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Antibacterial Activity of *Origanum majorana* and *Curcuma longa* Extracts against Multiple Drug-resistant Pathogenic *E. coli* and Methicillin-resistant *Staphylococcus aureus* Isolates Recovered from Meat Products

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ABSTRACT

The presence of antimicrobial-resistant foodborne pathogens in our food can threaten our life. Thus, great attention was paid to find a potentially effective, safe, and natural antimicrobial agent. Therefore, this study carried out this study to in-vitro investigate the potential use of *Origanum majorana* and *Curcuma longa* as natural antimicrobial food additives to control multidrug-resistant foodborne pathogens isolated from minced beef and beef burger samples. Herein, we examined 100 raw meat product samples, i.e., 50 each of fresh minced beef and frozen beef burger samples, randomly collected from butcher's shops in Egypt, for the presence of pathogenic *E. coli* and *S. aureus*. Pathogenic *E. coli* was detected in 36 % of examined minced beef samples, while we failed to isolate it from beef burger. On the other hand, *coa*gulase-positive *S. aureus* was found in 26 and 10 % of minced beef and beef burger samples, respectively. The multiple-drug resistant (MDR) isolates of *E. coli* and *S. aureus* were identified using 11 and 6 commercial antimicrobial discs, respectively. MDR isolates were selected for molecular identification based on virulence and anti-microbial resistance genes. Molecularly, *eaeA* gene was detected in 100% of identified pathogenic *E. coli* strains, while *stx1* was detected in one strain only. Whereas *mecA* and *coa* were detected in 100% of *coa*gulase-positive *S. aureus* isolates, The antimicrobial effectiveness of *Origanum majorana* (OM) and *Curcuma longa* (CL) ethanolic extracts against isolated MDR pathogens were evaluated. OM and CL were potentially effective against MDR *coa*gulase-positive *S. aureus* with variable inhibition zones ranged from 2mm to25mm. While they did not inhibit pathogenic *E. coli* strains (O158, O157, O114, O142, O44, O86, O25). Extracts of OM and CL were proved to be potentially effective against MDR *coa*gulase-positive *S. aureus*, and can be used as a natural alternative food preservative to control *S. aureus* growth in food in place of chemical antimicrobial agents.

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Introduction

More than two million people die each year because of foodborne diseases (Winias, 2011). Foodborne illnesses and food poisoning affect public health globally as they lead to countless premature deaths, several health complications, and massive losses in productivity (WHO, 2007). The situation worsens in developing countries due to difficulties in achieving optimum hygienic food handling practices (Schlundt *et al.*, 2004; Newell *et al.*, 2010; Badi *et al.*, 2018).

Staphylococcus (S.) *aureus* is a major foodborne pathogen that causes a wide variety of diseases fluctuating in severity from somewhat skin infection to more severe diseases (Lowy, 1998). *S. aureus* can grow at 15 °C to 45 °C, and at high salt concentration reaches to 15% (Behling *et al.*, 2010). S aureus is associated with 241000 illnesses of foodborne disease in USA (Scallan *et al.*, 2011; Wu *et al.*, 2018), which gives rise to foodborne illness such as vomiting, gastroenteritis, and even systemic shock in primates (Hennekinne *et al.*, 2012), Also it can adapt to the changes in the environmental conditions and rapidly acquires resistant to approximately all antibiotics (Mccallum *et al.*, 2010). Several studies have reported MDR S aureus in food poisoning outbreaks and it was isolated from several food products (Gharsa *et al.*, 2012; Papadopoulos *et al.*, 2018). Furthermore, *E. coli* has been implicated in countless foodborne illness outbreaks. Foodborne illnesses and gastrointestinal diseases reaching epidemic proportions were related to pathogenic *E. coli* strains (Fratamico *et al.*, 2014), such as hemorrhagic colitis and hemolytic uremic syndrome, which are severe complications that can lead to death (Markland *et al.*, 2015).

The misusage of antimicrobials in animal and poultry production leads to the evolution of multiple antibiotic-resistant bacteria during the last few decades, besides it clues to the spread of resistant foodborne pathogens (Teuber, 2001; Zdolec, 2016).

The food contamination with antibiotic-resistant bacteria could be a major threat to public health because antibiotic resistance can be transferred to other pathogenic bacteria, causing a compromise in the treatment of severe infections

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(Hassan *et al*., 2019). On the other hand, the continual application of chemical preservatives caused an accumulation of chemical hazards in the food and feed chain (Akinyemi *et al*., 2006)

During the last two decades, growing evidence that plants are fabulously rich with antimicrobial natural substances acting as protective systems against biotic and abiotic stresses (Nabavi *et al*., 2015). Moreover, secondary plant metabolites are mostly known as generally recognized as safe compounds for food products (Simoes *et al*., 2009).

