Studies on isolation and inhibitory effect on food-borne pathogens from raw milk in Egypt by natural oils

Mahasen Mohamed Ahmed Ebaya[1], Maha Abdo Mohamed AL-Ashmawy[2], Mohamed Mohamed EL-Sokkary[3], Khaled Hussein Abd El Galil[4] and Rasha Mohamed Barwa[3*]

[1]Department of Pharmacy, Specialized Medical Hospital (SMH), Mansoura University, Mansoura, Mansoura, Egypt
[2]Department of Food Hygiene and Control, Faculty of Veterinary Medicine, Mansoura University, Mansoura, Egypt
[3]Department of Microbiology, Faculty of Pharmacy, Mansoura University, Mansoura, Egypt
[4]Department of Pharmaceutical Sciences, Faculty of Pharmacy, Beirut Arab University, Beirut, Lebanon

Abstract: This study sheds the light on the presence of (some) food-borne pathogens in raw market milk in Mansoura city, (Egypt) using several techniques for isolation and identification including serology and PCR. It determines, further, the susceptibility of the isolated pathogens to some antimicrobial agents and natural oils, including watercress, basil, parsley, and hot green pepper oils. From 100 milk samples, 22 *Escherichia coli* isolates harboured *stx1*, *stx2* and/or *eae* genes. Additionally, 17 *Listeria monocytogenes* (*L. monocytogenes*) isolates harboured *hylA* gene. Moreover, other related pathogens such as *Shigella flexneri* and *Klebsiella pneumoniae* were also detected. Antimicrobial susceptibility testing showed that *E. coli* strains were (completely) resistant to amoxicillin and sulfamethoxazole-trimethoprim but highly sensitive to gentamicin. *L. monocytogenes* strains showed complete resistance against oxytetracycline while the highest percentage of sensitivity was observed against norfloxacin. This study has also proved the following: *L. monocytogenes* was susceptible to all of the investigated oils, *Klebsiella pneumoniae* was sensitive to two types of oils, but *E. coli* and *Shigella flexneri* were resistant to all oils. In conclusion, it is risky to consume unpasteurized milk. Further, some natural oils (e.g. parsley and hot green pepper oils) can successfully be used as food additives to control the presence of some pathogens in milk.

Keywords: Antimicrobial agents, *E. coli*, *L. monocytogenes*, natural oils, raw milk.

INTRODUCTION

Milk and dairy products constitute some of basic components of human diet. Although, raw milk serves as a favourable growth medium for different microorganisms (Claeys *et al.*, 2013), its consumption has been popular.

Diarrheagenic *E. coli* strains, the commonly identified food-borne pathogens (Caine *et al.*, 2014), include shiga-toxin producing *E. coli* (STEC) that has the ability to produce one or more types of shiga-toxins such as shiga-toxin 1 encoded by *stx1* and shiga-toxin 2 encoded by *stx2* genes (Hussein and Sakuma, 2005). Additionally, protein intimin, which is encoded by the *eae* gene, represents one of the virulence factors commonly found in pathogenic STEC isolates and is responsible to the form of the lesions in gastrointestinal tract (IT) epithelial cells (Paton and Paton, 1998). The STEC transferred to healthy human, through contaminated raw milk, can cause many consequences including hemorrhagic colitis (HC), haemolytic-uraemic syndrome (HUS), hemorrhagic diarrhea and thrombotic thrombocytopenic purpura (TTP) (Gyles and Fairbrother, 2010).

Similarly, the consumption of milk contaminated with *L. monocytogenes*, another food-borne pathogen, can cause serious diseases such as listeriosis that affects particularly the risk populations and manifested clinically by septicaemia, encephalitis and meningitis (Pesavento *et al.*, 2010).

Nowadays, polymerase chain reaction (PCR) techniques are widely used for detection of pathogens in food due to its high specificity, rapidity and accuracy (Toze, 1999). Additionally, it is possible to detect simultaneously several genes including virulence genes via multiplex PCR by using more than one primer for amplification of DNA regions encoding different genes in each targeted bacterial strain (Touron *et al.*, 2005).

Food additives can be used for controlling food-borne pathogens. Since the synthetic food additives have been considered to be potentially harmful to human health, plant products attract the researchers' attention for their use as natural alternative antimicrobials.

Essential oils, obtained from aromatic plants and commonly added for enhancing the food taste and flavour, have been known to have antimicrobial properties (Burt, 2004). Watercress, parsley and basil oils are three examples of natural oils that were acclaimed as Ancient Egyptian and Arabian traditional medicine. In addition, hot green pepper is often used for preservation of some traditional dairy products in Egypt. Watercress and

**Corresponding author:* e-mail: rasha2000@gmail.com

parsley are widely used as salad ingredients and food additives. Traditionally, basil has been added to food as a flavouring agent and also has been used in some industries as medicines and perfumery (Telci *et al.*, 2006).

Therefore, this work aimed initially to isolate some food-borne pathogens (e.g. STEC, other related pathogens, and *L. monocytogenes*) from raw market milk, followed by their antimicrobial susceptibility, and reaching the final target i.e. the determination of some natural oils having antimicrobial effect.

MATERIALS AND METHODS

Samples collection

One hundred raw market milk samples were randomly and aseptically collected from different supermarkets in Mansoura city, Dakahlia (Egypt), and were delivered to the laboratory to follow studies.

Isolation of some pathogens from raw milk

Isolation of STEC and other related pathogens was directly performed by platting 0.1ml of each agitated sample onto sorbitol MacConkey's agar supplemented with tellurite and cefixime (SMAC-TC) plates. The plates were then incubated at 37^{o}C for 24 h. Furthermore, enrichment technique was performed for the isolation; 25 ml of each agitated sample were inoculated into 225 ml of tryptone soya broth (TSB) and then incubated for 24 h at 37°C; 0.1ml of the incubated enriched broth suspension was plated onto SMAC-TC plates and incubated for 24h at 37^{o}C (Roberts *et al.*, 1995). Non sorbitol fermenter colony (NSFC) is characterized as it has almost colourless appearance while sorbitol fermenter colony (SFC) is pink. In both direct and enrichment techniques, at least 5 characteristic colonies were picked up and subcultured on tryptone soya agar (TSA) and incubated at 37°C for 18-24 h. Afterwards, biochemical characterization was applied for all isolates.

