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In vitro and *In vivo* Molecular Studies on The Effect of Some Antibiotics and *Allium sativum* Extract on Some *Escherichia coli* Serovars Isolated from Chickens

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Abstract

A total number of 16 *Escherichia coli* serovars were recovered from different colibacillosis diseased chicken organs. Characterization of the isolates was performed by biochemical and serological tests, biofilm formation, antimicrobial susceptibility assays, detection of virulence and antibiotic resistance genes by PCR. The biofilm formation (at OD_{595}) of all *E. coli* isolates showed a similar trend. eaeA gene was detected in 3 serovars O2:H6, O26:H11 and O44:H18 while, hly gene was detected in 2 serovars O2:H6 and O144:H21. The gene encoding for stx2 was detected in 2 serovars (O55:H7 and O146:H21) while the gene encoding stx1 was not detected in any serovars. Furthermore, all serovars were carrying the encoding blaTEM. blaSHV and blaCTX were detected in 75% and 18.75% of isolates respectively, while blaOXA-1 was not detected in all serovars. Although all isolates were multi-resistant against most of β-lactams antibiotics, they were susceptible to 20 to 80 mg ml^{-1} of garlic extract. Finally, we demonstrated that a five-oral administration of garlic extract to baby chicks colonized with *E. coli* reduces the mortalities and significantly increase the body weight. The efficacy of garlic extract as the antibacterial natural product was sufficient as a bactericidal alternative against the multidrug-resistant *E. coli* isolates.

Keywords: *Allium sativum*, Antibiotic-resistant genes, Biofilm, Virulence genes

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Introduction

Escherichia coli is a gram-negative, facultatively anaerobic bacterium, short rods-bacilli in shape, that belongs to the family Enterobacteriaceae. *E. coli* can colonize in the gastrointestinal tract of mammals and birds (Hamoudi and Aggad, 2008). Avian pathogenic *E. coli* strains are responsible for various diseases, including colibacillosis (Calnek et al., 1997) In laying chickens and breeders, colibacillosis causes oviduct inflammation that lead to in decreased egg production and sporadic mortality (Barnes, 2003). The early infections would increase the avian susceptibility to Avian Pathogenic *Escherichia coli* (APEC) strains due to the deciliation of the upper respiratory cells (Nagaraja et al., 1984). This infection is also referred to as aero sac and usually occurs between birds with 2 to 12 weeks of age, with mortality rates up to 20% (Dho-Moulin and Fairbrother, 1999). Many virulence factors have been associated with APEC strains and they explained its ability to cause disease such as toxins, verotoxin, attaching and effecting mechanisms (*eae*A) (Pass et al., 2000). Most bacterial pathogens associated with human diseases can be transmitted directly from animals to humans or indirectly during food of animal origin (Newell et al., 2010). The progress in molecular techniques such as polymerase chain reaction (PCR) has made it easy to identify and quantify the virulence genes in *E. coli* (Ahmed et al., 2007). The appearance of antimicrobial resistance in bacteria has been well known as a serious problem all over the world (Cohen, 2000). In Enterobacteriaceae, ESBL-producing *E. coli* represent an growing difficulty for public health (ECDC, 2011). Garlic (*Allium sativum*) is a strong antibacterial agent against both Gram-positive and Gram-negative bacteria such as *E. coli*, *Salmonella spp*. effective against those strains that have become resistant to antibiotics (Ross et al., 2001, Indu et al., 2006). The antibacterial compound of *A. sativum* was identified as diallylthiosulphinate and named allicin (Balestra et al., 2009). Allicin was reported to inhibit the acetyl CoA-forming system leading to inhibition of DNA and protein synthesis and also inhibiting RNA synthesis (Cutler and Wilson, 2004).

In this study, colibacillosis was screened after post-mortem examination of diseased chickens. Biofilm formation, virulence and antibiotic-resistant genes in multi-drug resistant *E. coli* were also studied. Finally, the efficacy of garlic extract as an antibacterial agent was studied against the isolated serovars and for treating experimentally *E. coli* induced colibacillosis in chickens.

Material and methods

I. Sampling, isolation, and identification of E. coli:

A total of one hundred and twenty-five unhealthy chicken's samples were collected between February to August (2016) from diverse governmental and private poultry farms in Qena province, Egypt. Samples were between 1- 3 weeks old with an average weight of 75 - 450 g. Diseased organ samples were chosen according to post-mortem examination and colibacillosis symptoms. For *E. coli* isolation, a 0.5 g of each sample was aseptically immersed in selenite cysteine broth (SCB) medium (Oxoid®) and incubated at 37°C for 24 hours. Via a sterile wire loop, a loopful of the incubated broth culture was then streaked onto eosin methylene blue agar (Oxoid®) plates as a selective medium then incubated at 37°C for 24 hours. Suspected colonies of *E. coli* were picked up and identified by classical biochemical method

(Gram staining, citrate, indole, urease, triple sugar, and lysine) (Reller and Mirrett, 1975, Ewing, 1973., Quinn, 2002). Isolates that were mainly identified by biochemical tests were then serologically identified according to (Hamoudi and Aggad, 2008) by using rapid diagnosis *E. coli* antisera sets (Denka Seiken Co., Japan) for diagnosis of enteropathogenic types. CFU (colony forming units) were also determined as a respect to the original weight.

II. Detection of virulence, antibiotic resistance genes, and biofilm formation:

The detection of eight genes encoding enterotoxins and antibiotic resistance was performed using fourteen primers sets. Extraction of DNA was carried according to QIAamp DNA mini kit instructions. genes encoding different virulence factors stx1, stx2 (Dipineto et al., 2006), hly (Piva et al., 2003) and eaeA (Bisi-Johnson et al., 2011). For antibiotic resistance genes, genes for blaTEM, blaSHV, blaOXA-1 (Colom et al., 2003) and blaCTX (Archambault et al., 2006) were analyzed by PCR (Table 1). Static biofilm were performed in microtiter plates by crystal violet staining essentially as previously published (Seper et al., 2011) After modifications by (Salem et al., 2015). Briefly, the respective isolates (16 serovars) were grown overnight (ON) on tryptic agar plates (Oxoid®). Suspended in tryptic broth, adjusted to an OD_{595} of 0.02. 130 µl of each isolate dilution were placed in a 96 well microtiter plate (U Bottom, Sterilin) for 24 h at 37°C. Wells were subsequently rinsed with d H_2O biofilm was stained by 0.1% crystal violet for 10 min. Then, wells were subsequently rinsed with d H_2O, solubilized in 96% ethanol and the OD_{595} was measured using Infinite® F50 Robotic (Austrich) Microplate Reader to quantify the amount of biofilm.

