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Antibacterial activity of some plant extracts on *Escherichia coli* with special reference to its resistance pattern

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Abstract

The present work was planned to isolate, characterize and evaluate the prevalence of *E. coli* serotypes in 180 samples (herbs, water and chicken) as well as detection of haemolysin production, Congo Red binding activity, serum activity and antimicrobial susceptibility test. Forty four samples out of 180 samples were found to be positive to *E. coli* with a percentage of 24.4%. The serological identification showed that O125, O112 and O86 were the most prevalent serotype among the 44 isolated strains with percentages of 27%, 25% and 18% respectively. The study showed that all 44 *E-coli* isolates had the ability to bind with C.R. dye gave red colonies and were sensitive to bactericidal effect of human and sheep serum (100%). The results of haemolysis showed that the isolates belonged to O112 and 0114 serotypes had a hemolytic activity and the other serotypes had no hemolytic activity. The result of antibiotics sensitivity indicated that *E. coli* strains were highly resistant to penicillin and erythromycin (100%), followed by gentamicin, ampicillin, oxytetracycline and nalidixic acid with percentages of 81.8%, 75%, 47.7% and 15.9 %respectively. All *E. coli* strains were found to be moderately sensitive to ceftazidime (100%) and sensitive to ciprofloxacin (70.5%). The result of ethanolic plant extract showed that Coriander, Ginger, bay leaf, Black pepper, Chilly, Parsley and Turmeric did not show any bactericidal activity against *E. coli* strains. While Hibiscus, Thyme and Cinnamon showed intermediate bactericidal activity in concentration 100% .Moreover, Black cumin showed low bactericidal activity against herbs and water isolates only with 100% concentration.

Keywords: *E. coli*; serotype; antibiotic activity; plant extract; antibacterial activity.

1. Introduction

E. coli is a facultative anaerobic bacteria which is found in the gastrointestinal tract of mammals. It is never a predominant gut bacterium, but it can account for as many as 1% of colonic bacteria [1]. *E. coli* is a major pathogen of worldwide importance in commercially raised poultry, contributing significantly to economic losses in both turkeys and chickens. *E. coli* has

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been associated with a variety of diseases in birds including enteritis, arthritis, omphalitis, coligranuloma, septicemia, salpingitis, and complicated air sacculitis [2]. Ten to fifteen percent of the intestinal coliforms in chickens are of pathogenic serotypes [3]. Most strains of *E. coli* are harmless commensal organisms, but any *E. coli* can cause disease if it penetrates the gut mucosa and enterd the blood stream [4].

Grazing of herds on lands adjacent to sources of fresh water can lead to the accidental contamination of rivers, lakes and estuaries due to fecal runoff precipitated by heavy periods of rains. Fecal contamination of food and potable water sources with pathogenic *E. coli* can lead to devastating outbreaks of diarrheal disease [8, 9, 7, 32, 37]. The microbiological quality of dried herbs which were obtained from markets indicated that the fecal coliforms and *E.coli* levels exceeded the regulation levels [5, 6]. The use of antimicrobial agents in any venue, including therapeutically in human and veterinary medicine or as prophylaxis for growth promotion in animal husbandry, ultimately exerts selective pressure favorable for the propagation of antimicrobial resistant bacteria. The long term use of antimicrobials for therapy and growth promotion in animals selects for drug resistance in gram negative pathogens [10, 11]. There are several antimicrobials that have been approved for treatment of *E. coli* infections in broiler chickens including tetracycline and streptomycin [12]. Since the introduction of antibiotics, there has been tremendous increase in the resistance of diverse bacterial pathogens [13, 14]. This shift in susceptibility greatly affects our ability to successfully treat patients empirically. Plant derived products have been used for medicinal purposes for centuries. At present, it is estimated that about 80% of the world population rely on botanical preparations as medicines to meet their health needs. Herbs and spices are generally considered safe and proved to be effective against certain ailments. In recent years, in view of their beneficial effects, use of spices/herbs has been gradually increasing in developed countries also [15, 16]. Nowadays using antibiotics to subside infection produces adverse toxicity to host organs, tissues and cells. The toxicity produced by the antimicrobial agents can be cured or prevented or antagonize with herbs [16]. Herbal molecules are safe, will overcome the resistance produced by the pathogens since they are in combined form or in pooled form of more than one molecule in the protoplasm of the plant cell. Some herbs have antibacterial and antifungal properties which will be useful to clinical use [17, 18, 19]. The antimicrobial activity of herbs may differ between strains within the same species of bacteria so it is essential that the antibacterial effects of crude ethanolic extracts of herbs against several serotypes of *E.coli* to be investigated. Therefore, the objective of this study is to determine the antibacterial property of some ethanolic herbal extracts for a possible future application as natural anti-*E.coli* agents.