For *L. monocytogenes*, raw milk (0.1ml) was directly platted onto selective agar plates of Oxford medium supplemented with Listeria selective supplement and incubated for 48h at 37°C. For enrichment of *Listeria* spp., 25 ml of each agitated sample was aseptically added to 225ml of TSB and incubated for 24 h at 37°C. After the enrichment step, 10 ml of the incubated homogenate were transferred into 90 ml of Listeria selective broth base supplemented with Listeria selective enrichment supplement and incubated for 48 h at 37°C; 100 µl of incubated homogenate was streaked onto Oxford agar plates supplemented with Listeria selective supplement and incubated for 48 h at 37°C (Roberts *et al.*, 1995). After incubation, the appearance of colonies with grey-green colour surrounded by black zones of asculine hydrolysis represented *L. monocytogenes*. The characteristic Listeria colonies were picked from all (Oxford agar) plates, purified using TSA, and all the isolates were biochemically characterized as *L. monocytogenes.*

Serological identification for STEC and other related pathogens

A serological identification of different isolates was done by rapid diagnostic *E. coli* antisera sets (Denka Seiken Co., Tokyo, Japan). Firstly, by slide agglutination; each isolate was tested for its agglutination for the diagnosis by OK polyvalent sera. Further serotyping was performed with appropriate OK monovalent sera once the pathogenic type was determined by the use of polyvalent sera (Kok *et al.*, 1996).

Molecular detection

Screening was carried out by using PCR technique for detection of common virulence genes including *stx1*, *stx2* and *eae* genes for STEC and other related pathogens and *hlyA* gene for *L. monocytogenes* using the oligonucleotide primer sets (table 1). DNA templates for each type of bacterium were prepared by transferring 2-3 bacterial colonies, which were overnight grown on nutrient agar plates, into 50 µl nuclease free water tube and heating at 90°C for 5 min. Then, centrifuged at 13000 rpm for 1 min and the resulting supernatant was used. For each gene, uniplex PCR was performed in 25 µl reaction volume consisted of 12.5µl of Dream Tag Green PCR Master Mix (Fermentas), 1µl (10 µM) of forward primer, 1 µl (10 µM) of reverse primer, and 1µl of template DNA. On the other hand, multiplex PCR for *stx2* and *eae* genes, in STEC and other related pathogens, was performed in 25 µl as total volume containing 12.5 µl of Dream Tag Green PCR Master Mix (Fermentas), 0.5 µl (10 µM) of forward primer, 0.5µl (10 µM) of reverse primer of each gene, and 2 µl of template DNA. In addition, multiplex PCR was applied for the simultaneous amplification of *eae* gene of presumed STEC and other related pathogens and *hlyA* gene of *L. monocytogenes* strains in 25 µl reactions with 12.5 µl Dream Tag Green PCR Master Mix, 1 µl of template DNA of each bacterial isolate, 0.5 µl (10 µM) of each primer pair of each gene. All PCR reactions were performed by Techne progene thermocycler under the following cycling conditions: an initial denaturation step at 95°C for 5 min followed by 40 cycles of denaturation at 95°C for 30 sec, annealing at 60°C for 30 sec, and extension at 72°C for 1 min then a final extension at 72°C for 5 min. All amplified products of PCR were analyzed by gel electrophoresis in 1.2% agarose containing ethidium bromide (10 mg/ml), compared with 100 bp DNA molecular weight ladder, visualized under UV transilluminator and photographed.

Antimicrobial susceptibility testing

Antimicrobial discs were used to determine the susceptibility of *E. coli* (positive for whether *stx1*, *stx2* and/or *eae* genes) and *L. monocytogenes* isolates on

Mueller-Hinton agar by using agar disc diffusion method. Antimicrobial susceptibility testing was carried out according to Clinical and Laboratory Standards Institute guidelines (CLSI 2006a, 2006b). The antimicrobial agents used were amoxicillin (30 µg), ampicillin (10 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), erythromycin (15 µg), gentamicin (10 µg), kanamycin (30 µg), nalidixic acid (30 µg), neomycin (30 µg), norfloxacin (10 µg), oxytetracycline (30 µg), penicillin G (10 IU), streptomycin (10 µg) and sulfamethoxazole-trimethoprim (23.75/1.25 µg). For more informative results, the multiple antibiotic resistance index (MARs index= Number of resistance antibiotics/Total number of antibiotics tested) was calculated for each resistant pattern (Singh *et al.*, 2010).

Experimental design for controlling some isolated pathogens by using some natural oils

Natural oils tested for their antibacterial effects

Four types of natural oils-obtained from markets in Mansoura city, Egypt-included watercress (*Nasturtium officinale*), basil (*Ocimum basilicum*), parsley (*Petroselinum crispum*) and hot green pepper oils.

Bacterial isolates used in the experimental study

Four different bacterial strains included *L. monocytogenes* (positive for *hylA* gene), *Klebsiella pneumoniae* (positive for *stx2* and *eae* genes), *E. coli* O_{111}:H_4 and *Shigella flexnei* (both were positive for *stx1*, *stx2* and *eae* genes).

Preparation of the bacterial inoculums

Bacterial inocula were prepared from 24 h culture on the selective media agar plates (Oxford media supplemented with Listeria selective supplement for *L. monocytogenes* and SMAC-TC for *Klebsiella pneumoniae*, *E. coli* and Shigella species). Cultures were directly suspended in sterile water and the optical densities of the four bacterial suspensions were adjusted to 0.5 MacFarland standards; these suspensions were diluted to the appropriate bacterial concentrations needed in the experiment by transferring 10 µl of each suspension to 990 µl of sterile water.

Antibacterial assay

For each one of the 4 oils, 4 sets of 14 sterile experimental eppendorf tubes-a set for each bacterial strain-were prepared by adding gradually increasing amounts of oil as the following: 10, 20, 30, 40, 50, 100, 150, 200, 250, 300, 350, 400, 450 and 500 µl. For each set of tubes, 10 µl of one of the diluted bacterial suspensions were transferred to each tube; then, the volumes of all tubes were completed to 1000 µl with sterile milk (media). In all sets, a positive control tube was prepared for each experimental tube by replacing the amount of oil by an equal amount of sterile water; in addition, one negative control tube (1000 µl sterile milk only) and a tube of each type of tested oils (1000 µl) were included in our experiment to ensure sterility of the media and the oils.

At zero time of the experiment, all of the tubes were shaken well and 100 µl of each tube were platted on selective agar plates (Oxford media supplemented with Listeria selective supplement agar plates for *L. monocytogenes* and SMAC-TC agar plates for *Klebsiella pneumoniae*, *E. coli*, and *Shigella* species) followed by incubation of all plates and eppendorf tubes for 24 h at 37°C.