Table (1): Primers and probe sequences

Primer	Sequence	Amplified product
Stx1	ACACTGGATGATCTCAGTGG	614 bp
	CTGAATCCCCCTCCATTATG	
Stx2	CCATGACAACGGACAGCAGTT	779 bp
	CCTGTCAACTGAGCAGCACTTTG	
Hly	AACAAGGATAAGCACTGTTCTGGCT	1177 bp
	ACCATATAAGCGGTCATTCCCGTCA	
eaeA	ATG CTT AGT GCT GGT TTA GG	248 bp
	GCC TTC ATC ATT TCG CTT TC	
blaTEM	ATCAGCAATAAACCAGC	516 bp
	CCCCGAAGAACGTTTTC	
blaOXA-1	ATATCTCTACTGTTGCATCTCC	619 bp
	AAACCCTTCAAACCATCC	
blaSHV	AGGATTGACTGCCTTTTTG	392 bp
	ATTTGCTGATTTCGCTCG	
blaCTX	ATGTGCAGYACCAGTAARGTKATGGC	593 bp
	TGG CCA GAA CTG ACA GGC AAA	

III. Antimicrobial Susceptibility test:

The antibiogram of all the recovered isolates was also determined as described earlier by (Hamoudi and Aggad, 2008) using disc diffusion test (Bauer et al., 1966). The susceptibility of *E. coli* isolates was tested for 9 antibiotics from beta-lactam group (Bioanalyse®). The used antibiotics were ampicillin, ampicllin/sulbactam, cefoperazone/ sulbactam, carbencillin, ceftotaxime, ceftriaxon, ceftazidime, imipenem and meropenem.

IV. Determination of bioactive groups in garlic extract by FTIR and Ultra-high Performance Liquid Chromatography (UHPLC MS/MS):

For preparing garlic extract, 50 gm of fresh garlic were washed with sterile distilled water and blotted dry with paper towel. Samples were sliced into small pieces and blended using a blender. The crude of garlic soaked with 100 ml of commercial vinegar and placed for 1 h at room temperature with shaking using Bigger-bill shaker (USA) at 150 rpm for extraction of active ingredients. The mixture was sterilized using syringe filter holder equipped with a 0.45µ membrane filter, then kept at 4°C. The presence of bioactive functional groups in garlic material and garlic extract were determined as previously described (Salem et al., 2017) by Fourier transform infrared spectrometer (FTIR). The analysis was carried out using a Magna-FTIR 560 (USA) instrument at a resolution of 2 cm^{-1} ranging from 4,000 to 400 in KBr pellet using diffuse reflectance mode operated by Nicolet Omnic Software® as instructed by manufacturers. To confirm the results of FTIR, the extracted and purified garlic sample was subjected to UHPLCMS/MS. UHPLC MS/MS using an Agilent 1290 Infinity UHPLC System equipped with a 1290 Infinity sampler Quat. pump and a 1290 Infinity auto-sampler, and an Agilent 6420 triple quadrupole detector. All compounds were baseline separated on an separation acquity UPLC BEH shield RP column (18 1.7 µm 2.1 × 150 mm) using a binary solvent gradient (solvent A: 100% water; solvent B: non used; solvent C: 100% acetonitrile; solvent D: 100% water) The flow rate was 0.2 mL/min, and the injection volume was 20µl. MS and MS/MS experiments were performed on the Agilent 6420 triple quadrupole tandem mass spectrometer. The ion source was operated in ESI mode. Operation parameters of the ion source were as follows: gas temperature 320°C, gas flow 9 l/min, nebulizer 45 psi, Capillary 4000V.

V. Determination of the minimum inhibitory concentration of garlic extract:

Overnight cultures of *E. coli* were sub-cultured into tryptic soy broth (TSB). Samples of 100 µl bacterial growth were placed into 96-well plates plus 20 µl of appropriate serial dilutions (10-100%) of the original garlic extract.
Ampicillin/sulbactam (20%) was tested as a positive control. After 24 h incubation at 37°C, the MIC was the lowest concentration that inhibited bacterial growth. To confirm bacterial growth inhibition and lack of metabolic activity, 40µl of *p*-iodonitrotetrazolium violet (INT) (0.2 mg/mL, Sigma-Aldrich) was added to the microplate wells and reincubated at 37°C for 30 min (Eloff, 1998). The MIC in the INT assay was defined as the lowest concentration that prevented color change as described earlier by (Hamoudi and Aggad, 2008).

Concentration (MBC) testing was also determined, the bactericidal effect was defined as a 99.9% decrease in CFU (3 logs) in the starting inoculum during a 24 h incubation. The MBC was determined by

transferring 50 µl from each well of an overnight MIC plates (and /or higher) to sterile (TSA) fresh plates. Viable colonies were counted after 24 h at 37°C. The limit of detection for this assay was 10^1 CFU/mL (Sirelkhatim et al., 2015).

VI. In vivo studies:

Growth of *E. coli* (O2:H6) (field strain which were previously isolated and identified by serology and PCR) were grown overnight at 37°C in nutrient broth medium. Broth culture of *E. coli* (O2:H6) was then diluted with normal saline to obtain a concentration of 6x 10^8 CFU/ml (La Ragione et al., 2013). Seventy-five Cobb broiler chicks (one-day-old) were used to determine the protective dosage of garlic extract. The chicks were taken from a healthy breeder flock free from diseases. All birds were subjected to the ordinary vaccination program for broilers against New castle using live Hitchner B1 and La Sota vaccine strains at 6 and 17 days of age, respectively. Gumboro disease was applied using live intermediate strain (228 E) at 14 days of age. All vaccines were given via eye drop instillation. Chicks were fed balanced commercial starter and growing rations (21% and 18% protein, respectively) and water ad libitum and monitored under the care of full-time staff. According to South Valley University scientific ethics and animal care and use guidelines. Birds were housed in standard cages and kept in a controlled room conditions at 25 ± 1 °C and 50 ± 5 % relative humidity, with continuous medical care and observation until the end of the experiment. The Chicks were divided into three groups; group 1 served as control, groups 2 and 3 received only one-time intratracheal inoculation of *E. coli* (O2:H6). Every chick (except control group) was challenged with 1 ml of the inoculums orally (Okamoto et al., 2007) after 7 days age. Group 2 (infected but untreated chickens) received no treatment during the experiment. Groups 3 received treatments for five consecutive days (approx. 1 ml orally each day) of 80 mg ml^{-1} of garlic extract after 24 hours of infection with *E. coli*. All chicks were kept under daily observation for mortality, clinical signs and postmortem lesion. Body weight also was measured all over the experiment for all groups.