Therefore, a great concern has been paid to herbal plants, which are characterized as a rich source of natural bioactive elements with health-promoting activities and have no hazardous effect, as an alternative source of antimicrobial substances (Nabavi *et al*., 2015; Hassan and Cutter, 2020). Accordingly, several studies have demonstrated the antibacterial effect of herbal extracts such as thyme, cinnamon, Rhus coriaria, Punica granatum, Indigofera, daleoides. Citrus limon, Illium sativum, Punica grantum, and Persea americana against food poisoning pathogens (Delgado *et al*., 2004; Nasar-Abbas and Halkman, 2004; Mathabe *et al*., 2006; Verma *et al*., 2012; Akinpelu *et al*., 2015).

Turmeric is a spice that comes from the root of curcuma longa, a member of the ginger family, Zingiberaceae. It is a bright yellow in color and has been used as a coloring agent in food, as a spice, food preservative, and in medical applications (Luthra *et al*., 2001). Curcuminoids are the main bioactive portion of turmeric that include mainly curcumins, bisdemethoxycurcumin and demethoxycurcumin (Chainani-Wu, 2003). These bioactive compounds are ethanol and acetone-soluble but not water-soluble. They have antioxidant, anti-inflammatory, hypocholesterolemic, antibacterial, and antifungal effects (Peter, 1999). Furthermore, the presence of tannin, flavonoids, and saponin in turmeric plants enriching its medical effect. As tannin has been reported to hinder microbial growth by precipitation of microbial protein, iron destitution, and interaction with vital protein (Iniaghe *et al*., 2009).

Additionally, *Origanum majorana*, known as sweet majorana, which belongs to the family Lamiaceae is a perennial herbaceous plant. Origanum genus consists of 38 species among them 75% are restricted to the Eastern Mediterranean area. They are characterized by a wide range of volatile secondary metabolites. It is commercially used as a spice and condiment and used in perfumes (Vera and Chane-Ming, 1999). Marjoram plant and their extracts have relatively strong antioxidant and antibacterial activities due to their content of several bioactive compounds such as polyphenols, sabinene, and terpinene, which have been recognized as antioxidant and antibacterial compounds (Roby *et al*., 2013), in addition to flavonoids, steroid, and coumarins (Cseke *et al*., 2016).

Although the aforementioned promising properties of turmeric and majorana, the evaluation of their antibacterial activities against antibiotic-resistant bacterial meat isolates is infrequent. Therefore, the current study aimed at isolation and identification of pathogenic *E. coli* and *coa*gulase-positive *S. aureus* from two retail meat products (minced beef and beef burger) marketed in Egypt, as well as to investigate the antimicrobial resistance/susceptibility of isolates. Furthermore, to evaluate the antimicrobial efficiency of turmeric and marjoram extracts against isolated pathogenic strains.

Materials and methods

Chemicals and growth media

All bacterial growth media were obtained from Oxoid (Hampshire, United Kingdom) unless otherwise was mentioned. Novobiocin was purchased from Lab M (Lancashire, United Kingdom). Antimicrobial discs used for antimicrobial resistance testing were purchased from Oxoid (UK).

Investigating the prevalence of pathogenic E. coli and S. aureus in meat samples

Sample collection

Fifty fresh minced beef samples and 50 frozen beef burger samples were randomly collected from local butcher shops and supermarkets in El-minia and Beni-Suef governorates, Egypt, and transported immediately in an insulated icebox under aseptic condition to the laboratory of Bacteriology, Mycology and Immunology Faculty of Veterinary Medicine, Beni-Suef University, Egypt.

Isolation and Identification of bacterial pathogens

Regarding isolation of pathogenic *E. coli*, 25 g of each meat product sample was diluted with 225 mL from modified tryptone soya broth (mTSB) (Oxoid, UK) with novobiocin (Lab M) (UK) (10 mg/L) and homogenized in a homogenizer (JP Selecta SE-100) for 5 min at 2000 rpm. Then the broth containing the sample was incubated at 42 °C for 18 h. Subsequently, a loopful was streaked onto cefixime tellurite sorbitol MacConkey agar (CT-SMAC) plates and incubated at 37 °C for 24 h (ISO 16645, 2001). The identification was carried out by culture characteristics and bacterial films stained with Gram's technique. Identification of the recovered isolates was done according to Quinn *et al*., (2002). The colonies showing typical aspects of *E. coli* were confirmed by oxidase, indole, methyl red, and citrate utilization test. The cultures, displayed the characteristic biochemical pattern of *E. coli*, were kept for further serological identification.

All *E. coli* suspected isolates, based on the morphological and biochemical identification, were serologically identified based on the somatic antigen (O) by slide agglutination method using polyvalent and monovalent antisera.

Whereas, in the case of *S. aureus*, the isolation process was conducted according to the method described in Datta *et al*., (2012). Ten g from each meat product sample was taken into a sterile jar containing 90 mL of buffered peptone water, and homogenized for 3 min at 2000 r.p.m. Then the mixture was left to stand for 5 minutes at room temperature, and subsequently, each sample was subjected to 10-fold serial dilution then a 100 µL of each dilution was spread onto solid Baird parker agar medium supplemented with egg yolk tellurite, then was incubated aerobically at 37 °C for 48 h. Colonies that showed typical characters of *coa*gulase-positive *S. aureus* was subjected to Gram`s staining, and biochemical identification with catalase test, and *coa*gulase test (Quinn *et al*., 2002).

