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Tracking of Resistant *Salmonella* Species in Poultry Farms: New Method of Control Using Essential Oils Nano-Emulsion Conjugated with Antimicrobial Agents

H. S. H. Salam[a], A. N. Mohammed[b], A. R. Hosni[c]*, & A. A. E. Shehata[d]
[a]Department of Bacteriology, Mycology, and Immunology, Faculty of Veterinary Medicine, Beni-Suef University, Beni-Suef 62511, Egypt
[b]Department of Hygiene, Zoonoses, and Epidemiology, Faculty of Veterinary Medicine, Beni-Suef University, Beni-Suef 62511, Egypt
[c]Directorate of Veterinary Medicine, El-Fayoum, Egypt
[d]Department of Bacteriology, Animal Health Research Institute, El-Fayoum Laboratory, El-Fayoum, Agricultural Research Center, Egypt
*Corresponding author: amanyreda31@yahoo.com
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ABSTRACT

This work was designed to monitor and track *Salmonella* spp. in the different internal organs (heart, liver, spleen, and caecum) of 247 bird species (chickens *n*=176, chicks *n*=47, ducks *n*=15, and ducklings *n*=9) with variable ages in two governorates; El-Fayoum and Beni-Suef, Egypt. Besides assessing the antimicrobial activity of antibacterial agents, essential oils, oils nano-emulsion, and their interactions with each other against *salmonellae* isolates for their control at the farm level. All samples were collected aseptically for further microbiological and serological investigations. Moreover, the efficiency of essential oils and oils nano-emulsion (thymol, carvacrol, basil, and cinnamon) against recovered *Salmonellae* were tested using the agar dilution method. A total of fourteen *Salmonella* serotypes were detected from different investigated internal organs (heart, liver, and spleen), and the three most predominant serovars were *S. virchow* (17.14%), *S. infantis* (11.43%), and *S. anatum* (11.43%). The resistance profile of *Salmonella* spp. referred to 47.14%, 40.0%, 31.43%, 25.71%, 21.43%, 21.43%, and 21.43% against ampicillin, chloramphenicol, gentamicin, aztreonam, cefazolin, cefotaxime, and tobramycin, respectively. The ability of essential oils (carvacrol oil 0.01%, basil 0.1%, cinnamon 0.01%, and thymol oil 0.01%) to inhibit the growth of *Salmonellae* differed significantly at 34.29%, 17.14%, 11.43%, and 1.43%, respectively ($p<0.05$). Oppositely, essential oils nano-emulsion (thymol 0.01%, carvacrol 0.001%, basil 0.1%, and cinnamon 0.01%) showed no inhibitory effect on the growth of *Salmonella* species. In conclusion, the interactive action between essential oils and antimicrobial agents approved the ability to enhance the susceptibility of the resistant *Salmonella* isolates against gentamicin, tobramycin, chloramphenicol, and cefazolin. In addition, the interactive action between essential oils nano-emulsion and antimicrobial agents on resistant *Salmonella* isolates revealed a complete enhanced effect against cefotaxime and variable enhancement against aztreonam.

Keywords: Salmonella spp.; poultry farms; essential oils; multidrug resistance; nano-emulsion

INTRODUCTION

Nowadays, the poultry industry has a high economic implication and is considered an important source of animal protein. In addition, any diseases affecting poultry might cause death and subsequently may cause a drop in investment. Therefore, most of the poultry farms subjected to any bacterial, viral, and/or parasitic diseases must be treated, and so biosecurity and hygienic measures should be taken to ensure a successful production cycle in different farms (EFSA *et al*., 2019).

Different lesions in the internal organs of birds affected by *Salmonella* spp. were demonstrated (Hessen, 2006). Sometimes no lesions were found in acute deaths characterized by a septicemic picture in the internal organs.

Among different ages of birds, bacterial diseases could cause high complications in poultry farms. Moreover, the infection with *Salmonella* spp. among different ages of poultry compromises a severe drop in poultry production. Clinically, chicks affected by salmonellosis showed poor growth, weakness, ruffled feathers, closed eyes, droopy wings, weight loss, diarrhea, and dehydration. Furthermore, postmortem (P/M) lesions of salmonellosis are represented by the enlarged liver with necrosis and spleen, unabsorbed yolk sac, and enteritis with necrotic lesions in the mucosa (Hessen, 2006).

Salmonella infection causes significant economic losses in the poultry industry due to the costs of surveillance, investigation, treatment, and illness prevention (Shekhar, 2018). Furthermore, salmonellosis kills

birds and has a negative impact on their performances (Mshelbwala *et al.*, 2017)

Currently, there are 2600 *Salmonella* serotypes scattered worldwide (Guibourdenche *et al*., 2010). The different *Salmonella* serotypes commonly detected among poultry include *Salmonella typhimurium, Salmonella enteritidis, Salmonella infantis, Salmonella montevideo, Salmonella newport*, and *Salmonella pullorum* (Kumar *et al*., 2019). Moreover, *S. typhimurium* and *S. enteritidis* were recorded in the highest percentage among Specific-pathogen-free of poultry chickens besides *S.* Montevideo (Osman *et al*., 2010; Hendriksen *et al*., 2011). Ducks are potential reservoirs for *Salmonella* serovars, mainly *S. enteritidis* and *S. anatum* (Yang *et al*., 2019). On the other hand, mostly *S. enteritidis* and *S. typhimurium* had zoonotic importance as foodborne pathogens that cause food poisoning in humans (EFSA and ECDC, 2016).

Variable antimicrobial classes are used for the treatment of *Salmonella* serotypes in the veterinary field. Members of aminoglycosides usually have good antimicrobial effects on the treatment of *Salmonellae* (Hossain *et al*., 2015). The resistance pattern of Gram-negative bacteria to antibacterial agents is higher than that of the Gram-positive ones due to an additional outer membrane acting as an effective barrier for amphipathic agents (Cole *et al*., 2014; Elshafie *et al*., 2016). To overwhelm the resistance patterns of *Salmonellae* and the bad effect of withdrawal time of antibiotics, using natural herbs including some essential oils (Thymol, carvacrol, basil, and cinnamon), which have strong inhibitory effects on *Salmonella* serovars (Rattanachaikunsopon & Phumkhachorn, 2010; Sakkas & Papadopoulou, 2017) is recommended. Furthermore, the preparation of oil in nano-emulsion will potentiate the inhibitory effect of the low-size particles that facilitate the application of essential oils (Chouhan *et al*., 2017; Pathania *et al*., 2018). The current study was designed to determine the prevalence rate of *Salmonella* spp. in different poultry farms, assess the antimicrobial activity of antibacterial agents, some essential oils, and oils nano-emulsion. In addition, this study was also designed to evaluate the use and exploitation of essential oils and/or oil nano-emulsion in the presence of antimicrobial agents for enhancing their effectiveness to control *Salmonella* serotypes in the birds and their environments.

MATERIALS AND METHODS

Ethical Approval

According to the animal ethics guidelines, this current study was approved by Institutional Animal Care and Use Committee (IACUC) with reference No. 021-156.

Study Area and Period

This study was conducted on different birds in variable poultry farms (*n*=23) located in Beni-Suef and El-Fayoum Provinces, Egypt, from May 2017 to December 2020. The investigated birds were freshly dead and/or diseased from gastrointestinal manifestations, mostly with whitish diarrhea in the examined poultry farms during the study period. The hygienic measures inside the investigated farms were slightly moderate.