VII. Statistical analysis:

The variability degree of results was expressed in form of Means ± Standard Deviation (Mean ±S.D). The data were statistically analyzed by one-way ANOVA analysis of variance using prism computer program. It was done to compare between control and other treated groups. The difference was considered statistically significant when $P< 0.05$ %.

Results

I. Clinical picture, necropsy findings and incidence of E. coli among chickens:

A total of 125 diseased chicken samples represented the prevalence of *E. coli* isolated from different organs of chickens (Table 2). Symptoms of examined chickens were respiratory distress, nasal discharge, poor growth, loss of appetite, weakness, and sudden death without premonitory signs. On necropsy, the chicks died during the first few days of life after developing septicemia that caused high mortality with few or no lesions. The post-mortem lesions examination in chicks showed perihepatitis, air sacuilitis, congested liver and pericarditis (Fig. 1). Total number of positive organs, infected with colibacillosis, were 41 (32.8%) out of 125 examined chickens. From all the infected organs, cecum showed the highest prevalence of *E. coli* isolates indicated as cfu (111 X 10^4) followed by heart and yolk

sac, (258 and 134 X 10^3, respectively). Both air sac and spleen did not show any cfu for *E. coli* colonies (Table 2).

Table (2): Prevalence of *E. coli* isolated from different organs of chickens

Infected organs[a]	Number of		cfu[d]
	organs[b]	(+ ve *E. coli*)[c]	
Liver	55	12	$40x10^3$
Heart	37	9	$258 x10^3$
Lung	7	5	$132 x10^3$
Yolk sac	16	10	$134 x10^5$
Nasal cavity	3	1	$100 x10^5$
Intestine	3	3	$122 x10^5$
Air sac	1	0	0
Spleen	2	0	0
Cecum	1	1	$1110 x10^3$
Total	**125**	**41**	

[a] : Infected organs with colibacillosis selected according to post-mortem examination, [b]: number of selected organs, [c]: number of positive organs infected with colibacillosis, [d]: average of colony forming unit of *E. coli* prevalence in each organ.

Fig. 1. Symptoms of avian colibacillosis of the isolated *E. coli* from chickens. (a) air saculitis; (b) congested liver; (c) nasal discharge; (d) perihepatitis; (e) pericarditis.

II. Biochemical reactions:

The suspected positive cultures of *E. coli* showed the characteristic colonies on the selective eosin methylene blue agar media as metallic green reverse. Morphological characterization revealed that the isolates were Gram-negative, short rod bacilli. The biochemical reactions showed similar negative results in utilization of urea and citrate. All isolates were exclusively positive for indole production and lysine hydrolysis. For sugar fermentation and hydrogen peroxide production, the isolates showed in yellowish slant (yellow butt without H_2S production) in Triple Sugar Iron (TSI) media that confirming the expected growing of *E. coli* isolates

III. Serotyping of E. coli isolates:

Table (3) shows the results of serological examination of the identified *E. coli*. In general, *E. coli* O78 and O2:H6 showed greatest prevalence among other isolated serovars.

IV. Detection of virulence and antibiotic-resistant genes by PCR and static biofilm assay:

E. coli isolates were screened for four virulence genes (stx1, stx2, eaeA, and hly) and four antibiotic resistant genes (blaTEM, blaSHV, blaOXA-1 and blaCTX) by using PCR. No *E. coli* carrying the gene encoding stx1. In 2 serovars (O55:H7 and O146:H21) the gene encoding for stx2 was detected. eaeA gene was detected in 3 serovars O2:H6, O26:H11 and O44:H18 while, hly gene was detected in 2 serovars O2:H6 and O144:H21 (Fig. 2a; Table 3). Furthermore, the results showed that (100%) of isolates were carrying the encoding blaTEM. blaSHV and blaCTX were detected in 75% and 18.75% of isolates respectively, while blaOXA-1 was not detected in all serovars (Fig. 2b, c; Table 4). All serovars were biofilm producers with weak formation ability (Table 3).

V. Antimicrobial susceptibility test:

The antibiotic resistance of *E. coli* against the used 9 antibiotics with different disc potency was determined by disc diffusion method and the results showed that all *E. coli* isolates were resistant to ampicillin, high percentage (93%) exhibited resistance to ampicillin/sulbactam.
Ceftazidime and carbenicillin resistance were observed in almost 30% and 18.6% respectively, furthermore lower percentage of resistance (7.7 and 4.6%) was identified to cefoperazone/sulbactam, ceftriaxone and cefotaxime, respectively. All isolated showed sensitivity to imipenem and meropenem (Table 4).