After a moderately shaking step, 100 µl of each incubated tube were plated and the plates were incubated at 37°C for 24 h. The bacterial count in each incubated plate was determined to calculate percentage of reduction of bacterial count in each experimental plate relatively to its positive control plate by the following equation:

[(Positive control plate count-Experimental plate count)/ Positive control plate count] x 100

The lowest amount of oil that reduced relatively the bacterial count and the amount of oil that made the maximum reduction of this count were the results of high concern.

RESULTS

Isolation and identification of STEC and other related pathogens

In the current study, from 17 samples with a prevalence of 17% (17/100), 13 STEC and other related strains were isolated by direct technique while another 55 strains were isolated by enrichment technique. All of 68 isolates were biochemically and serologically identified. Consequently, it was proved that the enrichment technique was more productive than direct one especially for *E. coli* O_{111}:H_4, O_{55}:H_7 and O_{26} serogroups. Thirty one of sixty eight isolates were identified as belonging to the genus *Escherichia*; they were isolated from 15% of milk samples.

The present study revealed that O_{111}:H_4 was the most predominantly isolated serotype (22.5%) followed by O_{26} (12.9%), and O_{124} (12.9%). Twelve different serogroups of *E. coli* including ETEC (O_{125}:H_{21} and O_{128}:H_2), EHEC (O_{111}:H_4, O_{26}, O_{172}, and O_4), EPEC (O_{55}:H_7, O_{86}, O_1:H_7, O_{114}:H_{21}, and O_{119}:H_6), and EIEC (O_{124}) were detected.

Detection of stx1, stx2 and eae genes in different isolates

In this work, among 31 *E. coli* isolates, 22 (70.9%) exhibited whether *stx1*, *stx2* and/or *eae* genes (Fig. 1); these genes were respectively detected in 11 (34.1%), 12 (38.7%), and 20 (64.5%) of the *E. coli* isolates. Of 14 STEC isolates (carrying whether *stx1* and/or *stx2* genes), both *stx1* and *stx2* genes were detected in 9 isolates (64.2%), *stx2* gene was detected in 3 isolates (21.4%), and *stx1*gene was identified in 2 isolates (14%). In the present study, 64.5% of isolated *E. coli* exhibited *eae* genes. Moreover, both *stx2* and *eae* genes were amplified in

Table 1: Oligonucleotide primers for amplifying the targeted genes in *E. coli* and *L. monocytogenes*

Gene	Primer sequences (5'-3')	Product size (base pair)	Reference
eae	GCAACATGACCGATGACAAG ACCTCTGCCGTTCCATAATG	180	This study
stx1	TCCTGGTACAACTGCGGTTAC ACGCACTCTTCCATCTACCG	505	AL-Ashmawy, 2013
stx2	CTGGCGTTAATGGAGTTCAGTGG CCTGTCGCCAGTTATCTGACA	318	AL-Ashmawy, 2013
hlyA	ACTTCGGCGCAATCAGTGA TTGCAACTGCTCTTTAGTAACAGCTT	136	AL-Ashmawy *et al.*, 2014

Table 2: Antimicrobial resistance profile of *E. coli* exhibited whether *stx1*, *stx2* and/or *eae* genes (n=22)

E.coli serotype	No of isolates	Antimicrobial resistance profile	MAR index
O_{111} : H_4	1	AMX, SXT, P, E, N,OT, K, AMP, S, NA, NOR, C	0.857
	1	AMX, SXT, P, E, N,OT, K, AMP, S, NA, NOR	0.786
	1	AMX, SXT, P, E, N,OT, K, AMP, S, NA	0.714
	3	AMX, SXT, P, E, N,OT, K, AMP, S	0.642
	1	AMX, SXT, P, E, N,OT, K, AMP	0.571
O_{26}	1	AMX, SXT, P, E, N,OT, K, AMP, S, NA, NOR, C, CIP, CN	1
	1	AMX, SXT, P, E, N,OT, K, AMP, S, NA, NOR, C	0.857
	1	AMX, SXT, P, E, N,OT, K, AMP, S, NA	0.714
	1	AMX, SXT, P, E, N,OT, K, AMP, S	0.642
O_{55} : H_7	1	AMX, SXT, P, E, N,OT, K, AMP, S, NA, NOR	0.786
	2	AMX, SXT, P, E, N,OT, K, AMP, S	0.642
O_{125} :H_{21}	2	AMX, SXT, P, E, N,OT, K, AMP	0.571
O_{128} : H_2	2	AMX, SXT, P, E, N,OT, K	0.500
O_{172}	1	AMX, SXT, P, E, N,OT	0.428
	1	AMX, SXT, P, E	0.285
O_4	1	AMX, SXT, P	0.214
O_{119} : H_6	1	AMX, SXT	0.143
Average			0.607

MAR: Multiple Antimicrobial Resistance index; OT: Oxytetracycline; E: Erythromycin; NA: Nalidixic acid; P: Penicillin G; AMX: Amoxicillin; SXT: Sulfamethoxazole-trimethoprim; AMP: Ampicillin; S: Streptomycin; N: Neomycin; C: Chloramphenicol; NOR: Norfloxacin; CIP: Ciprofloxacin; K: Kanamycin; CN: Gentamicin.

35.4% of all *E. coli* isolates. Multiplex PCR was performed for the simultaneous detection of both *stx2* and *eae* genes (fig. 2).

Other related pathogenic isolates, harbouring *stx1*, *stx2* and/or *eae* genes, were also identified in this study such as *Shigella flexneri*, *Klebsiella pneumoniae*, *Serratia marcescens*, and *Enterobacter cloacae.*

Isolation and identification of L. monocytogenes

During the study, from 100 raw milk samples, 17 *L. monocytogenes* were isolated with a prevalence of 7% (7/100); these isolates were biochemically identified. Then, PCR identification of the specific gene was done (fig. 3). Four isolates were isolated by direct technique while 13 isolates were procured by enrichment technique. Accordingly, the enrichment technique showed more productivity than the direct one.

Multiplex PCR technique for *hlyA* gene of *L. monocytogenes* and *eae* gene of STEC and other related pathogens was performed for rapid detection (fig. 4).