Antimicrobial susceptibility testing (AST) of recovered isolates

All biochemically and serologically confirmed isolates of pathogenic *E. coli* and *S. aureus* were tested for their antimicrobial resistance pattern using the Kirby–Bauer disc diffusion technique. Antimicrobial sensitivity testing was done on a Muller Hinton agar medium using commercial antimicrobial discs soaked in tetracycline (TE, 30 µg), streptomycin (S, 10 µg), imipenem (IPM, 10 µg), sulphamethoxazole/trimethoprim (SXT, 25 µg), ciprofloxacin (CIP, 5 µg), gentamicin (GN, 10 µg), cefoxitin (FOX, 30 µg), amoxycillin/ clavulanic acid (AMC, 30 µg), ampicillin (AMP, 10 µg), cefotaxime–clavoran (CTX, 30 µg), and nalidixic acid (NA, 30 µg) for pathogenic *E. coli* isolates While in the case of *S. aureus* isolates, the resistance pattern was investigated using discs of tetracycline (TE, 30 µg), nalidixic acid (NA, 30 µg) erythromycin (E, 15 µg), azithromycin

(AZM, 15 µg), clindamycin (DA, 2 µg), and rifampin (RA, 5 µg). The degree of sensitivity was interpreted according to the Clinical Laboratory Standard Institute (CLSI, 2017), as well as multidrug-resistant (MDR) isolates were identified.

Molecular identification of virulence and antimicrobial resistance-associated genes in the recovered isolates

MDR isolates were selected for molecular identification of their resistance- and virulence-related genes using PCR. Genomic DNA was extracted from overnight bacterial cultures using Qiagen DNA extraction kit (Qiagen, Germany) according to the manufacturer's instructions. Specific primers obtained from Metabion (Germany) for each target gene were used for DNA amplification using uniplex PCR. The sequences of primers and sizes of amplified segments are listed in Table 1. Primers were utilized in a 25 µL reaction tube containing 12.5 µL of Emerald Amp Max PCR Master Mix (Takara, Japan), 1 µL of each primer of 20 pmol concentrations, 6 µL of DNA template, and 4.5 µL of nuclease-free water. The reactions were performed in an Applied Biosystem 2720 thermal cycler. Briefly, the initial denaturation step was done at 94 °C for 5 min, then followed by 35 cycles of 94 °C for 45 sec, afterwards, annealing was applied according to the conditions shown in Table 1. Subsequently, an extension step at 72 °C for 45 sec and a final extension step at 72 °C for 10 min was conducted. The products of PCR were separated by electrophoresis on 1.5% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature using gradients of 5V/cm. Twenty µL of the PCR products were loaded in each gel slot. The fragment sizes were determined using Gelpilot 100 bp and 100 bp plus DNA Ladders (Qiagen, Germany, GmbH) and gene ruler 100 bp ladder (Thermo Scientific, Germany). Afterwards, the gel was photographed by a gel documentation system (Alpha Innotech, Biometra).

Evaluating the antimicrobial activity of herbal extracts against isolated pathogens

Preparation of selected herbal extracts

According to the percolation method for herbal extraction, 100 g of dry leaf powder of each of turmeric (*Curcuma longa*) and marjoram (*Origanum majorana*) were added to a clean flask contains 1000 mL of ethanol 70% with thorough shacking. Afterward, the plant was kept soaked in ethanol 70% for 24 h and then percolated several times until the soaking solution be faint to clear in color. After that, the obtained extract was filtered through a number 1 filter paper (Whatman) and concentrated under reduced pressure in a rotary evaporator (Shimadzu, Germany) to remove the solvent, and then stored at 4 °C until use (Singh *et al*., 2017).

Determination of the antimicrobial activity of herbal extracts using well diffusion method

The well diffusion method previously described by Mostafa *et al*. (2018) was used. The dry herbal extracts of turmeric and marjoram were used to prepare different concentrations (1.25, 2.5, 5, and 10 %) of extract solution in ethanol 70% and distilled water, respectively, then were sterilized through filter 0.45 µm. Susceptibility of the isolated pathogens to plant extracts was determined using a well diffusion assay on Muller Hinton agar plates, according to described method on NCCLS manual (NCCLS, 2003). Bacterial cultures were adjusted to 0.5 McFarland turbidity (1-2 x 106 CFU /mL) and spread onto the entire surface of the agar plates, then allowed to air-dry for 10 min before wells (6 mm holes) were cut into the agar by using sterile plastic tips. Individual wells were filled with 50 µL of different concentrations of plant extract solution, before the plates being left to dry. Afterward, the plates were incubated at 37 °C for 24 h, the diameter of inhibition zones were recorded. Sterile distilled water was used as a control negative, while ampicillin (10 µg) and cefoxitin (30 µg) antimicrobial discs were used as control positive for *S. aureus* and *E. coli*, respectively. Each concentration was replicated at least three times.