2. Materials and Methods

2.1. Samples

A total of 180 samples (102 herbs samples: 17 basil, 20 dry mints, 14 marjoram, 36 chamomile,11 fennel and 4 calendula flower samples), 40 chicken samples and 38 irrigated water samples from different places and farms were collected and analyzed for detection and isolation of *E. coli*.

2.2. Isolation of E. coli *from herbs and chicken*

Ten grams of each sample were mixed well under aseptic conditions with 90 ml diluents (maximum recovery diluents) to prepare the initial suspension. By mean of a sterile pipette one ml of the initial suspension was transferred to nine ml of maximum recovery diluents to make serial dilution; vortex mixer was used to

Table 1. Interpretation of zone diameter of inhibition among the antimicrobial agents used [28].

Number	Antimicrobial agents	Zone diameter (mm)		
		Resistance (R)	Intermediate (M)	Sensitive (S)
1	Ampicillin (AMP10)	<11	12 - 13	>14
2	Ceftazidine (CAZ30)	<14	15 - 17	>18
3	Ciprofloxacin (CIP5)	<15	16 - 17	>18
4	Erythromycin (E15)	<11	12 - 14	>15
5	Gentamicin (CN10)	<14	15 - 18	>19
6	Nalidixic acid (NA30)	<16	17 - 21	>22
7	Oxytetracycline (OT30)	<14	15 - 18	>19
8	Penicillin G (P10)	<13	14 - 15	>16

Table 2. Natural medical plants used

1. Cinnamon (*Cinnamomum cassia*)
2. Coriander (*Coriandrum sativum*)
3. Turmeric (*Curcuma longa*)
4. Ginger (*Zingiber officinale*)
5. Bay leaf (*Laurus nobilis*)
6. Black Pepper (*Piper nigrum*)
7. Chilly
8. Parsley (*Petroselinum sativum*)
9. Hibiscus
10. Thyme (*Thymus vulgaris*)
11. Black cumin (*Nigella sativa*)

Table 3. The incidence of *E. coli* among the examined 180 samples.

Sample	Herbs						Water	Chicken	Total
	Basil	Dry mint	Marjor-am	Chamo-mile	Fennel	Calen-dula flower			
No. of examined sample	17	20	14	36	11	4	38	40	180
%	9.5	11	8	20	6	2	21	22.5	100
No. of positive samples	5	8	1	12	1	2	2	13	44
%	29.4	40	7.1	33.3	9.1	50	5.3	32.5	24.4

Table 4. The percentages of sensitive and resistant 44 *E. coli* strains against 8 antibiotics discs.

Antimicrobial agents	Resistantñ		Intermediate		Sensitive	
	N0	%	no	%	no	%
Ampicillin	33	75	11	25	-	0
Erythromicin	44	100	-	0	-	0
Ceftazidime	-	0	44	100	-	0
Penicillin	44	100	-	0	-	0
Ciprofloxacin	13	29.5	-	0	31	70.5
Gentamicin	36	81.8	8	18.2	-	0
Oxytetracycline	21	47.7	20	45.5	3	6.8
Nalidixic acid	7	15.9	37	84.1	-	0

mix the tube. Surface technique was used according to NMKL [20] and ISO [21]. By using a sterile pipette 0.1 ml was added to a sterile Petri dish containing VRB with MUG medium. The inoculum was spreaded carefully over the surface of plate without touching the sides of the dishes with sterile spreader. The plate was leaved for about 15 minute in the ambient temperature for the inoculum to be absorbed into the agar. The plate was inverted and incubated at 44.5°C ± 0.5 for 24 hr. *E-coli* was indicated by a precipitate giving blue florescence under U.V. lamp 365 nm.

2.3 Isolation of E. coli from water [22]

100 ml of water sample were filtrated using membrane filter with vacuum pump; the membrane filter was placed on the violet red bile lactose agar with MUG. After filtration the plate was incubated at 44.5ºC ± 0.5 for 24 hour then the membrane was examined and all characteristic colonies were counted.

2.4 Biochemical and serological identification of the isolates [23]

By inoculation of the isolates in different selective plates and by biochemical confirmation using biochemical galleries (Microbact 24E).

2.5. Detection of virulence factors

- **Congo Red (C.R.) binding test [24]**

  Congo red positive (C.R +) was indicated by the development of red colonies.

  Congo red negative (C.R -) was indicated by the development of white colonies.