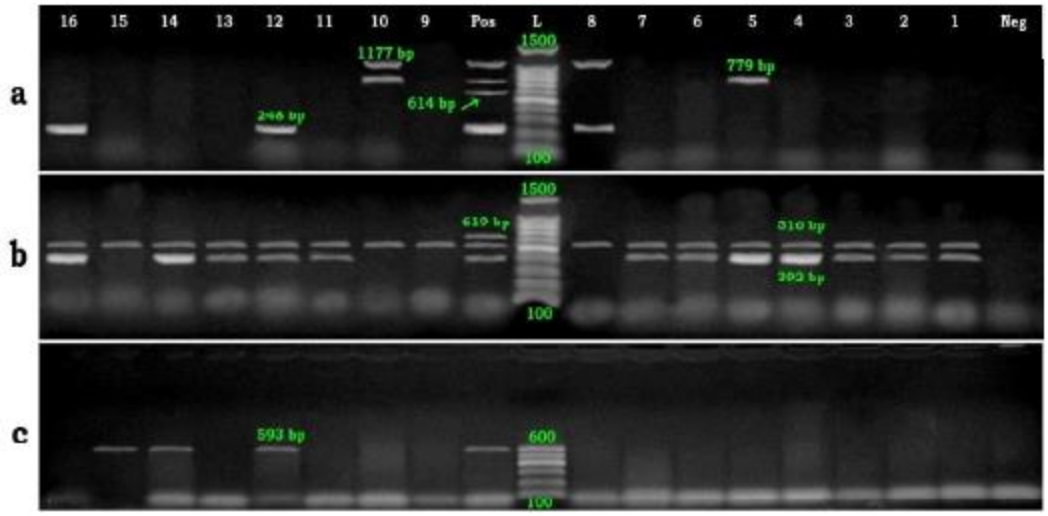

Fig. 2. 1.5% Agarose gel electrophoresis of multiplex PCR of: (a) stx1 (614 bp), stx2 (779 bp), eaeA (248 bp) and hlyA (1177 bp) virulence genes for characterization of *Enteropathogenic E. coli.* (b) *B*-lactamase resistance genes of *E. coli* species represented by blaOXA-1 (619 bp), blaSHV (392 bp) and blaTEM (516 bp). (c) blaCTX (593 bp) as antibiotic resistance genes of *Enteropathogenic E. Coli*. Lane L {a and b: Gel Pilot 100 bp plus ladder (cat. no. 239045) supplied from QIAGEN (USA); c: 100 bp ladder as molecular size DNA marker (cat. no. 239035) supplied from QIAGEN (USA). Lane pos.: control positive DNA confirmed by reference laboratory for quality control of poultry production; Lane neg: control negative. Lane 1: O127:H6; Lane 2: O153:H2; Lane 3: O1:H7; Lane 4: O91:H21; Lane 5: O55: H7; Lane 6: O78; Lane 7: O128: H2; Lane 8: O2:H6; Lane 9: O113:H4; Lane 10: O146:H21; Lane 11: O111:H2; Lane 12: O44:H18 Lane; 13: O124 Lane; 14: O158 ; Lane 15: O119:H6; Lane 16: O26:H11.

Table (3): The relation between virulence genes detected by PCR, different serotypes and biofilm formation of *E. coli* isolated from chickens

Serovars	No. of +ve serovars[a]	%[b]	Virulence genes[c]				Antibacterial efficacy	
			Stx1	Stx2	eaeA	hly	MIC/MBC[d]	OD_{595}[e]
O127:H6	1	2.3	-	-	-	-	30/35	+
O153:H2	4	9.3	-	-	-	-	20/70	+
O1:H7	2	4.6	-	-	-	-	20/20	+
O91:H21	3	6.9	-	-	-	-	15/45	+
O55:H7	2	4.6	-	+	-	-	20/80	+
O78	8	18.6	-	-	-	-	25/70	+
O128:H2	3	6.9	-	-	-	-	20/70	+
O2:H6	6	13.9	-	-	+	+	30/80	+
O113:H4	1	2.3	-	-	-	-	20/75	+
O146:H21	1	2.3	-	+	-	+	20/25	+
O111:H2	2	4.6	-	-	-	-	20/80	+
O44:H18	1	2.3	-	-	+	-	20/25	+
O124	2	4.6	-	-	-	-	20/75	+
O158	2	4.6	-	-	-	-	20/60	+
O119:H6	2	4.6	-	-	-	-	20/80	+
O26:H11	3	6.9	-	-	+	-	15/45	+

[a]: Number of isolated serovars from total selected infected organs; [b]: percentage of isolated serovars out from total number of isolated serovars (43); [c]: present (+), absent (-). [d]: minimal inhibitory concentration / minimal bactericidal concentration of garlic extract against isolated *E. coli* serovars represented in $mgml^{-1}$.[e]: low grade biofilm formation (+).

Table (4): Resistance genes detected in antibiotic resistant *E. coli* isolates obtained from chickens.

Phenotype of Antibiotic[a]				Genes detected by PCR	
B-lactams	R	M	S	Resistance genes	No. of positive serotype[b]
AM-10	43	0	0	*blaTEM*	16
SAM-20	40	3	0	*blaCTX*	3
CES-30	3	0	40	*blaSHV*	12
CAR-30	8	3	32	*blaOXA-1*	0
CTX-30	2	1	40		
CRO-30	3	1	39		
CAZ-30	13	1	29		
IPM-10	0	0	43		
MEM-10	0	0	43		

Antimicrobial susceptibility of *E .coli* isolates against *B*-lactams group.[a]:R: Resistant ;M: Moderate; S: Sensitive; AM*:* Ampicllin; SAM: Ampicllin/sulbactam; CES: Cefoperazone/sulbactam; CAR: Carbencillin; CTX: Ceftotaxime; CRO: Ceftriaxon; CAZ: Ceftazidime; IPM: Imipenem; MEM: Meropenem. 10, 20 and 30: antibiotic concentration. [b]: Number of positive serovars for each detected gene.

VI. Determination of bioactive groups in garlic extract:

The most important bioactive functional groups of garlic extract were determined by FTIR as S=O (sulphonyl group) that was present at 1417 and 1024 cm^{-1} (Supplementary Fig. S1). The results of UPLC-MS/MS showed the peaks with m/z values of 162, 177, 114, 234 and 142 were identified as allicin, alliin, allylsulfide, E-ajoene and vinyldithiin (Supplementary Fig. S2).

VII. Determination of MIC for garlic extract:

The colorimetric INT- formazon assay of garlic extract showed reproducible, effective antibacterial activity against all tested *E. coli* isolates. Garlic extract concentrations ranged from (15 to 30 mg ml^{-1}) were sufficient as minimum inhibitory concentrations for all the tested *E. coli* isolates with the highest MIC value of 150 and 175, mg ml^{-1} were recorded for (O127:H6 and O2:H6). In general, the efficiency of the extract as bactericidal (MBC) natural product against *E. coli* was from 20 to 80 mg ml^{-1} (Table 3).

