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Potential of Aqueous and Alcohol Extracts of *Quercus infectoria*, *Linusm usitatissium* and *Cinnamomum zeylanicium* as Antimicrobials and Curing of Antibiotic Resistance in *E. coli*

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Abstract: Eighty two clinical isolates were collected from Sulaymani hospitals. The isolates show resistance to more than five antibiotics. The ethanol extracts of *Quercus infectoria*, *Linusm usitatissium* and *Cinnamomum zeylanicium* were proved to be the most powerful against *E. coli* E45 and E62 isolates, than aqueous extracts. The extracts exhibited most of the antibiotics activity against these two isolates irrespective of their antibiotic resistance behaviour. A comparative evaluation of plasmid elimination from *E. coli* E62 clinical isolate by sub-MIC of plant extracts showed that these extracts could cure plasmids effectively at their respective sub-MIC concentration. Maximum plasmid cured was observed by sub-MIC of ethanol extract of *C. zeylanicum* at 1100 µg/mL cured two plasmids 15 and 45 kb, followed by sub-MIC of *L. usitatissimus* extract at 1100 µg/mL was cured 45 kb plasmid and sub-MIC of aqueous and ethanol of *Q. infectoria* were cured 15 kb plasmid from E62 isolate. Curing of plasmid DNA from *E. coli* isolate confirmed by determining the loss of resistance markers in cured cultures.

Key words: Antibiotic resistance, *Cinnamomum zeylanicum*, *E. coli*, *Linusm usitatissium*, *Quercus infectoria*

INTRODUCTION

Medical plants have been used as source of drug to combat diseases for several thousand years (Cragg *et al*., 1997; Craig, 1999). However, there has been remained scientific interest in plant extracts and their constituents for health care during the past two decades. The shift from synthetic chemical agents to plant - based products is primarily due to the frequent untoward effects seen with synthetic chemical agents (Dev, 1997). In recent years, the escalation of multidrug resistance in bacteria has gained worldwide attention du to the high impact on public health. Increased usage of antimicrobial agents to treat bacterial infections has lead to the emergence of multi drug resistant (MDR) strains (Bonnet, 2004). the increasing of MDR incidence in the genetic and mechanisms of resistance evolved by bacteria, as such information could lead to strategies for counter acting the effect of antimicrobial resistance.

Studies from different geographical areas, especially rarely studied areas such as the Middle East urgently needed, especially in our reagent, because antimicrobials are available to public and with different origin and low qualities are distributed. There fore the aim of this study was performed to evaluate the effects of aqueous and alcohol extracts of *Quercus infectoria, Linusm usitatissium* and *Cinnamomum zeylanicium* as antimicrobials and elimination of antibiotic resistance in *E. coli* isolated from different sources in Sulaymani hospitals.

MATERIALS AND METHODS

Bacterial isolates: Eighty two isolate of *E. coli* were randomly collected from several hospitals in Sulaymani city, Iraq. These isolates were recovered from various specimens, Urine, wound, vaginal, stool, csf and were considered to be the causative agent of the patients illness in most cases during march 2009 and September 2009. The study conducted in Biology Department, college of Science, University of Sulaymani, Iraq. Data concerning antibiotic usage against these isolates is not available. All isolates were identified by conventional technique (Murray *et al*., 1995), confirmed where necessary by the API20E system (Biomerieux, Marcy-I' Etoile, France) and stored in nutrient broth containing 20% glycerol at -20ºC until further analysis.

Bacterial strains: The standard strain of *E. coli* NN2219 serotype obtained from Dr. Shwan Rashid, Saarland University Germany, was used in this study. The organism was maintained on nutrient agar slants at 4ºC.

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Antimicrobial sensitivity test: The antimicrobial sensitivity phenotypes of all bacterial isolates were determined using dilution method in nutrient agar plates (Oxoid, Cambridge UK) according to (Atlas *et al*., 1995). The antimicrobials purchased from (sigma company, Germany), are Amoxicillin (Amo), Amikacin (Aic), Ceftazidime (Caz), Ciprofloxacin (Cip), Chloramphenicol (Chl), Clindamycin (Clc), Gatifloxacin (Gat), Glutamin (Gtm), Gentamycin (Gen), Nalidixic acid (Nal), Nitrofuration (Nif), Pipracillin (Pip), Rifampicin (Rif), Tetraciclin (Tet), Tobramycin (Tob), Trimethoprim (Tri). These antimicrobials were used at final concentrations or plant extracts were added to the medium after sterilization and cooling to 50°C, the medium were mixed and poured into Petri-dishes, then inoculated with isolated bacteria using streaking method, susceptibility or resistance were recorded after incubation for 24 h at 37ºC.