- **Serum resistance test [25]**

  Sheep and human blood was obtained from apparently healthy sheep and human and allowed to clot for 2 hours at room temperature. The serum was decanted, pooled and filtered through a membrane filter 0.45 µm. Buffer peptone water cultures of each *E. coli* isolate was incubated at 37ºC ± 1°C for 24 hours. Two tests were carried out; the first to detect the survival and the second to observe the growth of the organism in serum.

- **Detection of Haemolysin [26]**

  E. coli isolates were propagated on blood agar base supplemented with 5% washed sheep erythrocytes. Blood agar plates were then incubated at 37ºC for 24 hrs and colonies producing clear zones of hemolysis were then recorded as hemolysin positive.

2.6 Antibiotic susceptibility test [27]

The antimicrobial susceptibility testing was performed to the *E. coli* isolates using the disk diffusion technique .

2.7. Natural plant extract testing (Ethanolic extract) [29]

50 g of medical plants were added to 500 ml ethanol 70% and left for 2 days. Then the content was filtrated by using Buchner filter. The filtrate was added into Rota vapor to evaporate alcohol, and then left in the incubator at 44.5ºC to evaporate the remaining water. Concentrations of each extract (100, 50, and 25%) were prepared and the antimicrobial effect of each concentration was measured.

The hole plate diffusion technique was used according to Clark, *et al*. [30]. A Mueller Hinton agar plate was swabbed with a suspension of each *E. coli* isolate (44 isolates). The suspension was then matched with a McFarland 0.5 barium sulphate standard tube.

Three plugs of agar were removed from each agar plate using the back of a Pasteur pipette. Each concentration of every extract (100, 50, and 25%) was added into a hole and was allowed to diffuse at room temperature for 20 minutes. The plates were incubated aerobically overnight at 37°C. Each extract was tested against *E. coli* isolates and serotypes. The antimicrobial activity of 11 plant extracts was recorded as the mean diameter of the resulting inhibition zones of growth measured in millimeters.

The microbicidal activity was classified according to Baur, *et al*.(31) into resistant if the zone of inhibition in millimeter was less than 8, intermediate if the zone was 9-11 mm and sensitive if the zone was 12 or more.

3. Results

3.1. Incidence of E. coli among the examined herbs, water and chicken samples

A total of 180 samples from Basil, Dry mint, Marjoram, chamomile, Fennel, Calendula flower, water and chicken were examined for detection of *E. coli*. The result showed that 44 samples out of 180 samples were found to be *E. coli* with a percentage of 24.4% (table 3).

3.2. Identification of E. coli *isolates*

The results of the identification of 44 *E.coli* isolates indicated that 100% of *E .coli* showed characteristic metallic sheen colonies onto E.M.B agar medium, characteristic orange red colonies surrounded by a zone of precipitate onto HEA medium and characteristic yellow opaque colonies onto XLD agar medium among 44 florescence colonies onto violet red bile lactose MUG medium at 44ºC.

3.3. Serotyping of E. coli isolates recovered from herbs, water and chicken samples

The results of the serotyping of 44 *E-coli* isolates revealed that O125, O112 and O86 were the most prevalent serotypes among the 44 isolated samples with percentages of 27%, 25% and 18% respectively, followed by serotypes O127, O128, O114, O44 and O124 with percentage 11.5, 7%, 4.5%, 4.5% and 2.5% respectively.

3.4. Virulence tests of E. coli isolates

All 44 *E. coli* isolates were able to bind with Congo Red dye giving red colonies (100%).

More over, the result showed that all 44 *E. coli* isolates were sensitive to the bactericidal effect of both human and sheep serum (100%). On the other hand the results showed that the isolates with serotypes O112 and 0114 serotype had a hemolytic activity while the other serotypes had no hemolytic activity.

3.5. In vitro antibiotic sensitivity test using the disc diffusion technique on 44 strains of E. coli

All tested strains were highly resistant to penicillin and erythromycin with a percentage of 100%

and most of tested strains were resistant to gentamicin, ampicillin, oxytetracycline and nalidixic acid with percentages of 81.8%, 75%, 47.7% and 15.9% respectively. All tested strains were moderately sensitive to ceftazidime. On the other hand 70.5% of tested strains were sensitive to ciprofloxacin. Thirteen strains isolated from chickens had high resistance pattern against ampicillin, erythromycin, penicillin and ciprofloxacin with a percentage of 100% and gentamicin, oxytetracycline and nalidixic acid with a percentage of 92.3%, 53.8% and 38.5% respectively. Non of the chickens strain had resistance against ceftazidime.