VIII. In vivo study

Clinical signs and postmortem lesions:

All control chicks (G1) remain active throughout the experimental period. They appeared healthy displaying neither abnormal clinical signs nor postmortem lesions during the time of the experiment. G2 (infected with *E. coli*) showed clinical signs such as closed eye, diarrhea, depression, respiratory symptoms including gasping, rales and nasal discharge. The most postmortem lesions were congestion in all organs with pericarditis, perihepatitis, splenomegaly, air sacculitis, and pneumonia. Clinical signs of the infected chicks treated with garlic in G3 were less severe than those of infected chicks group G2. While survival chicks appeared with no post-mortem lesions (Fig. 3).

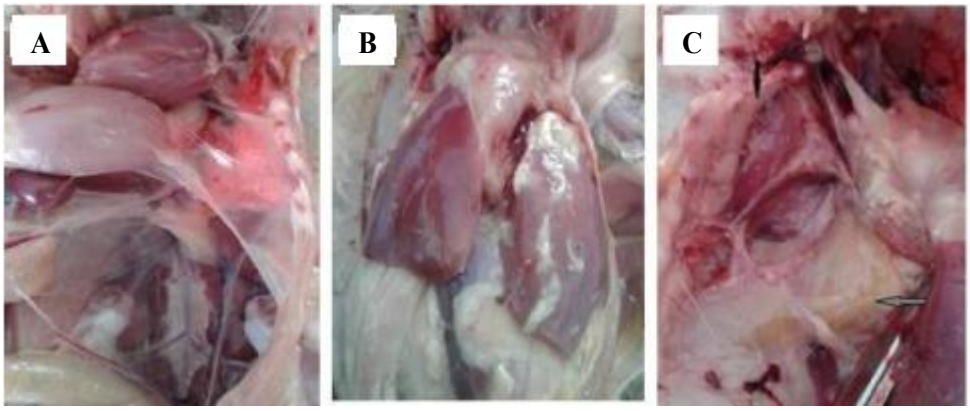

Fig. 3. Clinical signs of the infected chicks treated with garlic. Control chicks A: (G1 group) appeared healthy displaying neither abnormal clinical signs nor post- mortem lesions during the time of the experiment. B: (G2 group- infected with *E. coli*) showed lesions were congestion in all organs with pericarditis, perihepatitis, splenomegaly, C: air sacculitis and pneumonia.

IX. Mortality rate and Body weights:

Mortality rate was investigated, and the results indicated that G1 (control group) there was no mortality and all chicks were surviving. While for G2 mortality rate was 60 %. On the other hand, mortality decreased to 6.6% after treatment with garlic extract in G3. With regarding to body weight, there were increases in body weight

in G3 when compared with infected non-treated groups G2. There were significant decreases in body weight at 14 and 21 days age in G2 which infected with *E. coli* when compared with control while after treatment with garlic extract body weight increased significantly in G3. There was no change in body weight at 1 and 7 days because the infection begins after 7 days age (Fig. 4).

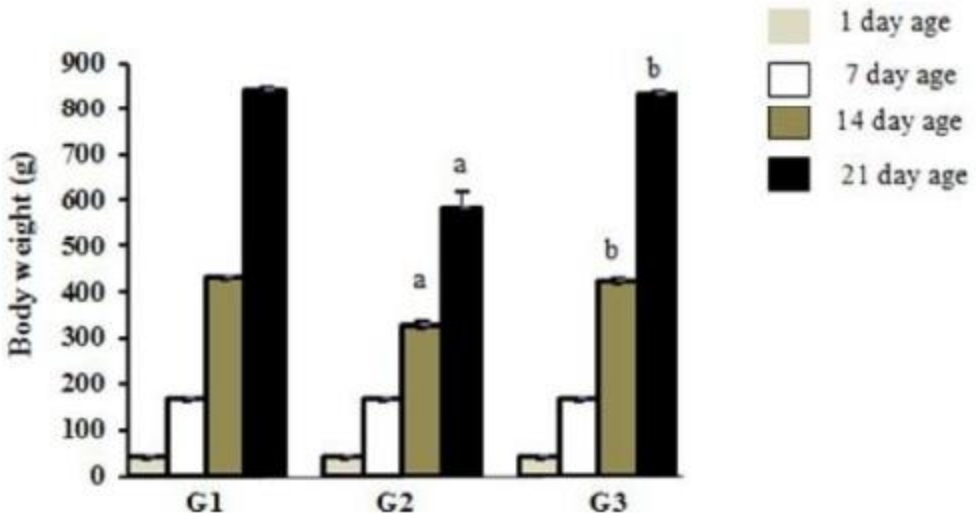

Fig. 4. Average body weight of chicks at 1, 7, 14 and 21 days age. G 1 (control), G 2 (*E. coli* infection) , G 3 (*E. coli* + garlic extract). a: significant difference when compared with control when P<0.05 %, b: significant difference when compared with group 2 when P<0.05 %, c: significant difference when compared with group 4 when P<0.0.5 %.

Discussion

Escherichia coli infections in birds cause many clinical manifestations which characterized by a respiratory disease that is frequently followed by a generalized infection which ended by death. Avian pathogenic *E. coli* (APEC) strains fall under the category of extraintestinal pathogenic *E. coli*, which are characterized by the possession of virulence factors that enable to live extra intestinal life (Johnson et al., 2006). Symptoms (clinical signs) and post-mortem lesions of colibacillosis infections there were high mortality, nasal discharge, retarded growth and decrease water intake. This is in agreement with (Barnes, 2008) who mentioned that clinical signs of colibacillosis are increased mortality, lameness, stunted growth, inactivity, lack of appetite and water consumption. Interestingly, the percentage of colibacillosis in our study was 33% with the highest prevalence reported for O78 serovar 8 (18.6%) out of the total isolated samples (Table 2, 3). These results in agreement with the results obtained by (Abd El Tawab, 2016) who reported that the percent of *E. coli* isolation from chickens were 38%. The mechanisms of APEC pathogenesis depends on a number of virulence factors which have been implicated in these extra-intestinal diseases in avian species, including adhesions, toxins, hemolysins and invasion genes (Ewers et al., 2009). The pathogenicity of *E. coli* strains is, in large part, due to the fact that they express genes for Shiga toxins (stx genes) and intimin, a virulence factor that is an outer membrane protein (eae)