Antimicrobial susceptibility testing

Susceptibility patterns of different *E. coli* isolates to several antimicrobial agents were illustrated in Fig. 5a. In the present study, *E. coli* isolates showed complete resistances to amoxicillin and sulfamethoxazole-trimethoprim however intermediate effects were detected to both nalidixic acid and chloramphenicol; the best sensitivity was also observed against gentamicin.

Susceptibility patterns of *L. monocytogenes* isolates against different antimicrobial agents are illustrated in Fig. 5b; complete resistance pattern of *L. monocytogenes* isolates to oxytetracycline was identified while high levels of resistance were detected against penicillin G and neomycin; moreover, intermediate effects against ampicillin and sulfamethoxazole-trimethoprim were observed; the high grade sensitivity was detected against norfloxacin.

In the current study, 86% of the *E. coli* isolates developed resistance to five or more antimicrobial agents (table 2); the highest multi-drug resistances were shown by serotypes O_{26} followed by O_{111}: H_4 and then O_{55}: H_7. A percentage of 82% of *L. monocytogenes* showed resistance to at least 5 antimicrobial agents in the current study (table 3).

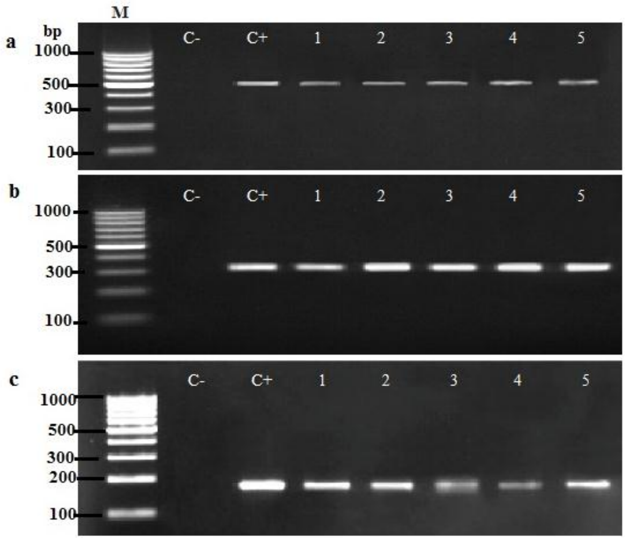


Fig. 1: Agarose gel electrophoresis of genes amplicons specific for shiga-toxin producing *E. coli* as detected by PCR method. Lane M: 100 bp DNA ladder, lane C-: negative control, lane C+: positive control (*E. coli* O157: H7, 16-CDC, Georgia - Meat), lanes (1-5) are representative runs for PCR results of 5 amplified DNA samples representing positive amplicon genes of: (a) *stx1* (b) *stx2* (c) *eae.*

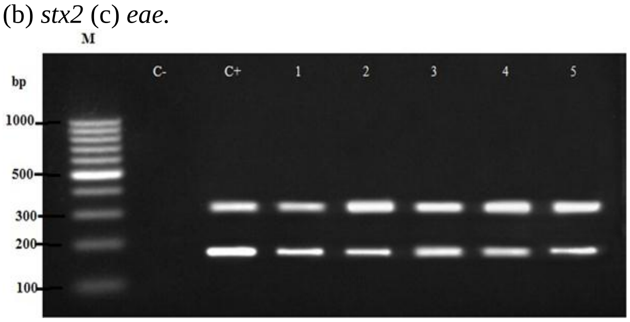


Fig. 2: Agarose gel electrophoresis of genes amplicons as detected by multiplex PCR, Lane M: 100 bp DNA ladder, lane C-: negative control for *eae* gene and *stx2* gene of STEC, lane C+: positive control (*E. coli* O_{157}: H_7, 16 - CDC, Georgia - Meat), Lanes (1-5) are representative runs for PCR results of 5 amplified DNA samples representing positive amplicons of both genes.

Experiment study of the antimicrobial effect of some natural oils

The natural oils used in this study included watercress, basil, parsley and hot green pepper oils. Our results showed that all of these oils exerted antimicrobial activity against *L. monocytogenes* while *Klebsiella pneumoniae* was affected only by parsley and hot green pepper oils; on the contrary, *E. coli* O_{111}:H_4 and *Shigella flexneri* resisted the effects of all the oils (tables 4 and 5). No growth was observed in the negative control and tested oils incubated plates while all positive control incubated plates showed growth.

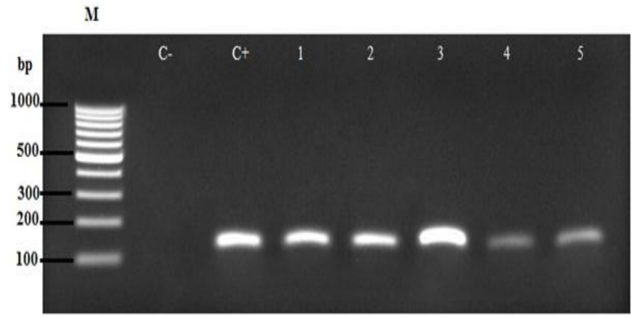


Fig. 3: Agarose gel electrophoresis of *hylA* gene amplicons specific for *L. monocytogenes* as detected by PCR method. Lane M: 100 bp DNA ladder, lane C-: negative control, lane C+: positive control (*L. monocytogenes*19115 - ATCC - human isolate), lanes (1-5) are representative runs for PCR results of 5 amplified DNA samples representing positive amplicon *hylA* gene.

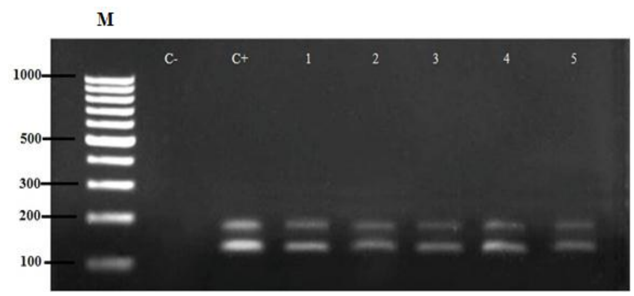


Fig. 4: Agarose gel electrophoresis of genes amplicons as detected by multiplex PCR, Lane M: 100 bp DNA ladder, lane C-: negative control for *hlyA* gene of *listeria monocytogenes* and *eae* gene of STEC, lane C+: positive control (*L. monocytogenes*19115 - ATCC - human isolate and *E. coli* O_{157}: H_7, 16 - CDC, Georgia - Meat) Lanes (1-5) are representative runs for PCR results of 5 amplified DNA samples representing positive amplicons of both genes.