Samples Collection

A total of 247 samples were collected aseptically from different internal organs (heart, liver, spleen, and caecum) of diseased and/or dead birds [chickens (*n*=176), chicks (*n*=47), ducks (*n*=15), and ducklings (*n*=9)]. Aseptically, pieces of the heart, liver, spleen, and caecum were taken by sterile scissors. Then, collected samples were classified *into* two groups, heart, liver, and spleen, as one sample, while caecum was a separate sample to reduce the possibility of cross-contamination between samples (Cox *et al*., 2007). After labeling, each sample was added to 5 mL peptone water broth in screw-capped test tubes and transferred to the lab in a sterile icebox. Moreover, pre-enrichment, differential, and selective medium were used for isolation of the *Salmonella* species. The microbiological isolation and identification were performed according to ISO 6579 (2002).

Isolation and Identification of *Salmonella* Species

All samples were transferred in screw-capped test tubes containing peptone water (1 g of samples on 9 mL peptone water) to the lab to be incubated at 37°C for 16-18h. Then, a loopful (100 µL) was taken from each incubated peptone water broth to 9.9 mL Rappaport Vassiliadis broth and incubated at 42°C for 24h. After that, a loopful from incubated Rappaport Vassiliadis broth was cultivated on MacConkey agar media, then pale colonies were taken and cultivated on XLD (Xylose lysine deoxycholate medium, Oxoid). Red colonies with a black center were cultivated on TSI (Triple sugar iron agar medium) and urease solution. Finally, colonies showed negative urease test and R/Y with black ppt. on TSI were kept on tryptone soya agar medium. All isolated *Salmonella* spp. strains were identified using biochemical tests include TSI, urease test, Oxidase test, Indole test, Methyl red test, Voges Proskauer test, Citrate test, and Lysine iron agar test according to methods described by Quinn *et al.* (2002).

Serotyping of Isolated *Salmonella* spp.

According to Grimont & Weill (2007), all *Salmonellae* were serologically identified in the Central Health Laboratories, Egypt, and Animal Health Research Institute Dokki, Giza, Egypt.

Assessing the Effectiveness of Antimicrobial Agents Against *Salmonella* spp. Preparation of All Testing Agents Antimicrobial Agents

Six antimicrobial classes of antibiotics were used: aminoglycosides (gentamicin, CN 10 µg and tobramycin, TOB 10 µg), first-generation cephalosporin (cefazolin, KZ 30 µg), third-generation cephalosporins

(cefotaxime, CTX 30 μg), phenicol (chloramphenicol, C 30 μg), penicillin (ampicillin, AMP 10 μg); monobactam (aztreonam, ATM 30 μg). The antimicrobial discs and Muller-Hinton agar were purchased from (Oxoid, UK).

Essential Oils Used

Four essential oils (EOs) include thymol, carvacrol, basil, and cinnamon oils, were used in the present study. Basil oil was extracted from the aerial parts of Ocimum basilicum collected from a flowering stage in the Naser region, Beni-Suef, Egypt, in March 2019. The method used to extract basil oil was hydro distillation using Clevenger type apparatus for 3 h (Elyemni *et al*., 2019). Anhydrous sodium sulfate was used during the dryness process, revealing the corresponding oil in 1.7% (W/W) then kept in a sealed vial at 4°C. The extraction process was performed in the herbarium of the Department of Botany, College of Science, Minia University, Egypt. The other three essential oils were purchased from (Sigma–Aldrich, St. Louis, MO, USA) and used in different concentrations using ethanol to enhance oil solubility (Chen *et al*., 2014). Thymol, carvacrol, and cinnamon oils were prepared at 0.1, 0.01, and 0.001% concentrations; meanwhile, basil oil was prepared at 1.0%, 0.1%, and 0.01% using the tenfold dilution method.

Essential Oils Nano-emulsion

Thymol, carvacrol, basil, and cinnamon oils nano-emulsion were performed using the Ultrasonication method as the method recommended by Pongsumpun *et al*. (2019). The formation of oil nano-emulsion was prepared in two steps. The first one is called "the oil phase" that includes the essential oil diluted into a certain concentration (thymol oil 0.01%, carvacrol 0.001%, basil 0.1%, and cinnamon 0.01%) then the second step was called "aqueous phase" that prepared by mixing Tween 80 3% in distilled water thereafter, adding "oil phase" slowly on the "aqueous phase" (W/W) with mixing vigorously on a magnetic stirrer (MSH- 20D, WiseStir) at 500 rpm, 25°C for 15 min. to prepare a coarse emulsion. Nano-emulsion was obtained after mixing in the ultrasonic bath (Ultrasons, P-Selecta). The sonication was operated at a fixed operation frequency of 43 kHz, power output of 210 W, and oscillation power at a high level. The ultrasound from the generator through a water bath was made at various temperatures (25°C) and exposure times (10 mins).

Characterization of Essential Oil Nano-emulsion

All used essential oils nano-emulsion were characterized using FT-IR (Fourier Transform Infrared Spectrum; a JEOL JEM 2000EX) and TEM (Transmission electron microscopy, JEOL-JEM -2100). FT-IR spectra were measured at the Faculty of Postgraduate Studies of Advanced Science, Beni-Suef University, Egypt, as shown in Figures 1, 2, 3, and 4. Meanwhile, the images of HR-TEM were measured at National Research Center (NRC), Egypt, as shown in (Figures 5, 6, 7, and 8) respectively.

Evaluating Methods

The antimicrobial sensitivity pattern of tested antimicrobial agents against recovered *Salmonella* serovars was tested using the disc diffusion method (CLSI, 2016). In contrast, seven antimicrobial discs representing various antimicrobial classes of antibiotics were used. The minimum inhibitory concentration of essential oils with *Salmonella* serotypes was detected using the agar dilution method (Rusenova & Parvanov, 2009). Furthermore, the agar dilution method as described by CLSI was used with some modification; a final concentration of 1% (v/v) diluted oil was poured into the agar medium (Muller-Hinton agar) after autoclaving and cooled at 50°C after that, plates were dried at 37°C for 30 min prior to spot inoculation with 2 μL aliquots of culture containing approximately 10^4 CFU of each organism. Inoculated plates were incubated at 37°C for 24h for *Salmonella* spp. The MICs were determined as the lowest concentration of oil inhibiting the visible growth of each organism on the agar plate. To evaluate the effectiveness of oil nano-emulsion, the tested concentration of oil nano-emulsion 1% was added to the agar medium (M.H.A.) by applying the agar diffusion method (Rusenova & Parvanov, 2009). After that, the interactive action between antimicrobial agents and both essential oils and oils nano-emulsion on resistant *Salmonella* serotypes showed the growth of *Salmonellae* using the disc diffusion method (CLSI, 2020) with some modifications in incorporation of essential oil 1% concentration (V/V) in the presence of seven antibiotic discs scattered on the agar medium after spreading of the microorganism.

Statistical Analysis

All data were prepared and analyzed using SPSS (statistical package for social sciences, version 22). The prevalence rate of *Salmonella* spp. and the frequency distribution of different serotypes of *Salmonella* isolates from the investigated birds and the effectiveness of different antimicrobial agents, tested essential oils, and oils nano-emulsions against all *Salmonella* serovars were analyzed using Chi-square test as a non-parametric test. The significance was confirmed at $p<0.05$ for both parametric and non-parametric tests.

