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NANOPARTICLES-PHENOLICS AS ANTI *SALMONELLA* TYPHIMURIUM

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ABSTRACT

The present study was designed to evaluate the activities of six phenolic compounds. Their activities against the potential foodborne pathogen *S.* Typhimurium were assessed using macro dilution and spectrophotometric methods. Their activities were in the order of thymol > benzoic acid > coumarin > cinnamic acid > curcumine > gallic acid. Thymol was bactericidal at a concentration of 0.08 mg /ml. With exception of curcumine, other phenolics revealed bactericidal effect in concentration varied from 1.25 to 10.00 mg/ml. Minimum inhibitory concentration (MICs) values by spectrophotometric method were significantly different compared to visual method in some antimicrobial assays. Coating fish with solutions of thymol or chitosan nanoparticle (CNPs) significantly reduced salmonella population. The nanostructured thymol CNPs capsule controlled the release of thymol and the effect in fish matrix continued significant during cold storage without adverse effect on pH value. The tested phenolics have the potential to be used in development of food coating technology. Also the formulated nanocapsule is promising in controlling the hazard of *S*. Typhimurium in fish.

Keywords: Phenolics —thymol- Salmonella – Nanoparticles encapsulation.

INTRODUCTION

Salmonellosis is a very common enteric infection which may be mild or severe life-threatening disease. The causative agent is Gram-negative bacterium belonging to the family *Enterobacteriaceae*. The major cause of human salmonellosis outbreaks in the United States and Europe is *Salmonella enterica* serovar Enteritidis (Gould *et al.,* 2013 and Collard *et al.,* 2008). The main routes of transmission are live stocks, consumption of contaminated food and human-to-human via the fecal-oral route (Tarabees *et al.,* 2017 and Kassem *et al.,* 2016).

Food safety is a shared goal for both consumers and food producers. Contamination with salmonella represents a threat to public health. It is one of the top 4 germs in the world with more

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hospitalizations and deaths than any other bacteria found in food (EFSA and ECDC, 2018). It has been repeatedly detected in a diverse variety of food products**.** Studies on food samples from street venders, butchers shops, retail markets and slaughterhouses (Ahmed and Shimamoto, 2014 and Ahmed *et al.,* 2014) as well as seafood (Bakr *et al.,* 2013) revealed salmonella. Besides, 68% of detected *Salmonella enterica* isolates showed multidrug resistance phenotypes which are of great health significance (Ahmed *et al.,* 2014).

Great efforts of research work have been directed toward the prevention of food borne diseases. Prevention demands critical antimicrobial strategies to decontaminate the food through its procession from farm till consumption (Jayasena and Jo, 2013). Researchers have been studied the inhibitory effect of extracts of spices (Ravichandran *et al., 2011* However, little is known about the potential use of the phenolic compounds which could be exploited by the food processors for use as natural preservatives.

Phenolics are bioactive substances occurring widely in food plants. The phenolic fraction of plant extracts has been linked to their antimicrobial activity as natural and safer alternatives to chemicals in food systems with the advantage of low cost. Their application as food preservatives on wide scale still faces limitations due to their high volatile character and sensitivity to oxygen (Hyldgaard *et al.,* 2012). Encapsulation of bioactive materials was reported to enhance their solubility and stability (Ghaderi-Ghahfarokhi *et al.,* 2016). In this respect, nanotechnology serves to manipulate matter at the nanometre scale, create and assemble substances at a molecular level with new and interesting properties. This study aims to evaluate the activity of some phenolics against *Salmonella* Typhimurium, and investigate the effect of nanoencapsulation of phenolic on its antibacterial efficiency in fish matrix.

MATERIALS AND METHODS

Bacterial strains

Salmonella Typhimurium- Reference strain (NCTC12023) was obtained from Animal Health Research Institute, Assiut, Egypt. The culture was activated by cultivation from the stock culture into Brain Heart Infusion Broth (BHIB), and incubated overnight at 35°C. Then subcultured by seeding growth to Xylose Lysine Deoxycholate agar (XLD agar, Himedia M031) and incubated 24 h at 35°C**.** Typical colonies were picked on Brain Heart Infusion Agar (BHIA) slants and incubated for 24h at 35°C as recommended by (Hsiao and Siebert, 1999).