To summarize, in the current work, the highest antimicrobial effect was recorded in parsley oil, and then in hot green pepper; followed by watercress and basil oils.

DISCUSSION

The more productivity of the enrichment technique than the direct one in our work may be attributed to that STEC may be present in stressed conditions such as food processing stress (preservatives, pH, temperature, etc.). Thus, it would be difficult to isolate these bacterial strains

Table 3: Antimicrobial resistance profile of *L. monocytogenes* (n=17)

No of isolates	Antimicrobial resistance profile	MAR index
1	OT, P, N, NA, K, C, E, AMX, S, SXT, CIP, AMP, CN, NOR	1
3	OT, P, N, NA, K, C, E, AMX, S, SXT, CIP, AMP	0.857
3	OT, P, N, NA, K, C, E, AMX, S, SXT, CIP	0.786
2	OT, P, N, NA, K, C, E, AMX, S, SXT	0.714
1	OT, P, N, NA, K, C, E, AMX, S	0.642
2	OT, P, N, NA, K, C, E, AMX	0.571
1	OT, P, N, NA, K, C	0.429
1	OT, P, N, NA, K	0.357
1	OT, P, N, NA	0.286
1	OT, P, N	0.214
1	OT	0.071
Average	0.618	

MAR: Multiple Antimicrobial Resistance index; OT: Oxytetracycline; E: Erythromycin; NA: Nalidixic acid; P: Penicillin G; AMX: Amoxicillin; SXT: Sulfamethoxazole-trimethoprim; AMP: Ampicillin; S: Streptomycin; N: Neomycin; C: Chloramphenicol; NOR: Norfloxacin; CIP: Ciprofloxacin; K: Kanamycin; CN: Gentamicin.

Table 4: Antibacterial activity of natural oils against the growth of *L. monocytogenes*

Type of natural oil	Quantity of natural oil (µl)													
	10	20	30	40	50	100	150	200	250	300	350	400	450	500
	Relative % of reduction of bacterial count													
Watercress	27.5	32.3	45.7	50.6	51.5	54.4	88.5	91.8	93.6	94.5	94.9	95.3	97.1	98.1
Basil	18	20.5	46.3	60.3	61.5	65.2	66.3	69.4	71.4	76	79.8	86	89.4	92.6
Parsley	18	24	50	55	60	69	77	79	83	89	91.5	93.5	94.5	96.6
Hot green pepper	44	48	54	61	69	70	77.7	81.1	89.1	91.5	92	93.1	94.5	96.1

without applying the enrichment step and false negative results may be obtained. For the enrichment *E. coli* O_{157} and other STEC serogroups, TSB is one of the most successful media (O'Sullivan *et al.*, 2007).

In our study, 31 isolates of the genus *Escherichia* were isolated from milk samples with prevalence 15%. A higher prevalence of *E. coli* were identified in other studies; 36.66% in Dakahlia (Gwida and EL-Gohary, 2013) and 57% in Tandojam city, Pakistan (Soomro *et al.*, 2002). The most important serogroups of *E. coli* are O_{157}:H_7, O_{26}, $O_{111,}$ O_{103}, O_{145} and O_{91} as stated by the Euro-pean Food Safety Authority report (EFSA, 2009). From these serogroups, O_{111}:H_4 was predominantly isolated followed by O_{26} in the current studyIn this work, both *stx1* and *stx2* genes, *stx2* gene, and *stx1*gene were respectively detected in 64.2%, 21.4%, and 14% of STEC isolates (carrying whether *stx1* and/or *stx2* genes). In another Indian study, the prevalence of STEC isolates containing both *stx*1 and *stx*2 genes was 54.8% while isolates only harbouring *stx1* gene represented a prevalence of 25.8% (Rasheed *et al.*, 2014). The high prevalence of *stx2* positive isolates in the current study may be of a public health concern because there is more important for *stx2* than *stx1* in HUS development (Nataro and Kaper, 1998). Outer membrane protein intimin represents one of the virulence factors commonly present in pathogenic STEC strains. In our study, 35.4% of all *E. coli* isolates were positive for both *stx2* and *eae* genes; the presence of both of these genes together could produce a synergistic effect (Boerlin *et al.*, 1999).

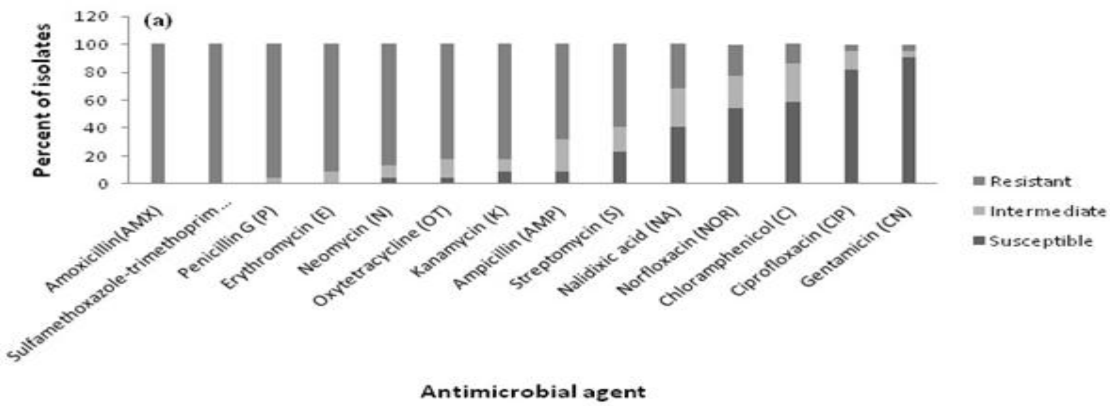
We can't ignore that *L. monocytogenes* often contaminating dairy products can produce serious diseases; this pathogen is characterized by the presence of *hlyA* gene encoding Listeriolysin O (LLO) (Mengaud *et al.*, 1988) that is considered as the most virulence factor associated with *L. monocytogenes* infection (Jay, 1996).
During our work, *L. monocytogenes* isolates were isolated with a prevalence of 7%. Several studies screening milk for the presence of *L. monocytogenes* were preformed. By way of illustration, it was previously reported in Cairo and Giza governorates, Egypt that *L. monocytogenes* was isolated from market raw milk samples with a prevalence of 2.55% (4/157) (Ahmed *et al.*, 2014). In contrast, other countries reported lower prevalence such as Iran and Austria where prevalence of 1.6% and 1.5% were respectively recorded (Moshtaghi and Mohammadpour, 2007; Deutz *et al.*, 1999).