(Beutin et al., 1995, Parreira and Gyles, 2002) identified a Stx- gene among *E. coli* strains isolated from chickens suffering from cellulitis, septicemia, and swollen head syndrome and from sick turkeys. Certain strains of Shiga toxin-producing *Escherichia coli* (STEC) are frequently identified as causative agents of life-threatening diseases in humans, such as hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS) (Nataro and Kaper, 1998). The majority of human diseases are associated with strains of STEC that produce either Shiga toxin 1 (Stx1) and/or Shiga toxin 2 (Stx2) which are encoded on lysogenic bacteriophages (Gyles, 2007). Our study revealed presence of Stx2 gene by 12.5 % and absence of Stx1 in all isolated serovars. These results were nearly agreed with those obtained (Al-Ajmi, 2011, Shimaa, 2013). Hemolysin is toxic to a range of host cells in ways that probably contribute to inflammation, tissue injury, and impaired host defenses (Scheffer et al., 1988). It is also highly cytotoxic to chicken embryo cell cultures (Chaturvedi et al., 1969). Hemolysin production is encoded by a four-gene operon termed hly (Goebel and Hedgpeth, 1982) a-hemolysin encoded by hlyA is an extracellular cytolytic protein toxin (Garcia et al., 2013). eaeA (intimin or *E. coli* attaching and effacing gene) (Franck et al., 1998). Our study revealed presence of these genes by 12.5 and 18.75%, respectively. These results came in accordance with those recorded (Ahmed et al., 2007). There is growing evidence suggesting that some genes involved in biofilm formation are also involved in adherence and colonization of host tissues (Latasa et al., 2005). (Naves et al., 2008) demonstrated that hlyA gene is one of the most common virulence genes involved in strong biofilm production. Intimin protein is responsible for the intimate adherence between bacteria and enterocyte membrane and encoded by eae gene (Al-Chalabi et al., 2010). In our study presence of eae and hly may explain the ability of *E. coli* to form biofilm. Non-judicious usage of antibiotics for therapeutic purpose or as growth promoters in poultry industry has led to selective pressure on various bacteria including *E. coli* and *Salmonella*. This resulted in emergence of multidrug-resistant strains which is a matter of serious concern for public health (Diarra et al., 2014). In this study, the presence of multidrug-resistant *E. coli* to *B*-lactams groups (Table 4) is attributable to the acquired ability of the strains to produce *B*-lactamase, which hydrolyzes *B*-lactams ring, rendering the entire compound inactive. At slaughter, resistant strains from the gut readily contaminate poultry carcasses, and as a result, poultry meats are often contaminated with resistant *E. coli* (Altekruse et al., 2009). In Enterobacteriaceae, resistance to antimicrobial agents is caused by the production of extended-spectrum beta-lactamases (ESBLs). Especially, ESBL-producing *Escherichia coli* which, represent an increasing problem for public health (Hamoudi and Aggad, 2008, ECDC, 2011). Among ESBLs, the CTX-M-type enzymes are most common and their number has increased rapidly during the last 10 years (Carattoli, 2013). (Overdevest et al., 2011) demonstrated that, the predominant ESBL genotype in chicken meat was *bla*CTX-M followed by *bla*TEM and *bla*SHV. In our study, 100% of isolates were carrying the encoding blaTEM. blaSHV and blaCTX were detected in 75% and 18.75% of isolates respectively. (El Tawab et al., 2015) studied the prevalence of *E. coli* in diseased chickens with its antibiogram pattern. And demonstrated that 73.3% of the isolates were resistant to a β-Lactam antibiotic (ampicillin) (Radwan et al., 2014) on their study on broiler chicken suffering from colibacillosis antibiogram profiles indicated the maximum resistance of

isolated *E. coli* strains to ampicillin (100%), this results in agreement with our results where all isolates were resistant to ampicillin. The spread of multidrug resistance among avian *E. coli* is usually endorsed to the selective pressure exerted by the antimicrobials included in broiler feed for the past years (Singer and Hofacre, 2006). (Daka and Awole, 2009) studied the antibacterial effect of garlic on diarrhea-causing bacteria and they revealed that dilute solutions of garlic can completely inhibit the growth of those pathogens (*Salmonella*, *Shigella* and *E. coli*). This is due to the action of a biologically active ingredient of *Allium sativum* (allicin) which acts primarily by blocking the sulfhydryl enzymes necessary for bacterial metabolism and by interfering with RNA synthesis (Radwan et al., 2014). In our study, the bactericidal efficacy was at 20 to 80 mg ml^{-1} of garlic against the isolated *E. coli* serovars (Table 3). The antibacterial effect of garlic apparently resulted from thiol-disulfide exchange reactions between sulfur compounds and free thiol groups of bacterial enzymes such as alcohol dehydrogenase, thioredoxin reductase, trypsin, other proteases, RNA and DNA polymerases (Jonkers et al., 1999). The bioactive functional groups detected in our garlic extract by FTIR along with XRF analysis indicated the presence of sulfur compounds (23.5±0.5 mol%) and sulfonyl groups ($S=O_3$) (40±1.2 mol%). (For more details, see the supplementary Figs. S1). This disruption can affect the cell essential metabolism and therefore the bacterial virulence and growth (Bakri and Douglas, 2005). Our results of UPLC-MS/MS for garlic extract confirmed five peaks with m/z values as 162, 177, 114, 234 and 142 and were identified as allicin, alliin, allylsulfide, E-ajoene and vinyldithiin (For more details, see the supplementary Figs. S2). During crushing, the mechanical breaking of the garlic bulb, the enzyme alliinase (lyase) is formed. Under its influence, alliin is disintegrated into allicin (an unstable product of strong garlic odor), pyruvic acid and ammonia. Allicin has bactericidal properties against Gram-positive and Gram-negative bacteria. Due to great instability, allicin disintegrates into among others: ajoene, vinyldithiin, allicin, DAS (diallyl sulfide), DADS (diallyl disulfide), DAT (diallyl trisulfide). These compounds, apart from vinyldithiin, have confirmed antimutagenic properties (Amagase et al., 2001). Vinyldithiin has diverse antibacterial and antiplatelet aggregation properties (Lutomski, 2001). Also, Ajoene (one of the garlic antibacterial compound) can inhibit the genes involved in pathogenicity controlled by quorum sensing (Jakobsen et al., 2012).