Results

Prevalence of E. coli and S. aureus in raw meat products

The data illustrated in Table 2 showed that 36% of examined minced beef contained *E. coli* strains, whereas the beef burger did not contain *E. coli* strains. On the other hand, 26% and 10% of minced beef and beef burger samples contained *coa*gulase-positive *S. aureus*, respectively.

Out of 50 examined minced beef samples, 18 isolates of pathogenic *E. coli* were obtained and serologically identified. The serological identification revealed that *E. coli* O157, O158, O114, O142, O44, O86, O25 represented 5.5%, 33.3%, 33.3%, 5.5%, 5.5%, 5.5% of *E. coli* isolates, respectively, while one isolate was untyped (Table 3).

Antimicrobial resistance/susceptibility of isolated pathogens

The result of *E. coli* sensitivity test in Table 4 showed that the isolates revealed various degrees of resistance, as 94.1%, 75.4%, 41.1%, 29.4%, 64.7%, 17.6%, 29.4%, 11.7%, of the isolates were resistant to tetracycline, streptomycin, sulphamethoxazole/trimethoprim, ciprofloxacin, gentamicin, amoxicillin/clavulanic acid, ampicillin, cefotaxime-clavoran, respectively.

On the other side, showed a high sensitivity pattern to

Table 1. Primer sequences of target genes, length of the amplified product, and annealing temperatures

Target microorganism	Target genes	Primers sequences (5' – 3')	Amplified segment (bp)	Resistance/ Virulence*	Annealing temperature (°C) / Time (sec)	References
E. coli	*eaeA*	ATGCTTAGTGCTGGTTTAGG GCCTTCATCATTTCGCTTTC	248 bp	(V)	51°C/30 sec.	Bisi-Johnson *et al*. (2011)
	stx1	ACACTGGATGATCTCAGTGG CTGAATCCCCCTCCATTATG	614 bp	(V)	58°C/40 sec.	Dipineto *et al*. (2006)
S. aureus	*coa*	ATAGAGATGCTGGTACAGG GCTTCCGATTGTTCGATGC	630 bp	(V)	55°C/40 sec.	Iyer and Kumosani (2011)
	mecA	GTAGAAATGACTGAACGTCCGATAA CCAATTCCACATTGTTTCGGTCTAA	310 bp	(R) Methicillin-resistant *S. aureus* (MRSA)	50°C/30 sec.	McClure *et al*. (2006)

* The role of the target gene, either antibiotic resistance or virulence activity. (V) means that the target gene is responsible for specific virulence activity of the strain. While (R) means that the target gene is responsible for antibiotic resistance.

Table 2. Prevalence of *E. coli* and *coa*gulase-positive *S. aureus* in minced beef and beef burger samples

Samples	*E. coli*		Coagulase-positive *S. aureus*	
	No. of positive samples	%	No. of positive samples	%
Minced beef (n=50)	18	36	13	26
Beef burger (n= 50)	0	0	5	10
Total	18	36	18	36

Table 3. Serotyping of 18 *E. coli* isolates recovered from minced beef samples

Serotypes	No.	%*	%**
E. coli O157	1	5.5	2
E. coli O158	6	33.3	12
E. coli O114	6	33.3	12
E. coli O142	1	5.5	2
E. coli O44	1	5.5	2
E. coli O86	1	5.5	2
E. coli O25	1	5.5	2
E. coli (untyped)	1	5.5	2
Total number of *E. coli* isolates	18	100%	36%

*Represents the percentage of *E. coli* serotype to the total number of *E. coli* isolates (n= 18). ** represents the percentage of *E. coli* serotype to the number of examined minced beef samples (n=50).

Table 4. Antimicrobial resistance of *E. coli* serotypes isolated from minced beef samples