The 29 strains isolated from herbs had resistance patterns against erythromycin and penicillin with a percentage of 100% and gentamicin, ampicillin, oxytetracycline and nalidixic acid with percentages of 75.9%, 65.5%, 48.3% and 6.9% respectively while non of the strains isolated from herbs had resistance against ciprofloxacin and ceftazidime. Moreover, the two strains isolated from water had resistance patterns against erythromycin, penicillin and gentamicin with a percentage of 100% and ampicillin with a percentage of 50% and non of strains isolated from water had resistance against ciprofloxacin, oxytetracycline, ceftazidime and nalidixic acid.

3.6. The antimicrobial effect of 11 plant extracts against 44 E. coli strains recovered from herbs, water and chicken samples.

The *in vitro* sensitivity test of 44 *E. coli* strains isolated from the examined herbs, water and chicken samples was done against 11 natural plant extracts. The results showed that ethanolic extracts of Coriander (*Coriandrum sativum*), Ginger (*Zingiber officinale*), bay leaf (*Laurus nobilis*), black pepper (*Piper nigrum*), Chilly, Parsley (*Petroselinum sativum*) and Turmeric (*Curcuma longa*) did not show any antibacterial activity against 44 *E. coli* strains with different concentrations of each extract (100, 50, and 25%). While Hibiscus, Thyme (*Thymus vulgaris*) and Cinnamon (*Cinnamomum cassia*) gave intermediate antimicrobial activity against the 44 *E. coli* strains in 100% concentration with zone diameters ranged between 9-11 mm while in 25 and 50% concentrations gave low antimicrobial activity against the 44 *E. coli* strains. Moreover, Black cumin (*Nigella sativa*) gave low antimicrobial activity against the 29 strains isolated from herbs and 2 strains isolated from water with concentration 100% only with zone diameter ranged between 3-5mm. It is of note that Black cumin (*Nigella sativa*) did not show any antibacterial activity against the13 samples isolated from chickens at any concentration.

4. Discussion

In the present investigation a total of 180 samples (102 herbs samples: (17 basil, 20 dry mint, 14 marjoram, 36 chamomile, 11 fennel and 4 calendula flower samples), 40 chicken samples from different organs, 38 irrigated water samples) from different places and farms were investigated bacteriologicaly to detect and isolate *E. coli.* As shown in Table (3) the incidence of *E. coli* among the examined samples was 44 out of 180 with a percentage of 24.4%. Moreover the incidences of *E. coli* in Basil, Dry mint, Marjoram, chamomile, Fennel, Calendula flower, Water and Chicken were 29.4%, 40%, 7.1%, 33.3%, 9.1%, 50%, 5.3% and 32.5% respectively. These results agreed with the results of Furlaneto [33] and Yadava, *et al.* [36] who isolated *E. coli* from herbs and the results indicated that the *E. coli* levels in basil (*Ocimum basilicum*) exceeded the regulation levels. Moreover, our results agreed also with McGowan *et al.* [34] and Srinivasan *et al.* [35] who isolated *E. coli* from water samples taken from environmental sites with percentages of 4% and 20% respectively.

The most prevalent serotypes among the 44 isolates were O125, O112 and O86 with a percentages of 27%, 25% and 18% respectively, followed by serotypes O127, O128, O114, O44 and O124 with percentages of 11.5, 7%, 4.5%, 4.5% and 2.5% respectively. These results were in agreement with Qazi, *et al*. [38] who isolated *E. coli* serogroups O86 from both fresh and frozen chicken. In contrast, most Indian and UK strains belonged to serogroup O86, O111 and O126 which have been associated with infantile diarrhea [24, 39, 40, 49]. The most common detected *E. coli* enteropathogens strains in patient with diarrhea were O114, O142, O127 O114 and O128 serogroups and also *E. coli* strains could be isolated from human diarrhea [41, 42, 46].

The ability to bind Congo Red (CR) dye in agar medium had been proposed as a marker for the invasive ability of several enteropathogens including *E. coli* [43, 47]. The data presented strongly suggested that all *E. coli* isolates were bounded with Congo Red dye and gave red colonies (100%), and our results agreed with those of Wani, *et al*. [44].

Our data revealed also that all tested strains were sensitive to the bactericidal effect of both human and sheep sera and this finding agreed with the finding of Maurer, *et al*.[52] who reported that most *E. coli* isolates (78%) were sensitive to killing by 12.5% human sera because oftheir sensitivity to human sera. The resistance to killing by complement was due to the O-antigen that caps the lipopolysaccharide of gram negative bacteria [53, 45].

The results of hemolytic activity reveled that the isolates belong to O112 and 0114 serotype had a hemolytic activity and the other serotype had no hemolytic activity with a percentage of 30%. These results were in agreement with Kon, *et al*.[54] who found that out of 94 *E. coli* tested 13.8% were haemolytic and the result indicated that a large proportion of *E. coli* O112, O114 were hemolytic (100%).