On the contrary to our observations illustrated in (fig. 5a), *E. coli* isolates from raw milk samples in Dehradun

Table 5: Antibacterial activity of natural oils against the growth of *Klebsiella pneumoniae*

Type of natural oil	Quantity of natural oil (µl)													
	10	20	30	40	50	100	150	200	250	300	350	400	450	500
	Relative % of reduction of bacterial count													
Parsley	*	*	*	*	*	*	*	*	*	*	*	33.7	55	85
Hot green pepper	*	*	*	*	10	13.6	24	37	44.6	45.8	50.9	54.3	55	66.9

*no reduction in bacterial count



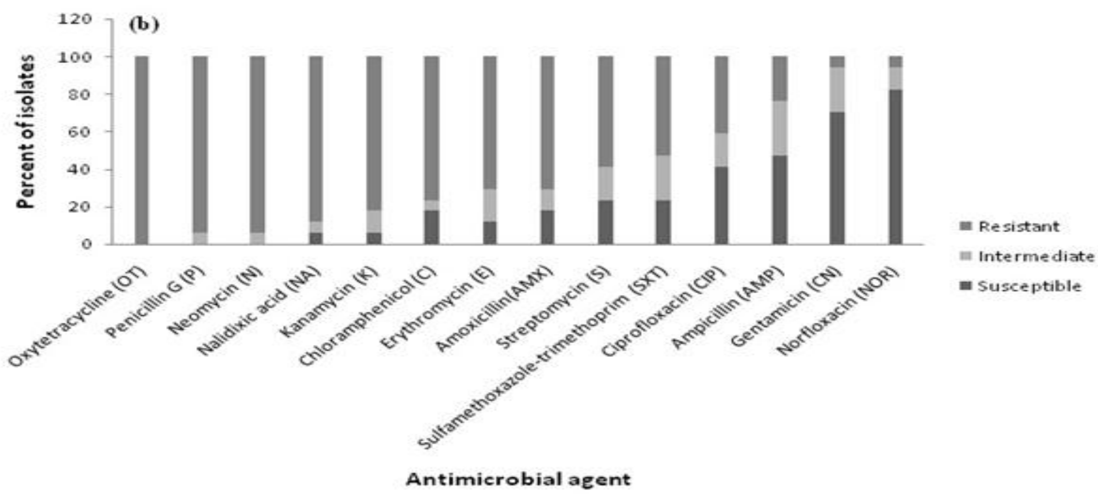


Fig. 5: Antimicrobial susceptibility pattern of (a) *E. coli* isolates exhibited whether *stx1*, *stx2* and/or *eae* genes (n=22) (b) *L. monocytogenes* (n=17).

exhibited sensitivity to streptomycin, erythromycin and penicillin; an intermediate effect was detected against chloramphenicol while a high level of resistance was identified against tetracycline (Pant *et al.*, 2013). In another study in Bangladesh, resistances were determined against tetracycline for all isolates of *E. coli* obtained from raw milk; in addition, 50% resistance against nalidixic acid was identified (Uddin *et al.*, 2011).

Susceptibility patterns of *L. monocytogenes* isolates against different antimicrobial agents (Fig. 5b) are in accordance with another study in Egypt (AL-Ashmawy *et al.*, 2014) in which *L. monocytogenes* isolates exhibited complete resistances against tetracycline, penicillin G and streptomycin; however, intermediate effects against ciprofloxacin and gentamicin were identified. In the same manner, in a previous study in Iran, all *L. monocytogenes* isolates from raw milk were sensitive to gentamicin and vancomycin while high resistance was witnessed against tetracycline and penicillin G (Jamali *et al.*, 2013).

The random use of antibiotics is considered as one of the main causes of multiple antibiotic resistance resulting in lowering the activity of commercially used antibiotics (Johnston *et al.*, 1983). In the present work, a percentage of 86% of the *E. coli* isolates showed resistance to five or more antimicrobial agents. While in Spain, 12% of non-

O_{157} *E. coli* strains, specifically serotypes O_{26}: H_{11}, exhibited resistance at least 5 antimicrobial agents (Mora *et al.*, 2005). In addition, *L. monocytogenes* is problematic and difficult to be treated due to its resistance to multiple antibiotics (Charpentier *et al.*, 1995). In our study, 82% of *L. monocytogenes* developed resistance to at least 5 antimicrobial agents. In another study in Egypt, 100% of *L. monocytogenes* isolates were multi-drug resistant for at least 8 antimicrobial agents (AL-Ashmawy *et al.*, 2014). In this respect, great awareness should be taken to reduce the misuse of antimicrobial agents.

Gram-positive bacteria are generally more sensitive than the Gram-negative (Hawkey, 1998); this may explain the sensitivity of *L. monocytogenes* to the antimicrobial effects of all the phyto oils used in this study while in case of *E. coli* and *Shigella* spp., high resistance patterns could be traced to the antimicrobial effects harboured by these oils.

In fact, the comparison between results of different studies-investigating the antimicrobial activities of plant oils-is highly intractable due to different methodologies used in these studies as well as the distinctions between herbal contents according to the geographical variations (Moghaddam *et al.*, 2011).

Our findings confirmed the better bioactivity and higher effectiveness of essential oils against Gram-positive bacteria than against the Gram-negative, this result is in agreement with those of other studies carried out on basil oil in different countries such as Iran (Prasad *et al.*, 1986), Spain (Lopez *et al.*, 2005) and Egypt (chenni *et al*., 2016). Our results agree also with another study reporting that parsley oil had only weakly or no inhibitory effect on *E. coli* (Elgayyar *et al.*, 2001). In the same way, the results of commercial oil of parsley and its aqueous and methanolic extracts displayed no antimicrobial activity against *E. coli* (El Astal *et al.*, 2005; Al-Talib *et al* 2016). In like manner, *E. coli* showed resistances to all the *n*-Hexane, acetone, ethanol, and aqueous extracts of watercress (Penecilla and Magno, 2001).