The pathogenicity of *E. coli* by oral inoculation of 7 day old chicks revealed that the most common clinical signs of colibacillosis in the infected groups were closed eye, diarrhea, depression, respiratory symptoms including gasping, rales and nasal discharge this finding in agreement with Barnes, 1994 (Barnes, 1994) who mentioned that the main clinical signs of naturally infected chicks with *E. coli* are reported as depression, loss of appetite, tendency to huddle respiratory distress, reduction of weight gain, dropped wing, closed eyes, cyanosis and labored breathing. At necroscopy, the most postmortem lesions were congestion in all organs with pericarditis, perihepatitis, splenomegaly, air sacculitis, and pneumonia. This in agreement with the results obtained by (Roy et al., 2006, Sharada and Ruban, 2010, Ewers, 2003.). Mortality rate in our study was 60%, these results in agreement (Calnek et al., 1997) who revealed that colibacillosis of the affected flocks may cause up to 75% mortality. In this study, the performance parameters of infected untreated groups

revealed significant decrease in body weight. Similar results were obtained by (Radwan et al., 2014). On the other hand, garlic treatment significantly increase body weight and decrease in mortality rate to 6.6 % (Figs 3 and 4).

Conclusion

The finding of 16 different *E. coli* serovars along with virulence genes (stx1, stx2, hly, and eaeA) is a great concern regarding colibacillosis infection and transmission. In addition, the association with antibiotic resistance genes (blaTEM, blaSHV, blaOXA and blaCTX) is more serious concern, because of the tendency to facilitate resistance. This is worrying as the management of colibacillosis, involving multi-drug resistant strains, is often hard to cure. Therefore, the efficacy of garlic was confirmed as strong antibacterial agent. Its active substances decreased also the ability of isolated *E. coli* for biofilm formation. Therefore, from the *In vivo* experiment results, garlic is recommended to improve food products as ideal antimicrobial agents as it has a selective toxicity as well as preventing further spread of resistant strains.

Ethical standards

This work was approved by South Valley University animal care and use committee (Approval No. 12-214). The use and care of experimental animals, used in this study, comply with the Egyptian animal welfare laws and policies.

Conflict of interest statement

The authors declare that they have no conflict of interest.

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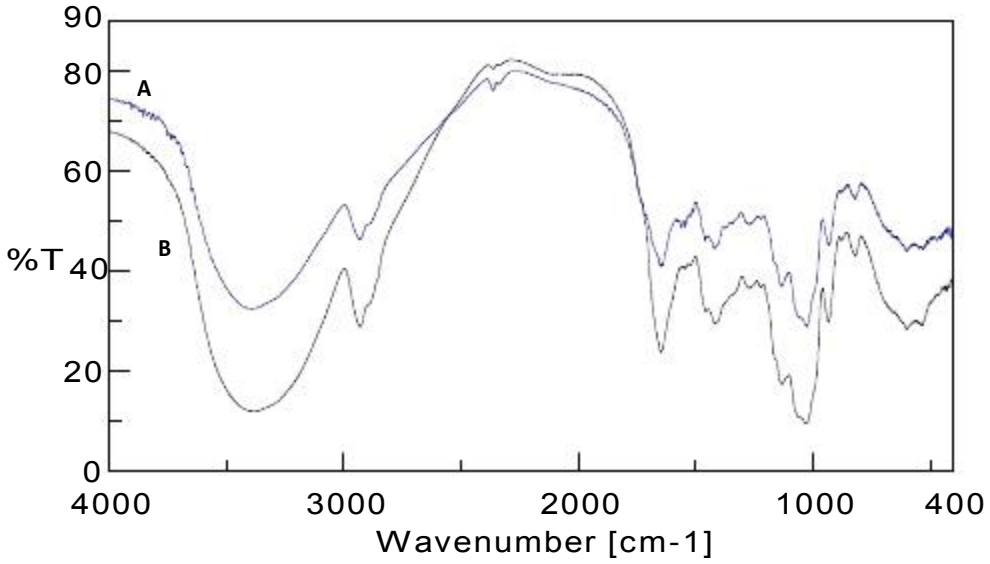
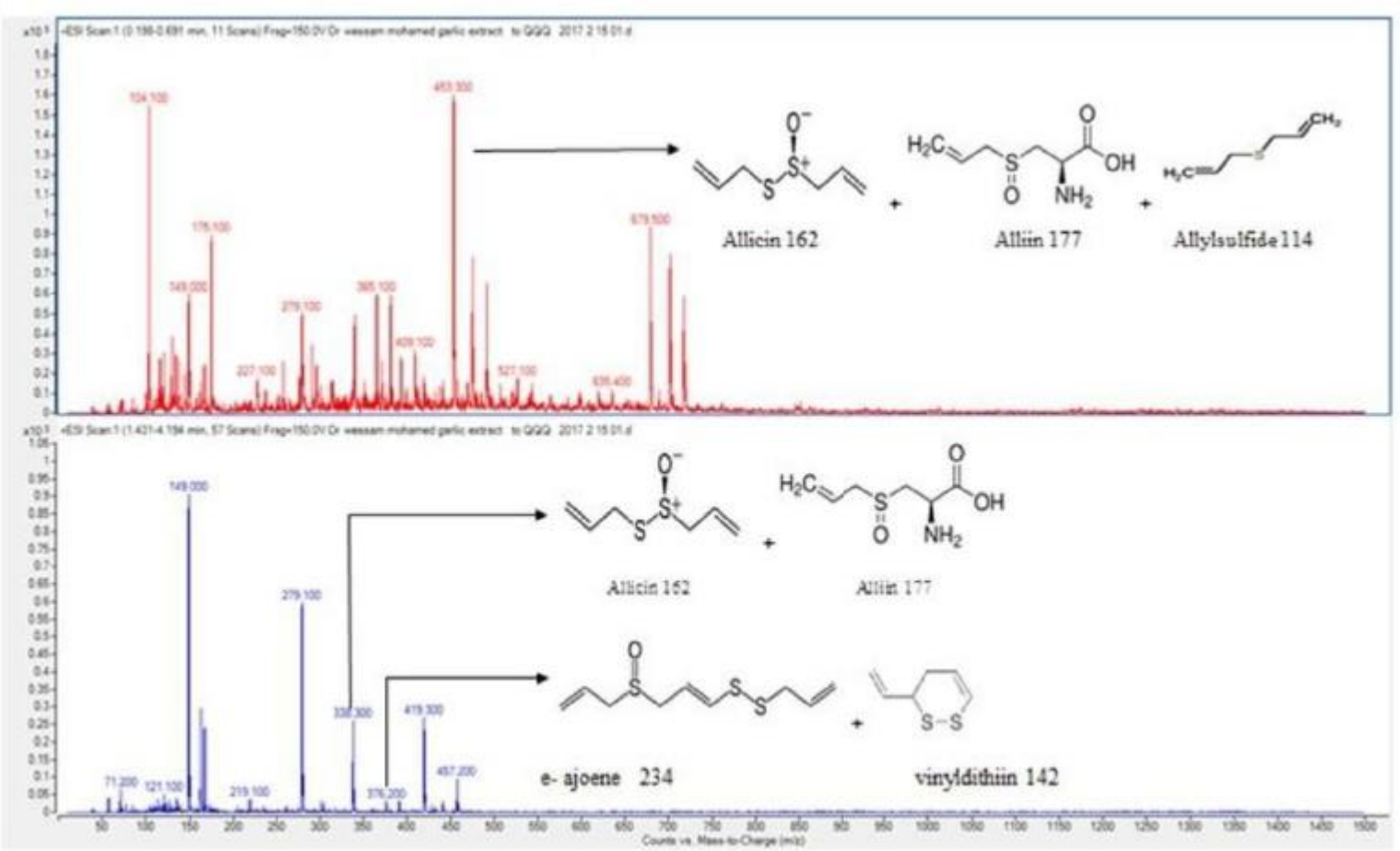