Serotypes (n=18)	Number of resistant serotypes (resistance %)										
	Antimicrobial agents										
	TE	S	IPM	SXT	CIP	GN	FOX	AMC	AMP	CTX	NA
E. coli O157 (1)	1(100)	1(100)	0(0)	1(100)	1(100)	0(0)	0(0)	1(100)	1(100)	0(0)	0(0)
E. coli O 158 (6)	6(100)	3(50)	0(0)	0(0)	0(0)	3(50)	0(0)	0(0)	0(0)	0(0)	0(0)
E. coli O114 (6)	6(100)	5(83.3)	0(0)	3(50)	2(33.3)	5(83.3)	0(0)	1(16.6)	3(50)	1(16.6)	0(0)
E. coli O 142 (1)	1(100)	1(100)	0(0)	1(100)	0(0)	1(100)	0(0)	0(0)	0(0)	1(100)	0(0)
E. coli O 44 (1)	1(100)	1(100)	0(0)	1(100)	1(100)	1(100)	0(0)	1(100)	1(100)	0(0)	0(0)
E. coli O86 (1)	1(100)	1(100)	0(0)	1(100)	1(100)	0(0)	0(0)	0(0)	0(0)	0(0)	1(100)
E. coli O25 (1)	0(0)	1(100)	0(0)	0(0)	0(0)	1(100)	0(0)	0(0)	0(0)	0(0)	0(0)
E. coli (untyped (1))	-	-	-	-	-	-	-	-	-	-	-
Total sensitive (%)	1 (5.8)	4 (23.5)	17 (100)	10 (58.8)	10 (58.8)	6 (35.2)	15 (88.2)	12 (70.5)	12 (70.5)	13 (76.4)	14 (82.3)
Total intermediate (%)	0 (0)	0 (0)	0 (0)	0 (0)	2 (11.7)	0 (0)	2 (11.7)	2 (11.7)	0 (0)	2 (11.7)	2 (11.7)
Total resistant (%)	16 (94.1)	13 (75.4)	0 (0)	7 (41.1)	5 (29.4)	11 (64.7)	0 (0)	3 (17.6)	5 (29.4	2 (11.7)	1 (5.8)

TE (tetracycline, 30 µg), S (streptomycin, 10 µg), IPM (imipenem, 10 µg), SXT (sulphamethoxazole/trimethoprim 25 µg), CIP (ciprofloxacin, 5 µg), GN (gentamicin, 10 µg), FOX (cefoxitin, 30 µg), AMC (amoxicillin/clavulanic acid, 30 µg), AMP (ampicillin, 10 µg), CTX (cefotaxime-clavoran, 30 µg), NA (nalidixic acid, 30 µg). (n =18) represents the total number of examined serotypes.

imipenem, nalidixic acid, cefoxitin with percentages 100, 82, 88.2, respectively. Multidrug-resistant strains were determined (O44, O114, O142, O86, O157, O158) and O25 strain, which was resistant for streptomycin (S), and gentamicin (GN)

The antibiotic sensitivity/susceptibility testing of *coa*gulase-positive *S. aureus* isolates showed that they were resistant to rifampin (88.8%), tetracycline (61.1%), azithromycin (66.6%), gentamicin (27.7%), erythromycin (72.2%), and clindamycin (83.3%).

Molecular identification of MDR isolates

Data illustrated in Table 6 shows the antimicrobial resistance pattern and virulence genes of 7 serotypes of pathogenic *E. coli*. They were selected based on their MDR pattern ranged from 0.27 (resistant to 3/11 antibiotics) to 0.63 (resistant to 7/11 antibiotics).

The virulent genes *eaeA* was detected in all isolated serotypes of pathogenic *E. coli*, while *stx1* was detected in *E. coli* O157 (Figure 1 and Table 6)

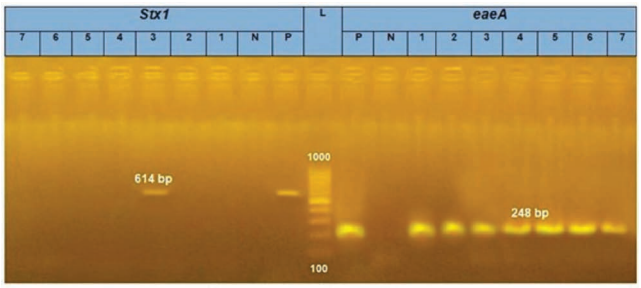

Fig. 1. Molecular identification of *stx1* and *eaeA* genes in *E. coli* isolates. *eaeA* gene and *stx1* gene are detected at 248 at 614 bp, respectively. P= control positive, N= csontrol negative.

S. aureus resistant pattern, resistant and virulent genes were showed in Table 6, four isolates of *S. aureus* isolated from minced beef and beef burger samples show a variable degree of MDR that ranged from 0.83 (resistant to 5/6 antibiotics) to 1(resistant to 6/6 antibiotics), *mecA* gene that cause a high-level of methicillin resistance in MRSA (methicillin-resistant *S. aureus*), and *coa* gene (Figure 2 and Table 6).

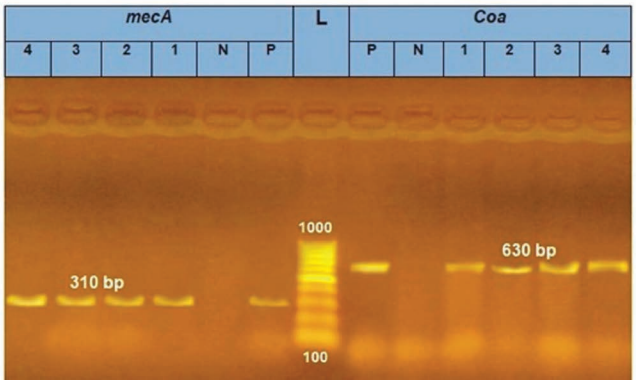

Fig. 2. Molecular identification of *mecA* and *coa* genes in *S. aureus* isolates. *mecA* gene and *coa* gene are detected at 310 and 630 bp respictively. P= control positive, N= control negative