RESULTS

The Prevalence Rate and Frequent Distribution of Different Serotypes of *Salmonella* spp.

The overall prevalence rate of *Salmonella* spp. among the investigated birds was 20.24% (50/247). The highest prevalence rates of salmonellosis significantly appeared in ducklings, followed by chicks, chickens, and ducks at $\chi 2= 15.21$, $p\leq 0.05$ (Table 1).

The different serotypes of *Salmonellae* (*n*=14) were obtained from each sample group and presented in Table 2. In Group A (*n*=35), including heart, liver, and spleen revealed that *S*. Virchow was significantly detected in the highest percentage at $\chi 2 = 12.34$, $p\leq 0.05$ followed by *S. infantis, S. anatum, S. paratyphi C, S.*

Table 1. Prevalence rate of *Salmonella* spp. species in different examined birds

Examined bird	Total		Prevalence rate (%)
	Examined No.	Positive No.	
Chickens	176	31	17.61[b]
Chicks	47	14	29.79
Ducks	15	2	13.33[ab]
Ducklings	9	3	33.33[a]
Total	247	50	20.24

Note: The frequency distribution of *Salmonella* species among different investigated birds are significantly differed in the column with different superscripts [a, b, & ab] ($p<0.05$).

Table 2. Frequent distribution of different serotypes of *Salmonella* spp. isolates

	Examined organs	Heart, liver, and spleen	Caecum
Total	Examined No.	247	247
	Positive No.	35	35
Distribution of different serotypes (No.%)	*S. virchow*	6 (17.14%)[a]	5 (14.29%)[a]
	S. anatum	4 (11.43%)	3 (8.57%)
	S. rehovot	2 (5.71%)	3 (8.57%)
	S. enteritidis	3 (8.57%)	2 (5.71%)
	S. salamae/ O6, 7: m, t	2 (5.71%)	1 (2.86%)
	*S. kedougou**	1 (2.86%)[b]	2 (5.71%)[b]
	S. salamae/ O6,7: g, m, {s}, t: e, n, x	1 (2.86%)	2 (5.71%)
	S. lumberhurst	2 (5.71%)	1 (2.86%)
	S. typhimurium	1 (2.86%)[b]	2 (5.71%)[b]
	S. fillmore	1 (2.86%)	2 (5.71%)
	S. montevideo	2 (5.71%)[c]	1 (2.86%)[c]
	S. paratyphi c	3 (8.57%)	4 (11.43%)
	S. infantis	4 (11.43%)	5 (14.29%)
	S. nanga	3 (8.57%)	2 (5.71%)

Note: Means in the same column with different superscripts differ significantly ($p<0.05$).

enteritidis, S. nanga, S. rechovot, S. salamae/ O6, 7: m, t, *S. lumberhurst, S. montevideo, S. kedougou, S. salamae*/ O6,7: g,m, {s}, t: e, n, x, *S. typhimurium*, and *S. fillmore*, respectively. In contrast, in group B (n=35) containing caecum showed that *S. virchow, S. infantis*, and *S. para-Typhi* C were found in the highest percentage, followed by *S. anatum, S. rechovot, S. enteritidis, S. fillmore, S. nanga, S. kedougou, S. salamae*/ O6,7: g,m, {s}, t: e, n, x, *S. typhimurium*, *S. salamae*/ O6, 7: m, t, *S. lumberhurst*, and *S. montevideo*, respectively.

FT-IR Spectrum and TEM Microscopy of Essential Oils Nano-Emulsion

The morphological characterization of thymol and carvacrol, basil, and cinnamon oil nano-emulsion were determined using FT-IR spectra, as shown in Figures 1, 2, 3, and 4. It has been found that FT-IR spectrum of thymol nano-emulsion clarified the widened and smooth peak was noticed at 3331.25 cm^{-1} that confirm hydrophilic (H-OH) interaction in nano-emulsion besides to the other noticed peaks at 1646.34, 1086.55, and 619.29 cm^{-1} as were shown in Figure 1b. Meanwhile, FT-IR spectra of carvacrol nano-emulsion exhibited the characteristic peaks that appeared at 3333.92, 1646.56, 1043.12, and 625.92 cm^{-1} (Figure 2b). On the other hand, basil essential oil showed variable peaks at 3379.45, 2923.95, 1514.98, 914.22, and 687.01 cm^{-1} (Figure 3a). In addition, FT-IR spectra of basil oil nano-emulsion appeared smooth peaks at 1669.16, 1118.83, 974.04, and 684.37 cm^{-1} (Figure 3b) that confirmed the formation of basil nano-emulsion. Moreover, FT-IR spectra of cinnamon oil (Figure 4a) showed the wide peak that appeared at 3350.96, 1647.34, 1047. 06, and 619.77 cm^{-1}. In addition, cinnamon nano-emulsion exhibited similar peaks at 3328.33, 1647.74, 1044.17, and 618.64 cm−1 (Figure 4b) that confirmed the success of nano-emulsion creation. Each thymol, carvacrol, basil, and cinnamon oil nano-emulsion were formulated by sonication for 20 min using thymol, carvacrol, water, and Tween 80 at a concentration of 3%.

In contrast, the TEM microscopy of thymol nano-emulsion showed the morphological shape of nanoparticles (Figure 5a) that appeared as a spherical particle distributed in the TEM field. Whilst on the opposite side, the average diameter of nanoparticles ranged from 24.02 nm to 54.0 nm (Figure 5b). Furthermore, the TEM image of carvacrol nano-emulsion showed the nanoparticles of spherical shape scattered into the nano-emulsion (Figure 6a). The nanoparticles' average size ranged from 0.03 µm to 0.13 µm (Figure 6b). TEM microscopy of basil nano-emulsion displayed the spherical and ovoid shapes of NPs scattered in the field of TEM (Figure 7a), with the average size ranged from 38.72 nm to 65.59 nm (Figure 7b). Finally, TEM microscopy of cinnamon nano-emulsion presented very fine spherical NPs in shape and had a raised surface (Figure 8a) besides the NPs size (Figure 8b) ranged from 53.21 nm to 54.30 nm in diameter.

The Effectiveness of Antimicrobial Agents Against *Salmonella* Serovars

The antimicrobial resistance profiles of *Salmonella* serovars are presented in Table 3. It has been found that *Salmonella* serovars exhibited their resistant patterns to ampicillin, chloramphenicol, gentamicin, followed by aztreonam, cefazolin, cefotaxime, and tobramycin, respectively.

To evaluate the effectiveness of thymol, carvacrol, basil, and cinnamon oils, MIC of essential oils and nano-emulsions against *Salmonella* serovars were detected and presented in Table 4. The complete inhibitory effect of thymol oil was clarified at a concentration of 0.1%. Whilst MIC of thymol oil for one *Salmonella* isolates at 0.01% concentration was 1.43%. Meanwhile, thymol oil nano-emulsion at 0.01% exhibited no effect on the growth inhibition of *Salmonellae*. On the other hand, all *Salmonella* serotypes were highly inhibited after using carvacrol oil 0.1% ($p<0.05$). The MIC of carvacrol was at 0.01% concentration in 34.29% *Salmonella* isolates. In contrast, carvacrol oil 0.001% in nano-emulsion showed that it did not affect all examined *Salmonella* serovars.