Supplementary Fig. S1. Fourier Transform Infrared Spectroscopy (FTIR) Spectrum of garlic (A) and garlic extract (B) showing bioactive functional groups. The FTIR spectrum obtained from original garlic and its extract showed absorption band at 3390, 2929, 1646, 1417, 1024 cm^{-1}. The peak position at 3390 cm^{-1} is due to OH-group of cellulose, hemicelluloses, lignin or phenol. While 2929 cm^{-1} reflect the presence of CH aliphatic and band at 1646 cm^{-1} corresponds to amidic carbonyl group (C=O). The most important bioactive functional groups of garlic extract were determined as S=O (sulphonyl or sulphonate) which present at 1417 and 1024 cm^{-1}.



Supplementary Fig. S2. Ultra-high-performance liquid chromatography/tandem mass spectrometry (UHPLC/MS/MS) of garlic extract showing important bioactive compounds.

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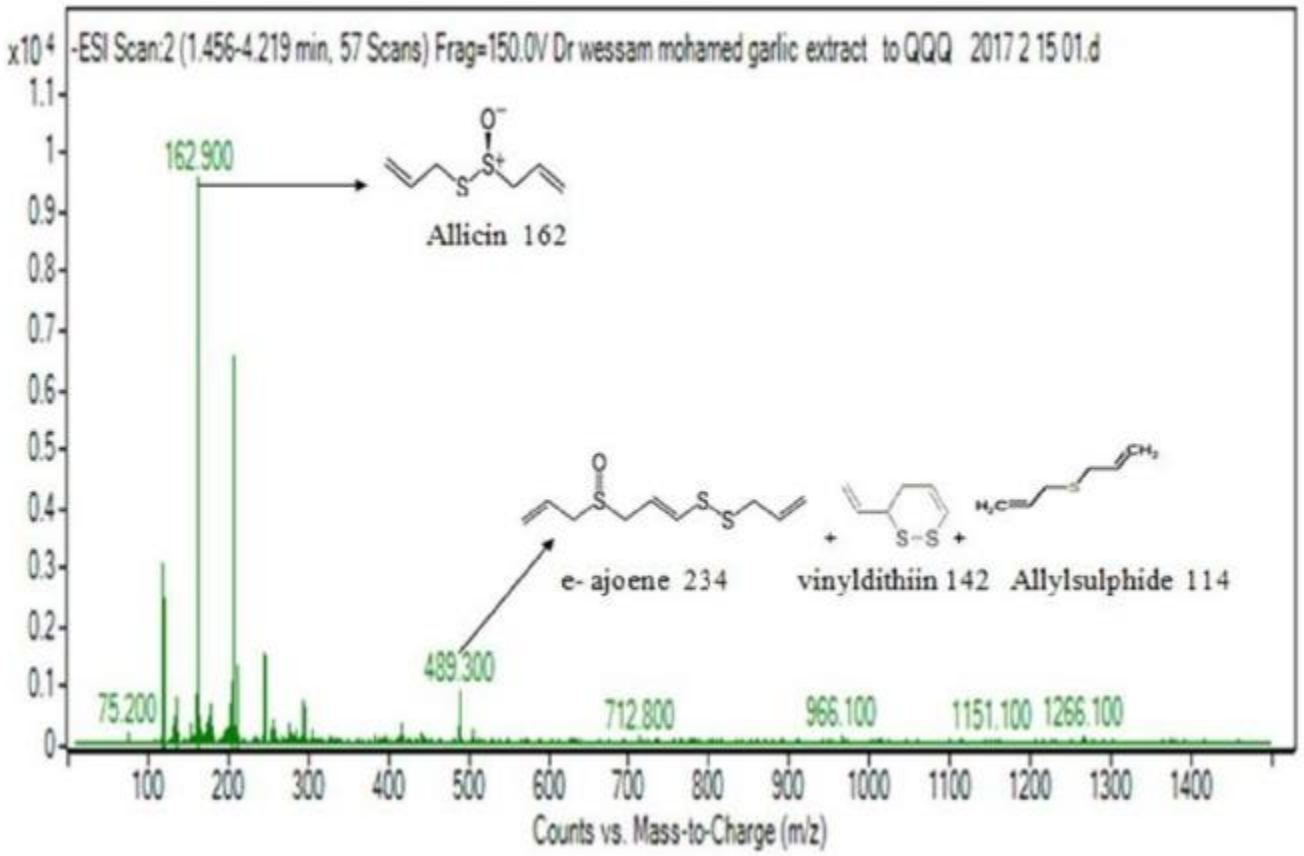

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