Antimicrobial activity of marjoram and turmeric ethanolic extracts against MDR E. coli and methicillin-resistant coagulase-positive S. aureus

Isolates recovered from minced beef and beef burger samples were investigated in-vitro using well diffusion method (Tables 7 and 8). Ampicillin and cefoxitin were used as positive controls for *S. aureus* and *E. coli* isolates, respectively, while sterile distilled water was used as a negative control. Apparently, both types of extracts have various degrees of antimicrobial activity against *S. aureus* isolates according to the type and concentration of the extract. Marjoram extract displayed higher effectiveness than turmeric, as the inhibition zone diameters were 2.69, 9.78, 14.63, and 22.06 mm for concentrations 1.25, 2.5, 5, and 10 % of marjoram extract, respectively. Whereas, in the case of turmeric extract, the inhibition zone

Table 5. Antimicrobial resistance of *coa*gulase-positive *S. aureus* isolates recovered from minced beef and beef burger samples

Samples	No. of *coa*gulase-positive *S. aureus* isolates =18	Number of resistant serotypes (resistance %)					
		Antimicrobial					
		RA	TE	AZM	GN	E	DA
Minced beef	13	12 (92.3)	10 (76.9)	10 (76.9)	4 (30.7)	10 (76.9)	10 (76.9)
Beef burger	5	4 (80)	1 (20)	2 (40)	1 (20)	3 (60)	5 (100)
Total sensitive (%)		1 (5.5)	6 (33.3)	0 (0)	13 (72.2)	0 (0)	0 (0)
Total Intermediate (%)		1 (5.5)	1 (5.5)	6 (33.3)	0 (0)	5 (27.7)	3 (16.6)
Total resistant (%)		16 (88.8)	11 (61.1)	12 (66.6)	5 (27.7)	13 (72.2)	15 (83.3)

TE (tetracycline 30 µg), NA (nalidixic acid 30 µg), E (erythromycin 15 µg), AZM (azithromycin 15 µg), DA (clindamycin 2 µg), RA (rifampin 5 µg), GN (gentamicin 10 µg).

Table 6. Resistance patterns, virulent genes, and antibiotic resistance-associated genes among MDR pathogenic *E. coli* serotypes and *S. aureus* (*coa*gulase-positive) isolated from minced beef and beef burger samples

Pathogen	Origin	Serotypes/ isolates	Resistance pattern	MDR ratio	Virulent genes			Antibiotic resistance genes
					coa	*stx1*	*eaeA*	*mecA*
Pathogenic *E. coli*	Minced beef	*E. coli* O114	TE, S, SXT, GN, AMP, CTX	0.54	NA	-	+	NA
	Minced beef	*E. coli* O44	TE, S, SXT, CIP, GN, AMC, AMP	0.63	NA	-	+	NA
	Minced beef	*E. coli* O 157	TE, S, SXT, CIP, AMC, AMP	0.54	NA	+	+	NA
	Minced beef	*E. coli* O 86	TE, S, SXT, CIP, NA	0.45	NA	-	+	NA
	Minced beef	*E. coli* O158	TE, S, GN	0.27	NA	-	+	NA
	Minced beef	*E. coli* O142	TE, S, SXT, GN, CTX	0.45	NA	-	+	NA
	Minced beef	*E. coli* O25	S, GN	0.18	NA	-	+	NA
S. aureus	Minced beef	Coagulase positive *Staph. aureus* isolate	RA, TE, AZM, GN, DA	0.83	+	NA	NA	+
	Minced beef	Coagulase positive *S. aureus* isolate	RA, TE, AZM, GN, E, DA	1	+	NA	NA	+
	Minced beef	Coagulase positive *S. aureus* isolate	RA, TE, AZM, GN, E, DA	1	+	NA	NA	+
	Beef burger	Coagulase positive *S. aureus* isolate	RA, TE, AZM, E, DA	0.83	+	NA	NA	+

MDR ratio (multiple drug resistance ratio), for instance, MDR 0.54 Means that this strain was resistant to 6 out of 11 antibiotics tested (6/11=0.54). *coa* gene (*coa*gulase gene), *mecA* (gene responsible for Methicillin-resistant *S. aureus* (MRSA), *eaeA* (*Escherichia coli* attaching and effacing gene), *stx1* (shiga toxin gene 1), TE (tetracycline, 30 µg), S (streptomycin, 10µg), IPM (imipenem, 10µg), SXT (sulphamethoxazole /trimethoprim, 25µg), CIP (ciprofloxacin, 5µg), GN (gentamicin, 10µg), FOX (cefoxitin, 30µg), AMC (amoxycillin/clavulanic acid, 30µg), AMP (ampicillin 10µg), CTX (cefotaxime-clavoran, 30µg), NA (nalidixic acid, 30µg). E (erythromycin 15µg), AZM (azithromycin, 15µg), DA (clindamycin, 2µg), RA (rifampin, 5µg).