Contrary to our observations, another study reported the effectiveness of basil oil against *E. coli* O_{157}:H_7 and *Salmonella* as Gram-negative pathogens (Di Pasqua *et al.*, 2005). Moreover, basil also inhibited *Klebsiella pneumoniae* growth (Dostalova *et al.*, 2014). It was also reported that essential oils of 5 varieties of basil have mild antibacterial activity against *L. monocytogenes* (Lachowicz *et al.*, 1998). Other studies also disagree with ours stating that parsley had antimicrobial effect on *E. coli* (Vokk *et al.*, 2011) while its oil has weak or no inhibitory effect against *L. monocytogenes* (Elgayyar *et al.*, 2001).

As a result, these investigated oils can be used as helpful ingredients in the manufacture of some conventional dairy products to ensure safety and to control the pathogenic bacteria in these products.

CONCLUSIONS

Our finding revealed that raw milk is considered to be hazardous for consumption and in manufacturing of some dairy products without heat treatment. Moreover, some plant oils show antibacterial activities against some food-borne pathogens. Consequently, the use of some commercial herbal oils as natural food additives for milk could be beneficial in controlling some of the contaminating pathogens. This usage may be healthier than the usage of synthetic food additives.

ACKNOWLEDGEMENTS

We thank staffs of the Department of Microbiology, Faculty of Pharmacy, Mansoura University and Department of Food Hygiene and Control, Faculty of Veterinary Medicine, Mansoura University for their great support.

REFERENCES

Ahmed HS, Hamed OM, Ghoneim NH and Reda WW (2014). Public health significance of *Listeria monocytogenes* as a food-borne pathogen. *J. Egypt Vet. Med. Assoc.*, **74**(1): 23-37.

AL-Ashmawy MAM (2013). Prevalence of enterobacteriaceae in table eggs with particular reference to enterovirulent *Escherichia coli* strains. *Int. J. Poult. Sci.*, **12**(7): 430-435.

AL-Ashmawy MAM, Gwida M M and Abdelgalil KH (2014). Prevalence, detection methods and antimicrobial susceptibility of *Listeria monocytogens* isolated from milk and soft cheeses and its zoonotic importance. *World Appl. Sci. J.*, **29**(7): 869-878.

Al-Talib H, Ali NDM, Suhaimi MH, Rosli SSN, Othman NH, Mansor NAS, Shah AKS, Ariffin NS and Al-Khateeb A (2016). Antimicrobial effect of Malaysian vegetables against enteric bacteria. *Asian Pac J Trop Biomed.*, **6**(3): 211-215.

Boerlin P, McEwen S, Boerlin-Petzold F, Wilson J and Johnson R *et al.* (1999). Associations between virulence factors of shiga toxin-producing *Escherichia coli* and disease in humans. *J. Clin. Microbiol.*, **37**(3): 497-503.

Burt S (2004). Essential oils: Their antimicrobial properties and potential applications in foods- a review. *Int. J. Food Microbiol.*, **94**(3): 223-253.

Caine L, Nwodo UU, Okoh AI, Ndip RN and Green E (2014). Occurrence of virulence genes associated with diarrheagenic *Escherichia coli* isolated from raw cow's milk from two commercial dairy farms in the Eastern Cape Province, South Africa. *Int. J. Environ. Res. Public Health*, **11**(11): 11950-11963.

Charpentier E, Gerbaud G, Jacquet C, Rocourt J and Courvalin P (1995). Incidence of antibiotic resistance in *Listeria* species. *J. Infect. Dis*., **172**(1): 277-281.

Chenni M, El Abed D, Rakotomanomana N, Fernandez X and Chemat F (2016). Comparative Study of Essential Oils Extracted from Egyptian Basil Leaves (Ocimum basilicum L.) Using Hydro-Distillation and Solvent-Free Microwave Extraction. *Molecules*, **21**(1): E113.

Claeys WL, Cardoen S, Daube G, Block JD, Dewettinck K, Dierick K, Zutter LD, Huyghebaert A, Imberechts H, Thiange P, Vandenplas Y and Herman L (2013). Raw or heated cow milkconsumption: Review of risks and benefits. *Food Cont.,* **31(1)**: 251-262.

CLSI (2006a). Methods for antimicrobial dilution and disk susceptibility testing of infrequently isolated or fastidious bacteria: Approved guideline, CLSI document M45-A. Wayne, PA.

CLSI (2006b). Performance standards for antimicrobial disc susceptibility tests: Approved Standard-9th ed., CLSI document M2-A9. Wayne, PA.

Deutz A, Pless P and Koefer J (1999). Examination of raw cows and ewes milk for human pathogens. *Ernaehrung,* **23**: 359-362.

Di Pasqua R, De Feo V, Villani F and Mauriello G (2005). *In vitro* antimicrobial activity of essential oils from Mediterranean *Apiaceae*, *Verbenaceae* and *Lamiaceae* against foodborne pathogens and spoilage bacteria. *Ann. Microbiol.,* **55**(2): 139-143.

Dostalova L, Detvanova L and Kalhotka L (2014). Antimicrobial activity of aqueous herbal extracts. *Mendel Net*, pp.403-406.

El Astal ZY, Ashour A and Kerrit AAM (2005). Antimicrobial activity of some medicinal plant extracts in Palestine. *Pak. J. Med. Sci.,* **21**(2): 187-193.

Elgayyar M, Draughon F, Golden A and Mount J (2001). Antimicrobial activity of essential oils from plants against selected pathogenic and saprophytic microorganisms. *J. Food Prot.,* **64**: 1019-1024.

European Food Safety Authority (2009). Technical specifications for the monitoring and reporting of verotoxigenic *Escherichia coli* (VTEC) on animals and food (VTEC surveys on animals and food) on request of EFSA. *EFSA. J.,* **7(11)**: 1366-1408.

Gwida MM and EL-Gohary FA (2013). Zoonotic bacterial pathogens isolated from raw milk with special reference to *Escherichia coli* and *Staphylococcus aureus* in Dakahlia Governorate, Egypt. **2**(4): 705.

Gyles CM and Fairbrother JM (2010). *Escherichia coli*. In: Gyles CL, Prescott JF, Songer G and Thoen CO editors. Pathogenesis of Bacterial Infections in Animals, Blackwell Publishing, Ames, Iowa, pp.267-308.

Hawkey PM (1998). The origins and molecular basis of antibiotic resistance. *Br. Med. J.,* **317**(7159): 657-660.

Hussein HS and Sakuma T (2005). Prevalence of shiga toxin-producing *Escherichia coli* in dairy cattle and their products. *J. Dair. Sci.,* **88**(2): 450-465.