Preparation of inoculum

Inoculums were prepared by seeding pure growth from the slants to XLD agar and incubated 24h at 35°C then four typical colonies were transferred to 10 ml BHIB and incubated for 18 h at 35°C. The optical density (OD_{625}) of the growth suspension was measured at 625 nm using spectrophotometer (Stat-fax 2100 spectrophotometer) where non-inoculated broth acts as blank. The bacterial suspension was diluted to approximate level OD_{625} of (0.08 – 012) that corresponds to 0.5 McFarland and confirmed by counting the number of cfu / ml on agar plates (McFarland 1907; Natta *et al.,* 2008).

1. Evaluation of antimicrobial activities of phenolic compounds:

1.1. Determination of minimum inhibitory concentrations (MICs) against *S.* Typhimurium by macro (visual) and micro (spectrophotometric) dilution methods:

Thymol, gallic acid, curcumine, coumarin, cinnamic acid, benzoic acid were obtained from El-Goumhouria Company for Trading

Chemicals, Cairo, Egypt. All are (≥98.5% purity) according to manufacture label. Separate stock solutions (10 mg/ml) each of phenolic compounds were prepared in BHIB with dimethylsulfoxide (DMSO, Sigma–Aldrich Co., USA) at 5% initial concentration as a solvent. Then, double fold serial dilutions (DFSD) of these compounds were prepared separately from stock solutions using BHIB to obtain concentrations of 0.004, 0.009, 0.019, 0.039, 0.078, 0.156, 0.312, 0.625, 1.250, 2.500, 5.000 and 10.000 mg/ml. MICs were determined in tubes and in sterile 96 well flat bottomed polystyrene microtitre plates as recommended with the Clinical and Laboratory Standards Institute (CLSI, 2012). In spectrophotometric method, each well was inoculated with 5 µl of target bacterial suspension (calculated 1.5 $x10^8$ cfu /ml) and 300 µl of fresh prepared DFSD of phenolic compound under investigation. BHIB alone was included to detect any cross contamination during shaking or handling. The phenolic compound dilutions without bacteria were used as a blank. Wells contains bacteria without phenolic compound were used as positive control.

To determine MICs using visual method, the same dilutions and inoculum concentration of target bacteria were used but wells were replaced by Wassermann tubes. After gentile mixing, the inoculated tubes and microtitre plate were incubated (mostly without agitation) at 35°C for 24 h. Optical density readings of test microplates were obtained using a microplate reader while tubes were examined by naked eye. The experiments were performed in triplicates.

In the spectrophotometric method, the lowest concentration of phenolic compound with OD_{600} reading equal to blank OD_{600} was considered the MIC (Pacheco-Ordaz *et al.,* 2017) while in visual method, the lowest concentration of clear tubes (no turbidity) was recorded as MIC (CLSI, 2012). The growth inhibition percent (GI %) was calculated using the equation recommended by (Liu *et al.,* 2017) where :

$$GI\,(\%) = OD_{\text{bacteria}} - (OD_{\text{(bacteria+ antimicrobial)}} - OD_{\text{antimicrobial}}) \times 100/\, OD_{\text{bacteria}}$$

OD (bacteria): is the OD_{600} for the positive control, OD (bacteria+ antimicrobial): is the OD_{600} for the sample treated with phenolic compound and OD (antimicrobial): is the OD_{600} for the negative control.

1.2. Determination of minimum lethal concentrations (MLCs) against *S.* Typhimurium:

The MLCs were assessed in accordance to (CLSI, 2012) where 0.1 ml from each tube that not revealed apparent growth were surface spread onto tryptic soya agar. Plates were incubated for 24 h at 35°C. The lowest concentration of tested material showing no growth after incubation was considered as the MLC.