Table 7. The antibacterial activity of marjoram and turmeric extract against *S. aureus* isolates cocktail recovered from minced beef and beef burger samples

Herbal extract type	Extract concentrations	Inhibition zone diameter (mm)			
		Minimum	Maximum	Mean	Standard error
Marjoram extract	1.25%	0	5	2.69	0.27
	2.50%	2	15	9.78	0.54
	5%	10	18	14.63	0.49
	10%	16	25	22.06	0.41
Turmeric extract	1.25%	0	3	1.39	0.24
	2.50%	0	5	1.9	0.22
	5%	0	8	3.63	0.29
	10%	10	25	19.21	0.64
Sterile distilled water (negative control)		0	0	0	0
Ampicillin (positive control)10 µg		45	50	49.7	0.57

Table 8. The antibacterial activity of marjoram and turmeric extract against MDR *E. coli* isolates cocktail recovered from minced beef samples

Herbal extract type	Extract concentrations	Inhibition zone diameter (mm)			
		Minimum	Maximum	Mean	Standard error
Marjoram extract	1.25%	0	0	0	0
	2.50%	0	0	0	0
	5%	0	0	0	0
	10%	0	0	0	0
Turmeric extract	1.25%	0	0	0	0
	2.50%	0	0	0	0
	5%	0	0	0	0
	10%	0	0	0	0
Sterile distilled water (negative control)		0	0	0	0
Cefoxitin (positive control)30 µg		16	30	22.1	0.52

diameters accounted for 1.39, 1.9, 3.63, and 19.21 mm for concentrations 1.25, 2.5, 5, and 10 %, respectively (Table 7). On the other hand, both marjoram and turmeric extracts did not show any antimicrobial effect against MDR *E. coli* isolates (Table 8).

Discussion

The food contamination with antimicrobial-resistant bacteria could be a major threat to public health because the antimicrobial resistance can be transferred to other pathogenic bacteria, causing a compromise in the treatment of severe infections (Akinyemi *et al*., 2006) MDR strains (Multidrug-resistant) are defined as those pathogens which have resistance to ≥ 3 groups of antibacterial agents (Magiorakos *et al*., 2012).

In the present study, a total of 100 samples of minced beef and beef burger, 50 each, were examined for the presence of *E. coli* and *coa*gulase-positive *S. aureus*. *E. coli* was recovered from 18 out of 50 minced beef samples (36%). These findings were similar to that reported by Ezzat *et al*., (2014), Gouda, (1991), who identified *E. coli* in minced beef by 28%, and 33%, respectively. While Abd El Tawab *et al*., (2015) recorded *E. coli* strains in 52% of minced beef samples.

Serological identification of 18 strains of *E. coli* isolated from minced beef samples revealed the presence of O157, O158, O114, O142, O44, O86, and O25 with percentages of 5.5%, 33.3%, 33.3%,5.5%, 5.5%, 5.5%, and 5.5%, respectively, while one sample isolate was untyped. The molecular identification of seven different MDR serotypes showed the presence of *eaeA* (*Escherichia coli* attaching and effacing gene that shown to be essential for the production of the attaching and effacing character of EPEC) (Jerse *et al*., 1990), intimin gene which is a remarkable gene for EHEC (Caprioli *et al*., 2005) in all serotypes. Furthermore, *stx1* (Shiga toxin type 1 gene), which is one of the most virulent factors of enterohemorrhagic *E. coli* (EHEC) that nearly identical to the toxin of S. dysenteriae type 1 (Caprioli *et al*., 2005) was detected in *E. coli* O157 serotype. These results indicate that minced beef samples contain pathogenic *E. coli* strains that could threaten public health.

On the contrary, there was no *E. coli* isolated from frozen beef burger samples. Similarly, Wehab and Hegazy *et al*. (2007) failed to isolate *E. coli* from beef burger samples. Moreover, a study reported by Cagney *et al*., (2004) illustrated that unpackaged beef burger samples were free of *E. coli* O157:H7. On the other hand, Shaltout *et al*., (2017) reported that 91 out of 105 beef burger samples were accepted as free of *E. coli* isolates. Additionally, Zaki-Eman (2003) and Ramadan (2015) found *E. coli* isolates with high rates in frozen beef burger samples.

The differences in the obtained results of beef burger in comparing to these studies could be attributed to the differences in the locality and sample origin and accordingly processing procedures, hygienic practices, storage condition, and packaging conditions, as all-beef burger samples collected during the current study were frozen packaged samples collected from supermarkets which originally processed in meat processing plants with good hygienic and manufacturing practices. Conversely, high levels of *E. coli* reported in fresh minced beef samples in the current study could be ascribed to the contamination of beef at traditional slaughterhouses during animal slaughtering and carcass preparation such as contamination by gastrointestinal content during dressing and evisceration, as well as contamination by water during rinsing (Duffy *et al*., 2003). In addition to carcass transportation to the butcher`s shop and further mincing step under unsanitary condition including poor cleaning and sanitizing and poor personal hygiene could increase the contamination level of fresh minced beef.