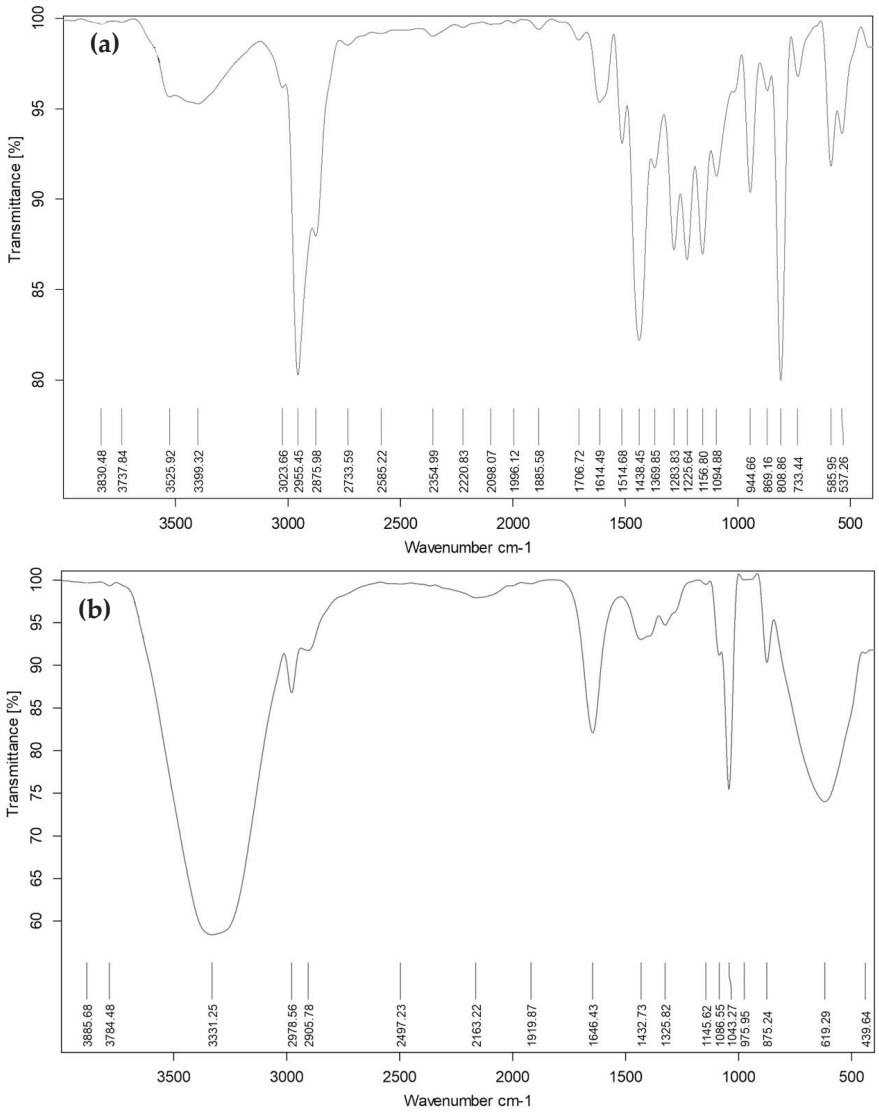

Figure 1. FT-IR spectra of thymol oil (a) at wavelength between 2955.45-585.95 cm^{-1} and thymol nano-emulsion (b) at strong infrared wavelength (3331.25-619.29 cm^{-1}).

Table 3. Antibiotics sensitivity testing against all *Salmonella* spp. isolated from the investigated birds

Antimicrobial agents	Tested concentration (μg)	Antibiotics sensitivity testing against *Salmonella* spp.					
		Sensitive		Intermediate		Resistant	
		No.	%	No.	%	No.	%
Aminoglycosides							
Gentamicin (CN)	10	42.0	60.0	6.0	8.57	22.0	31.43
Tobramycin (TOB)	10	46.0	65.71	9.0	12.86[a]	15.0	21.43[b]
Phenicol's							
Chloramphenicol (C)	30	39.0	55.71	3.0	4.29[b]	28.0	40.0
Penicillin's							
Ampicillin (AMP)	10	35.0	50.0[b]	2.0	2.86[c]	33.0	47.14[a]
Cephems (parenteral) including cephalosporins							
Cefotaxime (CTX)	30	48.0	68.57	7.0	10.0	15.0	21.43[b]
Cefazolin (KZ)	30	55.0	78.57[a]	0.0	0.0	15.0	21.43[b]
Monobactams							
Aztreonam (ATM)	**30**	**47.0**	**67.14**	**5.0**	**7.14**	**18.0**	**25.71**

Note: Total examined isolates (n=70); No.= Number of positive *Salmonellae*. The sensitivity pattern of *Salmonella* spp. serotypes to antimicrobial agents was significantly differed differed in the same column with different superscripts at $p<0.05$.

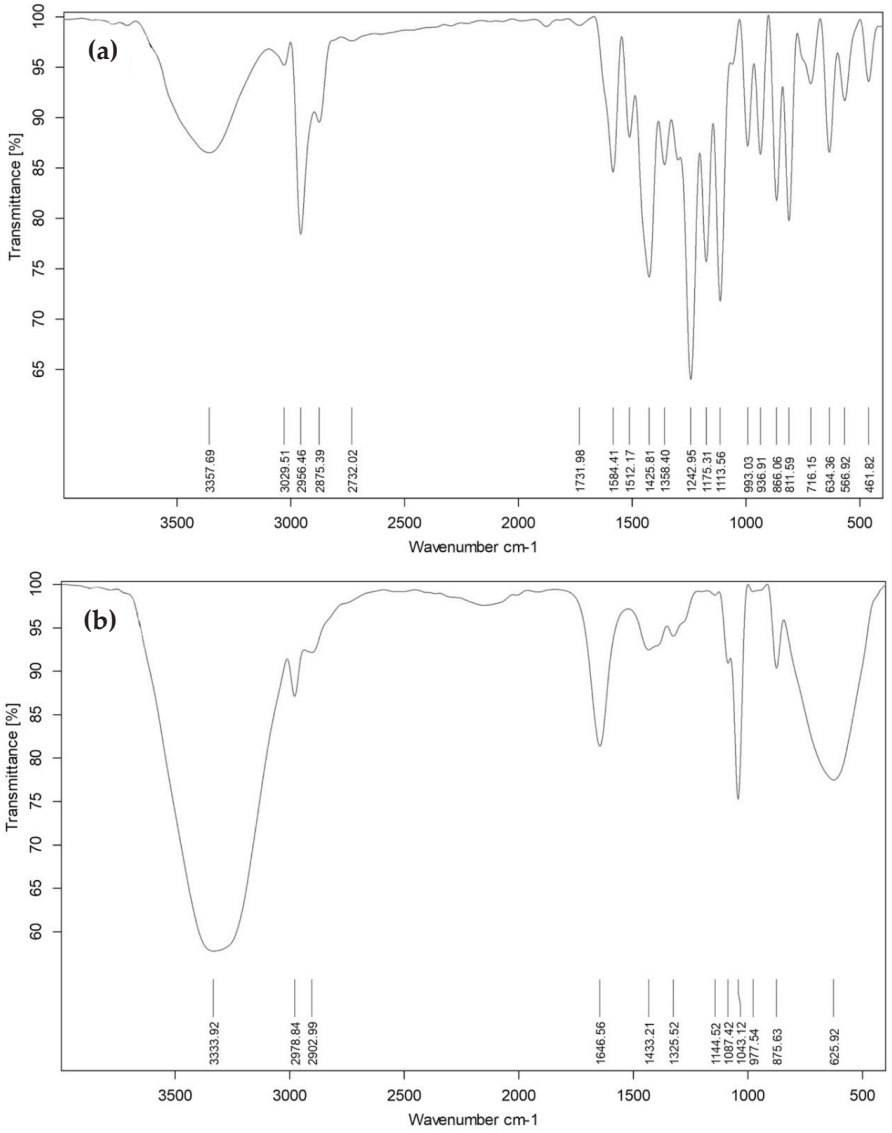

Figure 2. FT-IR spectra of carvacrol oil (a) at infrared wavelength 2996.45- 566.92 cm^{-1}, meanwhile infrared wave of carvacrol nano-emulsion (b) was 333.92-625.92 cm^{-1}.