Determination of *in vitro* antimicrobial effects: The aqueous and ethanol extracts of *Q. infectoria*, *C. zeylanicum* and *L. usitatissimum* were tested against two clinical isolates of *E. coli* E45 and E62 from patients with wound infection in Sulaymani hospitals.

Determination of Minimum Inhibition Concentration (MIC): The MIC of plant extracts was determined by a broth dilution method in test tubes. A standard inoculums contain 10^6 cuf/mL depending on slandered curve was added to a series of tubes containing increasing concentration of plant extracts being tested, and then incubated at 37ºC for 24 h. The MIC is quoted as the lowest concentration of plant extracts which inhibit the viable growth of microorganisms (Bauman, 2007).

Bacterial growth curve: *E. coli* clinical isolate was cultured in nutrient agar (NA) at 37ºC overnight, and then the bacterial suspension was subcultured (1:50) (vol/vol) in fresh Nutrient broth for another 12 h. The growth of bacteria at different time was determined with spectrophotometer by measuring the absorbance at 600 nm. For exact quantification of bacteria, bacterial suspension collected at different times were plated on NA and incubated for 24 h at 37ºC. The results of one of three experiments are reported.

Selection of medicinal plants: Fruits of *Q. infectoria*, seeds of *L. usitatissimum* and bark of *C. zeylanica* were purchased from an herbal shop in Sulaymani, Iraq. The seeds or plant bark were authenticated at the Herbarium of the Department of Biology Science Salahadeen University Erbil, Iraq.

Preparation of plant extracts: Fruit of *Q. infectoria*, seeds of *L. usitatissimum* and bark of *C. zeylanicum* were ground into slightly coarse powder using electric blender. Extracts were prepared by soaking three plants portions of 50 g of the dried powder separately in 250 mL of double distilled water and absolute ethanol using a conical flask plugged with cotton wool. The mixture was kept at room temperature for 72 h under continuous shaking. The viscous mixture was centrifuged for 5 min at 300 rpm to collect the supernatant. The aqueous supernatant and organic extracts were filtered separately through what man filter paper No. 2 under vacuum. The filtrates were evaporated to dryness by Rota vapor. Water bath was adjusted to 55ºC. The organic extract was kept over night under vacuum fame hood to obtain a constant dry weight. The extract weighed and stored in closed vessel at 4ºC in refrigerator for further use.

Elimination of antibiotic resistance: A small inoculum 10^4 bacteria per mL was grown overnight at 37ºC in nutrient broth containing sub inhibitory concentration of medical plant extract, giving in complete inhibition. The cultures were plated on agar, and isolated colonies tested for antibiotic resistance.

Plasmid extraction: Large-scale plasmid DNA preparation from *E. coli* was performed followed the method of Birnboim and Doly (1979).

Preparation of agarose gel electrophoresis: Method of Sambrook *et al.* (1989) was used for electrophoresis by using 0.75% agarose, 10 µL of ethedium bromide at final concentration 0.5 µg/mL was added, the mixture was poured onto gel tank surrounded by a gel former, the comb was inserted and the gel was allowed to set, the comb and surrounded cover removed and the gel smoked in a gel tank contain Tris-borate buffer. 15 µL of plasmid DNA samples were mixed with 3 µL of loading buffer dye finally 10 µL of this mixture were added to the wells, and then the gel tank was covered by lid. Gel was run at 60 volt for 2 h, the gel examined by ultraviolet illumination and photographed by Digital Camera.

RESULTS

A total of eighty two clinical isolates of *E. coli* were isolated from hospitals in Sulaymani city-Iraq. All isolates were screened to 16 antibiotics. Eighty one 98.78% of the isolates were resist to Clindamycin and 66 of isolates 80.48% were resist to Amoxicillin, and 65 isolates 79.26% were resist to Nalidexic acid and Piperacillin. The lowest resistance recorded toward Rifampicin 12.19%, Tobramycin 13.4% and Chloramphenicol 14.63% (Table 1).

The MIC value of aqueous extracts was 9000 µg/mL for *Q. infectoria* and *L. usitatissimum*, and 4500 µg/mL for *C. zeylanicum* water extract, while ethanol extract was 1200 µg/mL for *Q. infectoria* and *L. usitatissimum*

Table 1: Susceptability of *E. coli* isolates to different antibiotics

Antibiotics	No. of resistant isolates	Resistant (%)
Amo	66	80.48
Aic	25	30.48
Caz	40	48.78
Cip	15	18.29
Chl	12	14.63
Cic	81	98.78
Gat	15	18.29
Gtm	23	28.04
Gen	41	50.00
Nal	15	18.29
Nif	65	79.26
Pip	65	79.26
Rif	10	12.19
Tet	54	65.80
Tob	11	13.41
tri	40	48.78

Table 2: MIC value of aqueous and ethanol extracts of plants used against *E. coli* E45 and E62 isolates

Treatments	MIC µg/mL for E45	MIC µg/mL for E62
Aqueous extract of *Q. infectoria*	9000	9000
Ethanol extract of *Q. infectoria*	1200	1200
Aqoeous extract of *C. zeylanicum*	4500	4500
Ethanol extract of *C. zeylanicum*	3500	3500
Aquious extract of *L. usitatissimum*	9000	9000
Ethanol extract of *L. usitatissimum*	1200	1200

plant, and 3500 µg/mL for *C. zeylanicum* ethanol extract (Table 2).