The antimicrobial agents were valuable tools to treat clinical diseases and to maintain healthy and productive animals. In addition to the human health concerns, antimicrobial resistant pathogens also pose a severe and costly animal health problem [56, 51, 55, 57, 59].

E. coli isolates were becoming resistant to some antimicrobials agents that had been recommended to control *E. coli*. [50, 58, 62, 63].

The results in Table (4) showed that all tested strains were highly resistant to penicillin and erythromycin with percentages of 100%. These results agreed with those of Wani, *et al*. [44] who found that the *in vitro* sensitivity profiles revealed that all isolates were resistant to penicillin and erythromycin which indicated the need for judicious use of antibiotics. Moreover all tested strains were resistant to gentamicin, ampicillin, oxytetracycline and nalidixic acid with percentages of 81.8%, 75%, 47.7% and 15.9% respectively. Resistance of *E. coli* to ampicillin was also recorded by Quendnau, *et al*. [60] who recorded a high percentages of resistant to ampicillin (81%) and oxytetracycline (63%). On the other hand all tested strain were moderately sensitive to ceftazidine with a percentages of 100% and also all tested strains were sensitive to ciprofloxacin with a percentages of 70.5%. Saleem, *et al*. [65] found that 100 *E. coli* isolates isolated from livers and hearts of chickens on 25 poultry farms were sensitive to ciprofloxacin.

In the current study, the 13 strains isolated from chicken had high resistant patterns against ampicillin, erythromycin, penicillin and

ciprofloxacin with a percentagesof 100% and gentamicin, oxytetracycline and nalidixic acid with percentages of 92.3%, 53.8% and 38.5% respectively and this resistance of chicken strains were also observed in the *E. coli* isolated from chickens which were fed on rations containing antibiotics [61, 66, 67, 68, 69]. Moreover, Gauthaman, *et al* [70] stated that the indiscriminate use of antibiotics had provided a selective pressure for the emergence of drug resistant strains of bacteria associated with poultry products.

Herbs and spices were generally considered safe and proved to be effective against certain microorganisms [15, 48]. Nowadays using antibiotics to subside infection could produce adverse toxicity to host organs, tissues and cells. Some herbs had antibacterial and antifungal properties which would be useful to clinical use [17, 18].

The *in vitro* sensitivity tests of 44 *E. coli* strains were conducted against 11 natural plant ethanolic extracts. The ethanolic extraction method gave an antimicrobial activity of herbs more than in aqueous extracts [71]. Oils of some plants such as pepper, turmeric and cinnamon were not extracted because of very small amount of oils in plant materials [72]. The obtained results showed that the crude ethanolic extracts of Coriander (*Coriandrum sativum*), Ginger (*Zingiber officinale*), Laurel, Pepper (*Piper nigrum*), Chilly, Parsley (*Petroselinum sativum*) and Turmeric (*Curcuma longa*) did not show any antimicrobial activity against all strains with different concentration of each extract (100, 50, and 25%). The observed resistance of *E. coli* probably could be due to cell membrane permeability or due to other genetic factors [73]. These results coincided with those observed by Indu,et al [74] who said that Ginger and Pepper extract did not show any antibacterial activity against all serogroups of *E. coli* except O8 and 088 which gave moderate antibacterial properties. However, the crude ethanolic extracts of Hibiscus, Thyme (*Thymus vulgaris*) and Cinnamon (*Cinnamon zeylanicum*) gave intermediate microbicidal activity against the 44 *E. coli* strains in concentration 100% with zone diameter ranged between 9-11mm , while in 25 and 50% concentrations it gave low antibacterial activity against all *E. coli* strains. Thymus vulgaris had antibacterial properties because the plant was rich in tannins, alkaloids, flavonoids, terpenoids and essential oils [75, 76, 73, 77]. Black cumin (*Nigella sativa*) gave low antibacterial activity against the 29 herbs isolates and 2 water isolates with 100% concentration only with zone diameter ranged between 3-5 mm. Moreover, Black cumin (*Nigella sativa*) did not show any antibacterial activity against 13 chicken isolates with different concentration of each herbal extract (100, 50, and 25%). These results revealed that there is differences in the sensitivity to plant extracts among the serogroups of *E. coli* and that finding coincided with Indu, *et al.* [74].

In conclusion, Some herbs proved to have an antimicrobial activity against the isolated *E. coli* strains. Further studies should be conducted at the molecular level and genetic engineering to separate the active principles of herbs and amplify them in large quantities.

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