Table 4. The antimicrobial effect of different essential oils and oils nano-emulsion against *Salmonella* spp. isolates

Essential oils	Tested conc. (%)	Sensitivity of *Salmonella* spp. to tested oils and oils nano-emulsion			
		Sensitive		Resistant	
		No.	%	No.	%
Thymol oil	0.1	70	100	0.0	0.0
	0.01	1.0	1.43	69	98.57[a]
	0.001	0.0	0.0	70	100.0
Thymol oil nano-emulsion	0.01	0.0	0.0	70	100.0
Carvacrol oil	0.1	70	100.0	0.0	0.0
	0.01	24	34.29	46	65.71[c]
	0.001	0.0	0.0	70	100.0
Carvacrol oil nano-emulsion	0.001	0.0	0.0	70	100.0
Basil oil	1.0	70	100.0	0.0	0.0
	0.1	12	17.14	58	82.86[b]
	0.01	0.0	0.0	70	100.0
Basil oil nano-emulsion	0.1	0.0	0.0	70	100.0
Cinnamon oil	0.1%	70	100.0	0.0	0.0
	0.01%	8.0	11.43	62	88.57
	0.001%	0.0	0.0	70	100.0
Cinnamon oil nano-emulsion	0.01%	0.0	0.0	70	100.0

Note: Total examined isolates (n=70); No.= Number of positive *Salmonellae*. The sensitivity pattern of *Salmonella* spp. to tested oils and oils nano-emulsion was significantly declared in the same column with different superscripts at $p<0.05$.

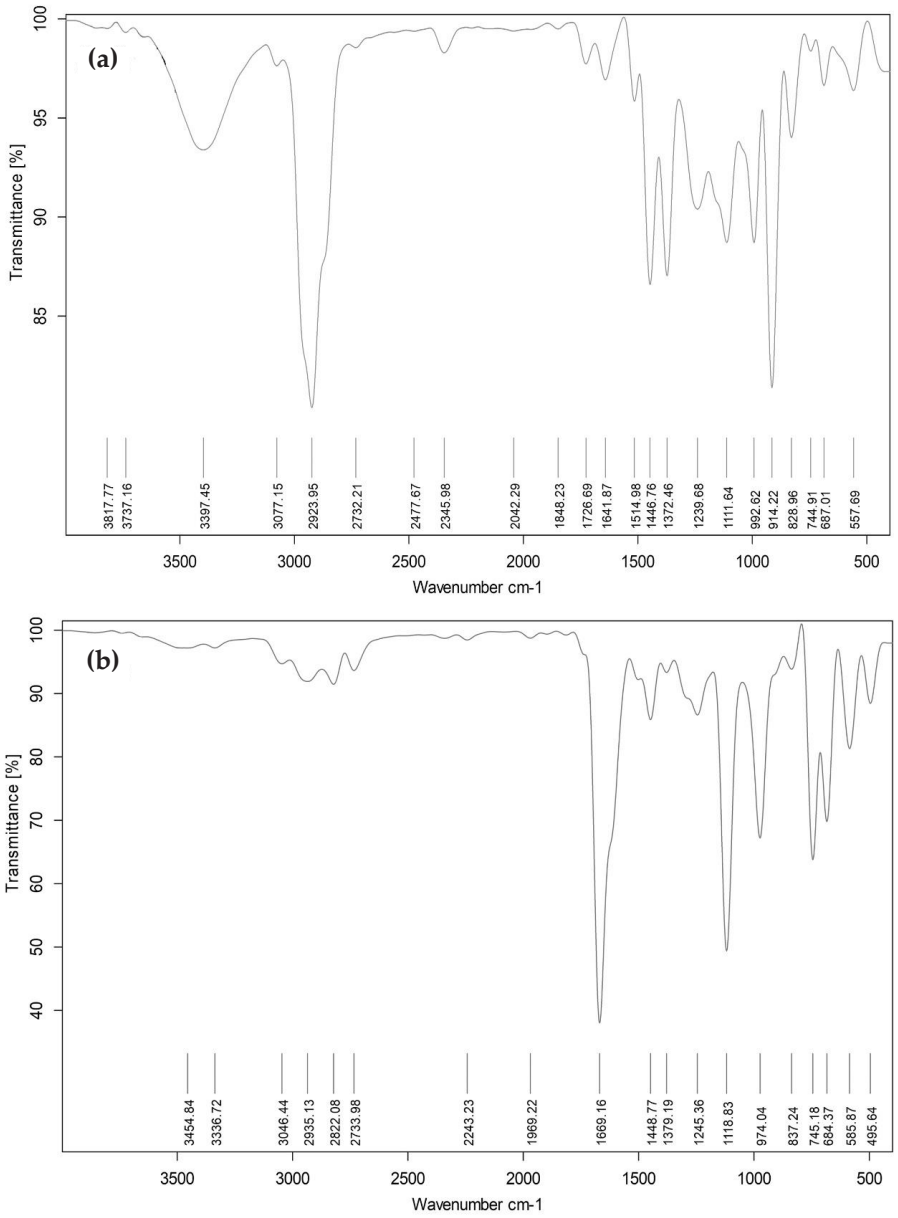

Figure 3. FT-IR spectra of basil oil (a) at infrared wavelength 2923.95-914.22 cm^{-1} and basil nano-emulsion (b) clarified at wavelength 1669.16-585.87 cm^{-1}.