Aqueous and ethanol extracts of these plants were tested for curing of antimicrobial resistance against two multidrug resistance clinical *E. coli* E45 and E62 isolates, and the preliminary assessment of the curing or elimination of antibiotic resistance of these two isolates reveled two basic outcomes (Table 3 and 4). First, the ethanol extracts at a concentration of sub-MIC

(Table 2) were proved to be more effective under the condition of the present investigation, comparing to aqueous extracts. Second, *L. usitatissimum* extracts proved to be more effective for elimination of antibiotic resistance than other two plant extracts, except sub-MIC of ethanol extract of *C. zeylanicum* was eliminated 90% resistance for *E. coli* E45 isolate. It is interested to note that sub-MIC of tested plant extracts apparently cured the plasmid from *E. coli* E62 isolates treated cells (Fig. 1). Sub-MIC of water and ethanol extracts of *Q. infectoria* cured 15kb plasmid from *E. coli* E62 isolate, while sub-MIC of ethanol extract for *C. zeylanicum* cured both 15 and 45 kb plasmids of treated *E. coli* E62 isolate cells. On the other hand when same isolate treated with sub-MIC of *L. usitissimum* aqueous extract only the 45 kb plasmid was lost. Curing of plasmid from *E. coli* E62 isolate was confirmed by determining the loss of resistance markers in cured cultures e.g., ethanol extract of *C. zeylanicum* at sub-MIC losses resistance to 12 antibiotics from 16 antibiotic markers (Table 4).

DISCUSSION

The clinical *E. coli* isolates appeared resistance phenotypes to five or more antibiotic agents were detected in all these isolates in Sulaymani hospitals, this may be due to widespread and discriminate use, and the antibiotics are available to the public, or the use of antibiotics in animal feeds is another factor to increasing antibiotic resistance. In this study, we screened and characterized antimicrobial resistance plasmids of *E. coli* isolates in Sulaymani hospitals. Similar studies have been reported from Italy (Mugnaioli *et al*., 2006), Brazil (Minarini *et al*., 2008), Purtugal (Machado *et al*., 2007) and Canada (Pitout *et al*., 2008), where the highest proportion were *E. coli* isolates. Hospital-acquired infection was mainly caused by *E. coli* isolates (Coque *et al*., 2008). Antibiotic resistance phenotypic profiles were noticed in previous studies, especially with bacterial isolates that showed close genetic determinants in other countries (Machado *et al*., 2006; Pitout *et al*., 2008; Minarini *et al*., 2008).

Broad spectrum activity of sub-MIC of these plant extracts on *E. coli* E45 and E62 isolates Table 3 and 4, e.g., where the sub-MIC of ethanol extract for *L. usitatissimum* and *C. zyelanicum* reduced resistance of these two isolates for all antibiotics except to Nal and Tet, this finding is an apparent indication that the plant

Table 3: Curing effect of sub-MIC for *Q. infectoria, C. zeylanicum* and *L. usitatissimum* extracts on *E.coli* E45 isolate

Antimicrobials	Aquious extract of *Q. infectoria* at 8000µg/mL	Ethanol Extract of *Q. infectoria* at 8000µg/mL	Aquious extract of *C. zeylanicum* at 4000 µg/mL	Ethanol extract of *C. zeylanicum* at 3000 µg/mL	Aquious extract of *L. usitatissimum* at 8000 µg/mL	Ethanol extract of *L. usitatissimum* at 1100 µg/mL
Amo	+	+	+	-	+	-
Chl	+	+	-	-	-	-
Clc	+	+	-	-	+	-
GeN	-	-	-	-	-	-
Gtm	+	+	-	-	+	-
Nal	+	-	-	+	+	+
Nif	+	+	-	-	+	-
Pip	+	+	+	-	+	-
Tet	+	+	+	+	+	-
Tri	+	-	+	-	+	-

+: The bacteria were resist, -: The bacteria were sensitive

Table 4: Curing effect of sub-MIC for *Q. infectoria, C. zeylanicum* and *L. usitatissimum* extracts on *E.coli* E62 isolate