Jamali H, Radmehr B and Thong K (2013). Prevalence, characterization and antimicrobial resistance of *Listeria* species and *Listeria monocytogenes* isolates from raw milk in farm bulk tanks. *Food Cont*., **34**(1): 121-125.

Jay JM (1996). Foodborne listeriosis. *In*: Modern Food Microbiology, Chapman and Hall, New York, USA, pp.478-506.

Johnston DW, Bruce J and Hill J (1983). Incidence of antibiotic-resistant *Escherichia coli* in milk produced in the west of Scotland. *J. Appl. Bacteriol.,* **54**(1): 77-83.

Kok TW, Worswick D and Gowans V (1996). Some serological techniques for microbial and viral infections. In: Collee JG, Marmion BP, Fraser AG and Simmons A editors. Mackie and McCartney Practical Medical Microbiology, Churchill Livingstone, New York, USA, pp.179-384.

Lachowicz KJ, Jones GP, Briggs DR, Bienvenu FE and Wan J *et al.* (1998). The synergistic preservative effects of the essential oils of sweet basil (*Ocimum basilicum* L.) against acid-tolerant food microflora. *Lett. Appl. Microbiol.,* **26**(3): 209-214.

Lopez P, Sanchez C, Batlle R and Nerin C (2005). Solid and vaporphase antimicrobial activities of six essential oils: Susceptibility of selected food-borne bacterial and fungal strains. *J. Agric. Food Chem*., **53**(17): 6939-6946.

Mengaud J, Vicente MF, Chenevert J, Pereira JM, Geoffroy C, Gicquel-Sanzey B, Baquero F, Perez-Diaz JC and Cossart P .(1988). Expression in *Escherichia coli* and sequence analysis of the listeriolysin O determinant of *Listeria monocytogenes*. *Infect. Immun.,* **56**(4): 766-772.

Moghaddam AMD, Shayegh J, Mikaili P and Sharaf JD (2011). Antimicrobial activity of essential oil extract of Ocimum basilicum L. leaves on a variety of pathogenic bacteria. *J. Med.Plants Res.,* **5**: 3453-3456.

Mora A, Blanco JE, Blanco M, Alonso MP, Dhabi G, Echeita A, González EA, Bernárdez MI and Blanco J. (2005). Antimicrobial resistance of Shiga toxin (verotoxin)-producing *Escherichia coli* O_{157}:H_7 and non-O_{157} strains isolated from humans, cattle, sheep and food in Spain. *Res. Microbiol.,* **156**(7): 793-806.

Moshtaghi H and Mohammadpour AA (2007). Incidence of *Listeria* spp. in raw milk in Shahrekord (Iran). *Foodborne Pathog. Dis.,* **4**(1): 107-110.

Nataro JP and Kaper JB (1998). Diarrheagenic *Escherichia coli*. *Clin. Microbiol. Rev.,* **11**(1): 142-201.

O'Sullivan J, Bolton DJ, Duffy G, Baylis C and Tozzoli R, Wasteson Y and S. Lofdahl S (editors) (2007). Verocytotoxigenic *E. coli*. *In*: Methods for Detection and Molecular Characterisation of Pathogenic *Escherichia coli*, Ashtown Food Research Centre, Dublin, pp.13-25.

Pant R, Nirwal S and Rai N (2013). Prevalence of antibiotic resistant bacteria and analysis of microbial quality of raw milk samples collected from different regions of Dehradun. *Int. J. PharmTech. Res.,* **5**(2): 804-810.

Paton JC and Paton AW (1998). Pathogenesis and diagnosis of Shiga toxin-producing *E. coli* infections. *Clin. Microbiol. Rev.,* **11**(3): 450-479.

Penecilla GL and Magno CP (2001). Antibacterial activity of extracts of twelve common medicinal plants from the Philippines. *J. Med. Plants Res.,* **5**(16): 3975-3981.

Pesavento G, Ducci B, Nieri D, Comodo N and Lo Nostro A (2010). Prevalence and antibiotic susceptibility of *Listeria* spp. isolated from raw meat and retail foods. *Food Cont.,* **21**(5): 708-713.

Prasad G, Kumar A, Singh AK, Bhattacharya AK, Singh K and Sharma VD (1986). Antimicrobial activity of essential oils of some *Ocimum* species and clove oil. *Fitoterapia.,* **57**(6): 429-432.

Rasheed MU, Jamil K, Thajuddin N, Pasupuleti M, Ahamed P and Muthukumaresan KP (2014). Distribution of the *stx1*, *stx2* and *hlyA* genes: Antibiotic profiling in shiga-toxigenic *E. coli* strains isolated from food sources. *Int. J. Curr. Microbiol. App. Sci.,* **3**(5): 348-361.

Roberts D, Hooper W and Greenwood M (1995). Isolation and enrichment of micro-organisms. *In*: Practical Food Microbiology, Public Health Laboratory Service, London, UK, pp.131-168.

Singh S, Yadav A, Singh S and Bharti P (2010). Prevalence of *Salmonella* in chicken eggs collected from poultry farms and marketing channels and their antimicrobial resistance. *Food Res. Int.,* **43**(8): 2027-2030.

Soomro AH, Arain MA, Khaskheli M and Bhutto B (2002). Isolation of *Escherichia coli* from raw milk and milk products in relation to public health sold under market conditions at Tandojam. *Pak. J. Nutr.,* **1**(3): 151-152.

Telci I, Bayram E, Yilmaz G and Avici B (2006). Variability in essential oil composition of Turkish Basils (*Ocimum basilicum* L.). *Biochem. Syst. Ecol.,* **34**(6): 489-497.

Touron A, Berthe T, Pawlak B and Petit F (2005). Detection of *Salmonella* in environmental water and sediment by a nested-multiplex polymerase chain reaction assay. *Res. Microbiol.,* **156**(4): 541-553.

Toze S (1999). PCR and the detection of microbial pathogens in water and wastewater. *Water Res*., **33**(17): 3545-3556.

Uddin MA, Motazzim-ul-Haque HM and Noor R (2011). Isolation and identification of pathogenic *Escherichia coli*, *Klebsiella spp.* and *Staphylococcus spp.* in raw milk samples collected from different areas of Dhaka city, Bangladesh. *Stamford J. Microbiol.,* **1**(1): 19-23.

Vokk R, Lõugas T, Mets K and Kravets M (2011). Dill (*Anethum graveolens* L.) and parsley (*Petroselinum crispum* (Mill.) Fuss) from Estonia: Seasonal differences in essential oil. *Agron. Res*, **9**(2): 515-520.