Whilst basil oil 1.0% was completely effective against *Salmonellae*. In addition, basil oil 0.1% was effective against 17.14% of the tested isolates. Otherwise, the effect of nano-emulsion form not appeared on *Salmonella* serovars under examination. *Salmonella* serovars were completely inhibited with cinnamon oil 0.1%, while 11.43% of *Salmonella* spp. were inhibited at 0.01% of cinnamon oil. However, the nano-emulsion form did not affect *Salmonella* isolates. Oppositely, the effect of essential oils and oils nano-emulsion interactive with antimicrobial agents on resistant *Salmonella* serotypes was clarified (Table 5). All resistant *Salmonella* serotypes to gentamicin, tobramycin, chloramphenicol and cefazolin became completely sensitive after using thymol, carvacrol, basil, and cinnamon oils in the media. On the other hand, 11.43% of *Salmonella* serotypes were still resistant to ampicillin after using essential oils (thymol, carvacrol, basil, and cinnamon oils). In addition, thymol, carvacrol, basil, and cinnamon oils enhanced the susceptibility of resistant *Salmonellae* to cefotaxime except in some isolates under test at 7.14%, 7.14%, 14.28%, and 7.14%, respectively. Moreover, they enhanced the susceptibility of resistant *Salmonellae* to aztreonam except in some isolates under test at 17.14%, 18.57%, 20%, and 18.57%, respectively. The interaction between antimicrobial agents and nano-emulsion form of essential oils revealed a complete enhanced effect of essential oils in nano-emulsion form to *Salmonellae* against cefotaxime. Furthermore, the lethal effect of thymol, carvacrol, basil, and cinnamon nano-emulsion was detected when interactive with aztreonam against *Salmonella* spp. except 83.33%, 76.92%, 71.43%, and 76.92%, respectively of the examined isolates. Whilst there is no enhancement in susceptibility of *Salmonella* serotypes to ampicillin as displayed in Table 6.

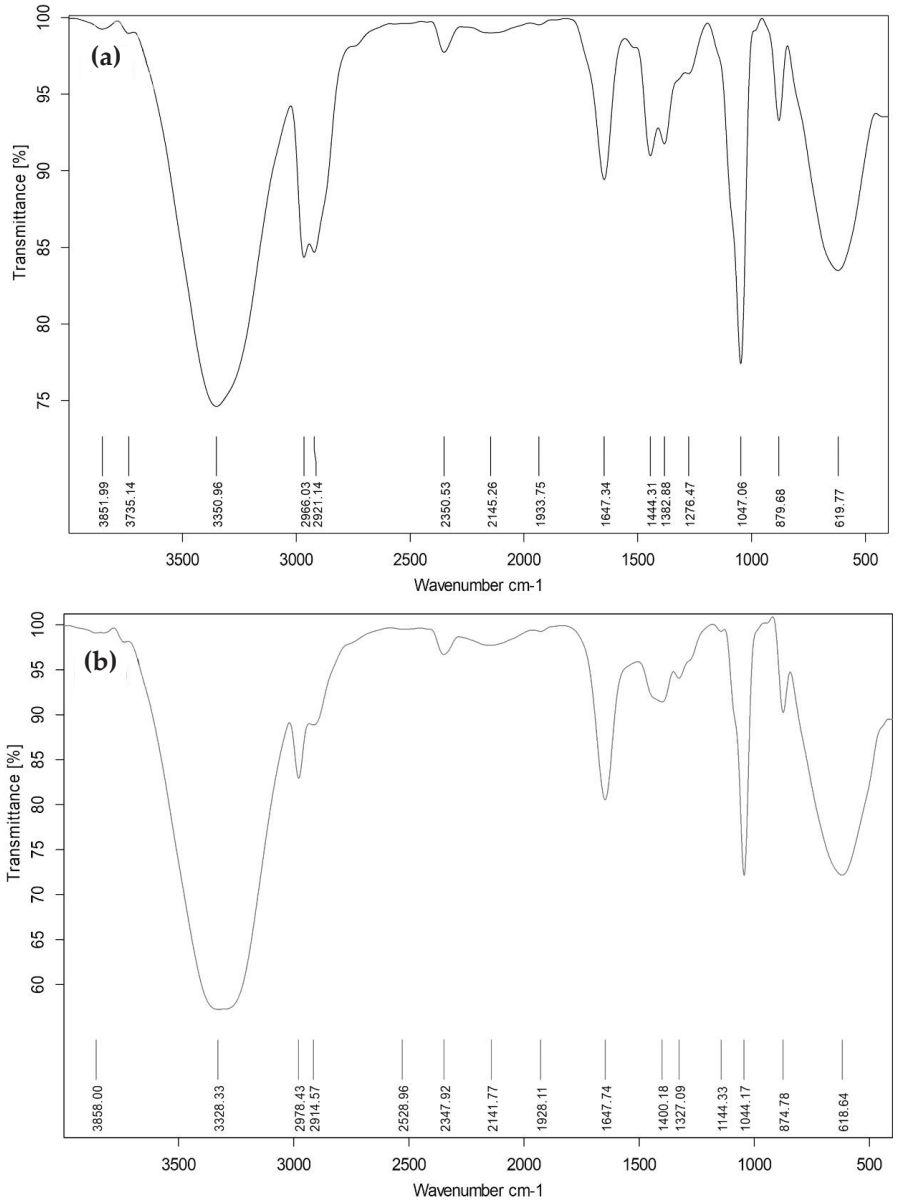

Figure 4. FT-IR spectra of cinnamon oil (a) at infrared wavelength 3350.96-619.71 cm^{-1} and infrared wavelength of cinnamon nano-emulsion (b) was 3328.33-618.64 cm^{-1}

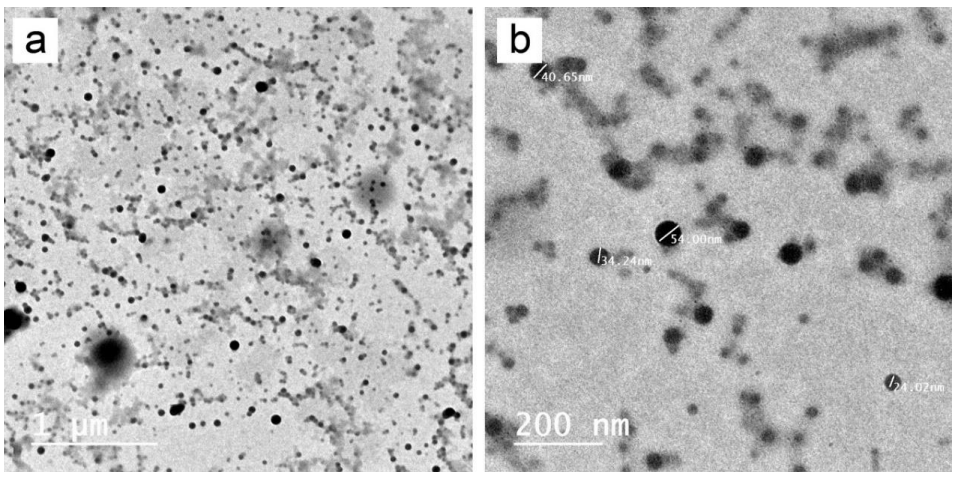

Figure 5. Transmission electron microscopy (TEM) of thymol oil nano emulsion (a-b) showed the morphological shape of nanoparticles (a) that appeared as a fine spherical particle distributed in the TEM field, and the average diameter of nanoparticles (b) is ranged from 24.02 to 54.0 nm