Regarding the levels of *coa*gulase-positive *S. aureus* isolates recovered from fresh minced beef (26%) and frozen beef burger (10%) samples, they were similar to that reported by Abd El Tawab *et al*., (2015) who isolated *S. aureus* from minced beef and beef burger (30 and 12.5%, respectively). Additionally, Shaltout *et al*., (2017) recovered *coa*gulase-positive *S. aureus* from frozen beef burger samples with 12.5% and from minced beef samples with 30%. On the other hand, Tarabees *et al*., (2015) demonstrated a high prevalence of *S. aureus* in minced beef (57.5%) and beef burger (20%) samples, whereas Wehab and Hegazy, (2007) failed to isolate *S. aureus* from beef burger sample. The molecular identification of selected MDR *S. aureus* isolates showed that all of them had the methicillin resistance gene (*mecA*) and the *coa*gulation accountable genes (*coa*), avirulence gene that enables *S. aureus* to generate abscess, persist in host tissue, and resist opsonophagocytic clearance by the host (Friedman and Ratard, 2007; Chandrakanth *et al*., 2010; Mcadow *et al*., 2012).

. The contamination of meat and meat product with *S. aureus* indicate poor personal hygiene and inadequate food safety training to food workers and handlers (Lues and Van Tonder, 2007)Spreading of antimicrobial resistance among foodborne pathogens play a role in increasing the clinical cases of resistant infection (Abdaslam *et al*., 2014).

Antimicrobial agents are used in veterinary medicine as a therapy, prophylaxis, and growth promoters, this may be the reason for the generation of resistant bacteria and multidrug resistance strains (Fallah *et al*., 2013; Rajagopal and Mini, 2013). The antimicrobial resistance/susceptibility testing of *E. coli* and *S. aureus* isolated revealed the existence of multidrug-resistant pathogens in raw minced beef and frozen beef burger samples which confirm the public health hazardous effects of these food items. Different carcinogenic and toxic changes caused by the use of chemical food additives have been shown by various studies, therefore, there is a growing concern to replace chemical food preservatives with natural

ones (Mariutti *et al*., 2011). In this context, several researchers demonstrated the antimicrobial activities of herbal extracts against foodborne pathogens (Alzoreky and Nakahara, 2003; Delgado *et al*., 2004; Verma *et al*., 2012; Akinpelu *et al*., 2015). However, there are scarcely found data on the effectiveness of marjoram and turmeric ethanolic extracts against MDR foodborne pathogenic isolates.

In the current study, marjoram and turmeric ethanolic extracts had various degrees of antimicrobial activity against *S. aureus* isolates according to the type and concentration of the extract. Marjoram extract displayed higher effectiveness than turmeric with a direct relationship between the concentration and inhibition zone diameters in both types. On the other hand, both marjoram and turmeric extracts did not show any antimicrobial effect against MDR *E. coli* isolates.

The variation in the obtained results than some previous reports could be attributed to the differences in herb provenance, accession, the climatic condition of cultivation and harvesting season as well as the differences in bacterial serotypes (Tiwari *et al*., 2009). Regarding the higher resistance of *E. coli* serotypes than *S. aureus* ones could be ascribed in the light of many previous pieces of research confirmed that Gram-negative bacteria are more resistant to natural herbal extracts than Gram-positive ones due to the difference in cell membrane structure (Burt, 2004; Stefanello *et al*., 2008; Rameshkumar *et al*., 2007)

Several studies agree with the results of marjoram extract against *S. aureus* and disagree with results against *E. coli* (Abdel-Massih and Abraham, 2014; Omara *et al*., 2014; Ouedrhiri *et al*., 2017)

Plants are a great source of active biological compounds in addition to a variety of secondary metabolites (Leeja and Thoppil, 2007). Marjoram extract has a strong microbicidal effect due to its content of phenols, essential oil, steroids, terpenoids, flavonoids, and coumarins (Cseke *et al*., 2016), these different compounds do not act separately but usually, they affect each other. The antibacterial mechanism of phenolics depends on their concentrations; a low concentration of phenols affects the enzymatic activity of bacterial cells especially energy-producing enzymes, while in high concentrations they lead to protein denaturation (Aminzare *et al*., 2016). Besides, other antimicrobial compounds in plants induce changes in bacterial cell permeability and consequently lead to loss of essential molecules, interfering with the normal function of the cell membrane, and change its structure and function.

Conclusion

Marjoram and turmeric ethanolic extracts have various degrees of antimicrobial activity against *S. aureus* isolates according to the type and concentration of the extract. Marjoram extract displays higher effectiveness than turmeric. On the other hand, both marjoram and turmeric extracts do not show any antimicrobial effect against MDR isolates. The obtained results could be of considerable value to the local regulatory authorities that necessitate an intervention to protect the public.

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Conflict of interest

The authors declare that there is no conflict of interest in this study.

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