition (mm)					
	Clove	Mint	Cinnamon	Coriander	Kalonji	Garlic
10	14.76	13.63	15.44	12.25	7.34	5.39
20	16.67	15.78	16.98	14.5	9.67	8.36
30	18.79	17.74	18.89	16.28	10.88	10.18

Data analysis through Fisher's exact test showed that there was significant relation ($p<0.05$) between the prevalence of *E. coli* in retail chicken meat samples and MDR pattern found in them. There also exist a significant difference in antibacterial activity produced by all herbs ($p<0.05$) except antibacterial activity of extracts of clove and cinnamon having no significant difference ($p>0.05$) using SPSS.18.

CONCLUSION

Our data demonstrate that the retailed chicken meat in Lahore city is highly contaminated with MDR *E. coli* and selected herbs have antibacterial activity against it. Clove, cinnamon and mint have more antibacterial activity as compared to coriander, kalonji and garlic. As these herbs are extensively used during eastern cooking method, these herbs can reduce bacterial load in chicken meat. Further there is a need for extensive study to establish co-relation between the amount of herbs and bacterial load on meat. Another advantage of using these herbs as antimicrobial agent is that, there is no harmful effect on body recorded so far and there is less chance of development of resistance in bacteria against these herbs.

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