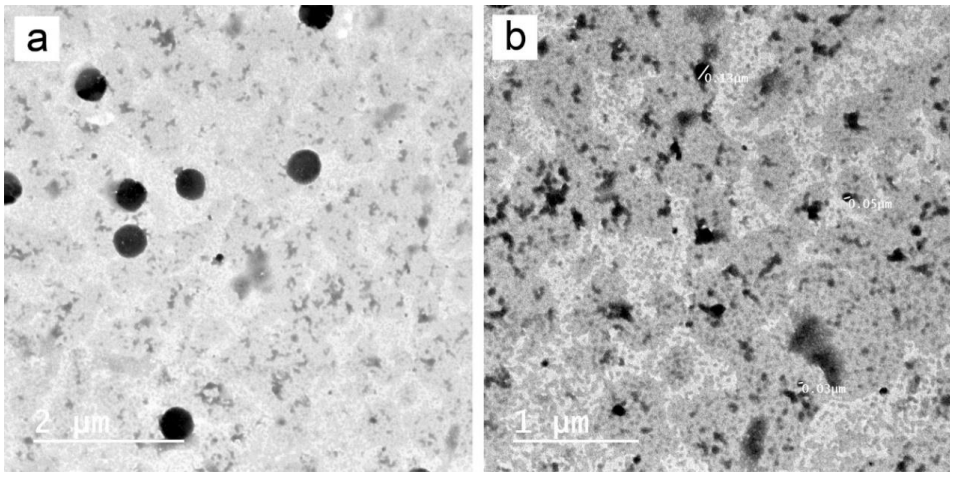

Figure 6. Transmission electron microscopy (TEM) of carvacrol oil nano-emulsion (a-b) revealed the nanoparticles of spherical shape scattered into the nano-emulsion (a), and the average size of nanoparticles is ranged from 0.03 to 0.13 µm as shown in (b).

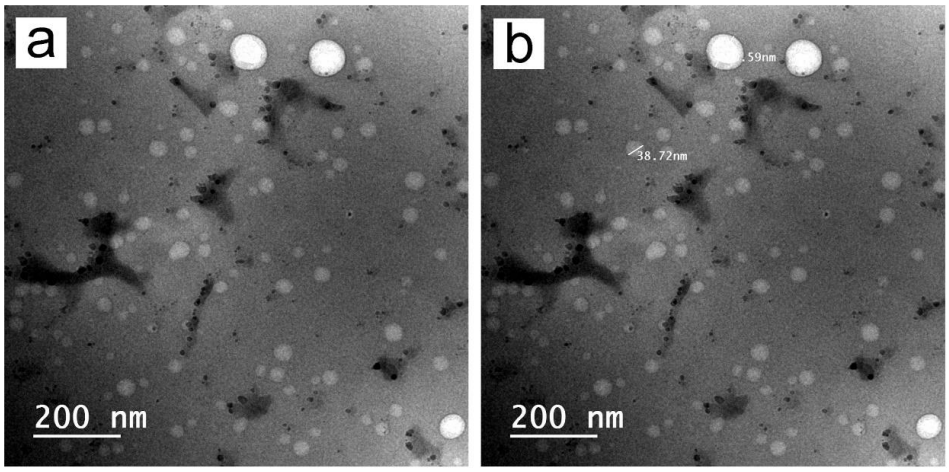

Figure 7. Transmission electron microscopy (TEM) of basil oil nano-emulsion (a-b) displayed the spherical and ovoid shape of NPs scattered in the field of TEM (a) with the average size is ranged from 38.72 to 65.59 nm (b).

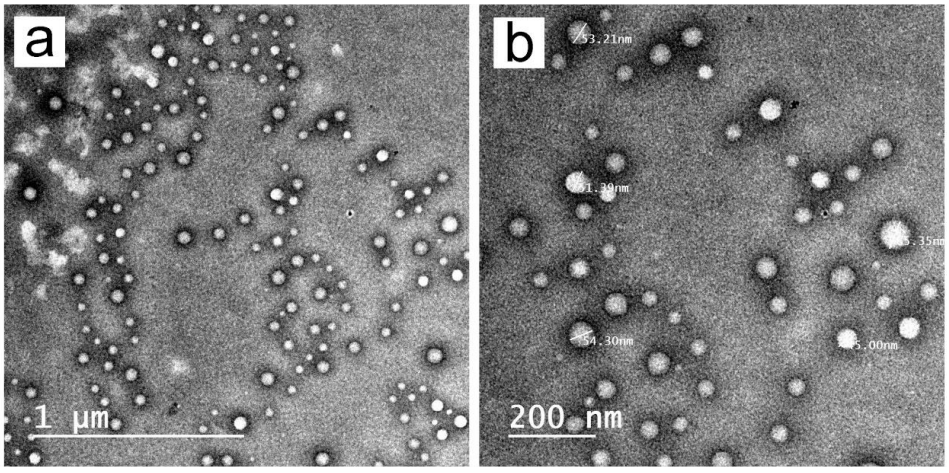

Figure 8. Transmission electron microscopy (TEM) of cinnamon oil nano-emulsion (a-b) presented very fine spherical NPs in shape and had a raised surface (a) besides the NPs size (b) is ranged from 53.21 to 54.30 nm in diameter.

Table 5. The interactive action between tested antimicrobial agents and essential oils on *Salmonella* spp. isolates

Tested antibiotic drugs (µg)		Efficiency of antibiotic alone			The interactive action between antibiotics drugs and tested oils on *Salmonella* spp. isolates (n=70)											
					Thymol oil 0.01%			Carvacrol oil 0.001%			Basil oil 0.01%			Cinnamon oil 0.001%		
		R	I	S	R	I	S	R	I	S	R	I	S	R	I	S
Gentamicin	No.	22	6	42	0	6	64	0	6	64	0	6	64	0	6	64
	%	31.43	8.57	60	0	8.57	91.43	0	8.57	91.43	0	8.57	91.43	0	8.57	91.43
Tobramycin	No.	15	9	46	0	9	61	0	9	61	0	9	61	0	9	61
	%	21.43	12.86	65.71	0	12.86	87.14	0	12.86	87.14	0	12.86	87.14	0	12.86	87.14
Chloramphenicol	No.	28	3	39	0	3	67	0	3	67	0	3	67	0	3	67
	%	40	4.29	55.71	0	4.29	95.71	0	4.29	95.71	0	4.29	95.71	0	4.29	95.71
Ampicillin	No.	33	2	35	8	1	61	8	0	62	8	0	62	8	0	62
	%	47.14	2.86	50	11.43	1.43	87.14	11.43	0	88.57	11.43	0	88.57	11.43	0	88.57
Cefotaxime	No.	15	7	48	5	2	63	5	3	62	10	2	58	5	2	63
	%	21.43	10	68.57	7.14	2.86	90	7.14	4.29	88.57	14.28	2.86	82.86	7.14	2.86	90
Cefazolin	No.	15	0	55	0	0	70	0	0	70	0	0	70	0	0	70
	%	21.43	0	78.57	0	0	100	0	0	100	0	0	100	0	0	100
Aztreonam	No.	18	5	47	12	1	57	13	0	57	14	0	56	13	2	55
	%	25.71	7.14	67.14	17.14	1.43	81.43	18.57	0	81.43	20	0	80	18.57	2.86	78.57

Note: R= Resistant; I= Intermediate; S= Sensitive.

DISCUSSION

The monitoring and tracking of different *Salmonella* serotypes in investigated poultry farms with different bird species and ages were considered the milestone for controlling bacterial infections. During this study, the highest prevalence rates of salmonellosis appeared in ducklings and chicks, followed by chickens and ducks that might be attributed to the absence of sanitation and hygienic measures among poultry farms (EFSA *et al*., 2019). Whilst Ahmed *et al*. (2019) found that the total prevalence rate of *Salmonella* infection was 6.4% in poultry farms. The prevalence rate of *Salmonellae* recovered from chicks was 7.5% (Sedeik *et al*., 2019); meanwhile, in chickens was 13.6% (Elsotohy, 2019). Oppositely, the prevalence rate of *Salmonella* spp. in ducks during this study was lower than recorded by Eid (2019). In addition, EFSA *et al*. (2019) recorded the distribution of variable *Salmonella* serovars among poultry, including *S. enteritidis*, *S. typhimurium*, *S. virchow*, and *S. infantis*.