Antimicrobials	Aquious extract of *Q. infectoria* at 8000 µg/mL	Ethanol Extract of *Q. infectoria* at 8000 µg/mL	Aquious extract of *C. zeylanicum* at 4000 µg/mL	Ethanol extract of *C. zeylanicum* at 3000 µg/mL	Aquious extract of *L. usitatissimum* at 8000 µg/mL	Ethanol extract of *L. usitatissimum* at 1100 µg/mL
Ami	-	-	-	-	-	-
Amo	+	+	+	-	+	+
Caz	+	+	-	-	-	+
Cip	+	-	-	-	+	-
Clc	+	+	-	-	+	-
Chl	+	+	-	-	+	-
Gat	-	-	+	-	+	-
Gen	-	+	+	+	-	-
Gtm	+	+	+	-	+	-
Nal	+	-	-	+	+	+
Nif	+	+	-	+	+	+
Pip	+	+	-	-	+	+
Rif	+	-	-	-	-	-
Tet	+	-	-	-	+	+
Tob	-	+	-	-	-	-
Tri	-	+	+	+	+	+

+: The bacteria were resist, -: The bacteria were sensitive

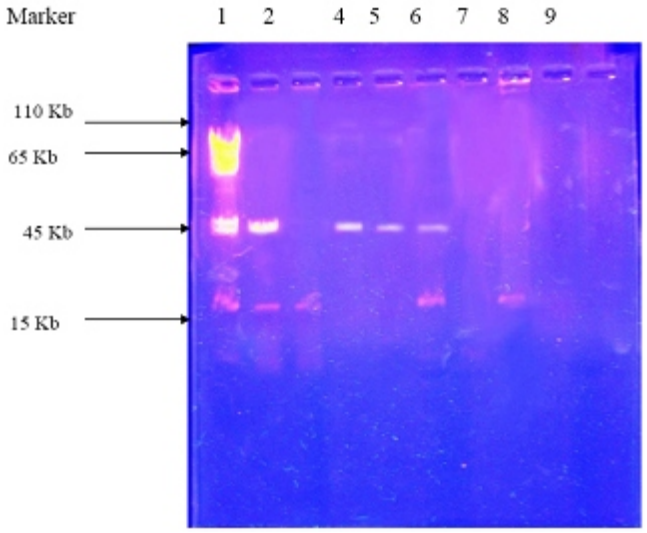

Fig 1: Plasmid profile of *E. coli* E 62 isolate after curing with *Q. infectoria, C. zeylamicum* and *L. usitatissimum* extracts and *E.coli* DH5alpha
Lane 1: *E.coli* plasmid marker NN2219 strain
Lane 2: *E. coli* E62 isolate
Lane 4: *E. coli* E 62 isolate after treating with sub-MIC of *Q. infectoria* aqueous extract
Lane 5: *E. coli* E 62 isolate after treating with sub-MIC of *Q. infectoria* ethanol extract
Lane 6: *E. coli* E 62 isolate after treating with sub-MIC of *C. zyelanicum* aqueous extract
Lane 7: *E. coli* E 62 isolate after treating with sub-MIC of *C. zyelanicum* ethanol extract
Lane 8: *E. coli* E 62 isolate after treating with sub-MIC of *L. usitatissimum* aqueous extract

extract might have a different mode of action than commonly used antibiotics. Antimicrobial activity of these plants, and active constituents were reported by other researchers, main constituents found in the galls of *Q. infectoria* are tannin, gallic acid and ellagic acid (Maged and Mhnad, 1988). Ethanol extract of *C. zeylanicum* proved to be active curing agent, and eliminated plasmids from treated isolates Fig. 1 lane 7, might be due to its active materials of cinamaldehyde which derived from sinnamon essential oil (Lee *et al.*, 2004), and coumarins, pinen, eugenol, methyle-euginol, cinnzelanin and cinnzelanol (Dewick, 2002).The active ingredients of the bark contain antimicrobial, antiseptic, antiviral, antispasmodic and anti fungal properties (Wurges, 2008).

Flaxseed (*L. usitatissimum*) and its derivative flaxseed oil/linseed oil are rich sources of the essential fatty acid, alpha-linolinic acid, omega-3 fatty acid, phenolic acid, lignans and hemecellulose.The important constituents of flaxseeds acting as curing plasmids from treated bacteria Fig. 1 lane 8 are lignans that act as phytoestrogens (Aldercreutz and Mazur, 1997). However,

in the crude extract synergistic or additive antimicrobial activity due to other phytoconstituents is not ruled out. The curing ability of these plant extracts might be due to these active constituent and inhibited plasmid replication selectively of sub-MIC concentrations.

Further activity-guided fractionation of crude extracts is needed to determine the effective fractions of these crude extracts. On the other hand data on *in vivo* toxicity of these plants extracts, stability of active components and protection against infections caused by drug-resistant bacteria are to be generated to determine the theraptic potential of these plant extracts.

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