The morphological characterization of thymol nano-emulsion by FT-IR revealing the widest peak at 3331.25 cm^{-1} with spherical particles and average size from 24.02 nm to 54.0 nm was detected through TEM in a parallel line with Wu *et al*. (2012) and Kumari *et al*. (2018). While thymol nano-emulsion using chitosan was characterized by nanoparticles ranged from 117-250 nm (Sotelo-boy's *et al*., 2015). In addition, carvacrol oil nano-emulsion exhibited spherical particles with 30-130 nm in size. Besides, wide peaks appeared at 3333.92 $cm^{-1,}$ similar to the result reported by Wu *et al*. (2012). Furthermore, FT-IR of cinnamon nano-emulsion revealed the widest peak at a frequency range of 450-4000 cm^{-1} with the highest peak at 3328.33 $cm^{-1,}$ and TEM microscopy showed spherical shape particles with diameter size ranged from 38.72 to 65.59 nm that was in line with the results reported by Ghosh *et al*. (2013). Additionally, Medhat *et al*. (2019) and Hussein *et al*. (2017) observed that the particles of carvacrol nano-emulsion are very fine and small (less than 50 nm in diameter), and the nano size of carvacrol was stabilized against aggregation. The effectiveness of oil nano-emulsion against *Salmonella* isolates exhibited lower decimal reduction concentrations than using essential oils in its form, which is a parallel way with Ahmed *et al*. (2017). The recovered *Salmonella* serotypes were exposed to six antimicrobial classes of antibiotics of public health concern to assess the susceptibility patterns. The obtained results showed that the higher resistance pattern of *Salmonella* spp. to both gentamicin and chloramphenicol was attributed to overuse and/ or misuse of antibiotics (Bertrand & Hocquet, 2011; Wibisono *et al*., 2020). While Badr *et al.* (2015) found that *Salmonella* serovars were completely sensitive to gentamicin and chloramphenicol in opposite to our results revealed resistance with 31.43% and 40% to the same antibiotics. Otherwise, the complete resistance of *Salmonellae* recorded by Ahmed *et al*. (2019) against ampicillin, cefotaxime, and aztreonam had a highly diverse opinion upon our resistance profile to the same antibiotics represented by 47.14%, 21.43%, and 25.71%, respectively. The sensitivity pattern of *Salmonella* spp. to four essential oils, including

Table 6. The sensitivity testing of resistant *Salmonella* spp. to essential oils, oils nano-emulsion, and antimicrobial agents

Essential oils/ nano-emulsion		The sensitivity pattern of resistant *Salmonella* spp. to tested essential oils, oils nano-emulsion, and antimicrobial agents		
		Ampicillin (AMP)	Cefotaxime (CTX)	Aztreonam (ATM)
		Resistant isolated No.	Resistant isolated No.	Resistant isolated No.
Thymol oil 0.01%	Alone	8.0	5.0	12.0
	Nano-emulsion	8.0	0.0	10.0
	%	100	0.0	83.33
Carvacrol 0.001%	Alone	8.0	5.0	13.0
	Nano-emulsion	8.0	0.0	10.0
	%	100	0.0	76.92
Basil 0.1%	Alone	8.0	10.0	14.0
	Nano-emulsion	8.0	0.0	10.0
	%	100	0.0	71.43
Cinnamon 0.01%	Alone	8.0	5.0	13.0
	Nano-emulsion	8.0	0.0	10.0
	%	100	0.0	76.92

thymol, carvacrol, basil, and cinnamon were tested to overcome the multidrug resistance pattern of *Salmonella* spp. isolates during the study and avoiding the residue hazards of antimicrobial agents in the poultry carcasses. Regarding the chemical structure of thymol and carvacrol, causing changes in the structure of Gram-negative bacteria, including *Salmonella* spp. (Guarda *et al*., 2011; Van de Vel *et al*., 2019). Furthermore, the antimicrobial activity of carvacrol and thymol oil was significantly high against *Salmonella* strains. These results were in parallel with Du *et al*. (2015) and Boskovic *et al*. (2016). Moreover, the antimicrobial activity of carvacrol against *Salmonellae* was higher than that of Ocimum basilicum and thymol oils, whereas these results agreed with the result reported by Soković *et al*. (2010). On the contrary, the antimicrobial activity of thymus and oregano oils was high which might be due to their high content of phenol components (Soković *et al*., 2010). The low water solubility of different essential oils leads to limitations in their antimicrobial activities. Therefore, using the disc diffusion method was not concerned in the current study with the essential oils used, whereas the study was agreed with Soković *et al*. (2010). Oppositely, the effect of essential oils and oils nano-emulsion interactive with antimicrobial agents on resistant *Salmonella* serotypes was clarified that all resistant *Salmonella* serotypes to gentamicin, tobramycin, chloramphenicol, and cefazolin became completely sensitive after using thymol, carvacrol, basil, and cinnamon oils in media. These results were in line with Idris *et al*. (2015), who found that thymol and carvacrol oils had a potent inhibitory effect against *Salmonella* spp. besides, the inhibitory effect of encapsulated nano thymol and carvacrol oil against *Salmonellae* was high. In this context, thymol, carvacrol, basil, and cinnamon oils revealed that the ability to enhance the susceptibility of resistant *Salmonellae* to cefotaxime except some *Salmonella* isolates as the results recorded by Ribeiro *et al*. (2020), who proved that the highest synergistic activity between basil and oregano oils conjugated with cefotaxime in inhibition of Gram-negative bacteria. In our study, the interaction between antimicrobial agents and oils nano-emulsion revealed a complete enhanced effect of oils nano-emulsion on *Salmonellae* against cefotaxime. Furthermore, the lethal effect of thymol, carvacrol, basil, and cinnamon nano-emulsion was detected when interactive with aztreonam against *Salmonella* spp. From the obtained results, it has been found that the interactive action between each essential oil, oil nano-emulsion with different antimicrobial agents was considered the upcoming promising for control of *Salmonella* infection at the farm level. In addition, reinforcing hygienic measures inside the investigated farms could minimize the risk of *Salmonella* infection. So, further studies are needed to evaluate the *in-vivo* usage of oils nano-emulsion for control of *Salmonella* serotypes and to ensure their suitability as an alternative to antimicrobial agents for the control of bacterial infections.

CONCLUSION

This study verified the wide distribution of different *Salmonella* serovars of zoonotic importance among poultry farms, including *S. virchow, S. typhimurium*, and *S. infantis.* Furthermore, the resistance pattern of *Salmonella* spp. to both gentamicin and chloramphenicol was higher than other tested antimicrobial agents. Using essential oils is operative sufficiently against *Salmonella* spp. infection to avoid multidrug resistance. Oppositely, essential oils nano-emulsion exhibited a low effect in growth inhibition of *Salmonellae.* Still, the interaction with antimicrobial agents helps enhance *Salmonellae* susceptibility to the different tested antimicrobial agents.

CONFLICT OF INTEREST

All authors declare that there is no conflict of interest during this work.

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