2. Evaluation of antimicrobial activity of chitosan, chitosan nanoparticles and phenolic-chitosan nanoparticles:

2.1. Preparation of nanoparticles (NPs):

Thymol was the most effective phenolic against *S.* Typhimurium in the present study so, it was chosen for loading on chitosan nanoparticles (CNPs). With slight modification to Medina *et al.* (2019), thymol loaded chitosan nanoparticles (TLCNPs) were prepared by diluting 1.9 g of citric acid in a volume of 100 ml of 1 mg/ml of thymol in water. Then, 300 mg of chitosan (degree of deacetylation within 75- 85% , Mw= 50,000-190,000 Da, Sigma–Aldrich Co. USA), was added to the mixture and stirred overnight. CNPs were prepared by diluting 300 mg of chitosan in citric acid solution (1.9 g/100 ml). The solutions of chitosan-thymol and chitosan were filtered using a 0.45 µm membrane and loaded into two 50-ml syringes mounted on an infusion pump. The rate of solution pumping was 1.8 ml/min over 50 ml of an aqueous solution of penta sodium tripolyphosphate (TPP, Sigma–Aldrich Co. USA) at 0.1% (w/v). The resulted suspension was centrifuged at 24,000 × g for 30 min. Then the collected supernatant of nanoparticles (NPs) was stored at 4 °C until use.

2.2. Characterization of nanoparticles:

The procedures recommended by Medina *et al.* (2019) for characterization was applied using Fourier Transformed Infrared Spectroscopy (FTIR) and zeta potential analysis.

2.3. Parameters of encapsulation

Following Medina *et al.* (2019), a supernatant sample of TLCNPs was dialysed against water for 150 min (using a dialysis tubing cellulose membrane with a molecular weight cut-off of 14,000). The resulted dialysate was then analysed using UV spectrophotometry at 273 nm following the thymol determination procedures described by Garsuch and Breitkreutz (2010) and Pan *et al.* (2014); where the dialysate obtained from the CNPs supernatant used as a blank. The lyophilization of dialysated sample was done in a plastic Petri dish with 13.5 cm diameter, covered with a layer of aluminium foil perforated at −55 °C and 6.7 Pa, for a duration of 2 days. Then, the sample was ground with the aid of a porcelain mortar and then stored at 4 °C. The encapsulation parameters were calculated as follows:

Efficiency of encapsulation (EF) % = Mass of thymol in the supernatant dialysated x100 / Initial thymol mass added

Loading capacity (LC) % = Mass of thymol in the supernatant dialysated x100 /Mass of the lyophilized sample.

Yield particles (YP) % = Mass of the lyophilized sample dialysated x100/ Mass of initial ingredients added

2.4. Evaluation of thymol release from TLCNPs:

As recommend by (Raj and Prabha, 2016),a weight of 0.1 mg of TLCNPs was suspended in a volume of 10 ml phosphate buffer saline (PBS) at various pH at controlled temperature of 37 ºC. The obtained suspension was placed in an incubated shaker and continually shacked at rate of 120 rpm for 1 h. Five milliliter aliquots were taken out of the dissolution medium at an intervals of (30 min), replaced by same volume of fresh PBS buffer, for keeping the volume of the release medium constant. The released amount of thymol was observed by UV spectrophotometer at 290 nm.

2.5. Evaluation of antimicrobial activities of chitosan, CNPs and TLCNPs

Double fold serial dilutions of the pure materials were carried out using BHIB. The MICs and MLCs against the *S*. Typhimurium were determined by visual and spectrophotometric methods using the same aforementioned techniques.

3. Antimicrobial activities of thymol and nanoparticles in fish matrix:

3.1. Preparation of fish:

Freshly caught farmed fish named tilapia (*Oreochromis niloticus*) were descaled, cleaned with tape water, filleted, deboned, portioned into nearly 2.5 cm × 2.5 cm pieces (10 g each) and used in the experiment.

3.2. Preparation of antimicrobial solutions:

The tested materials were prepared at their 1 MIC and 2 MIC using sterile distilled water for making dilutions with DMSO 5% for thymol and acetic acid 0.25% for CNPs and TLCNPs as solvents.

3.3. Inoculation of fish fillet:

A suitable number of fillets were surface inoculated with calculated inoculum of 10^5 cfu/g of *S*. Typhimurium according to (Lang *et al.,* 2004a, b) with slight modification. Inoculated fillets were left for one minute in Biosafety Class II laminar hood to help attachment of inoculum. Then fillets were soaked in antimicrobial solutions for 1 min and drained for 1 min. A group of inoculated fillets were dipped in sterile distilled water for 1 min (control). The treated as well as control fillets were sampled for zero time then stored at 4ºC.

3.4. Microbiological analysis:

Microbiological analyses were performed for treated and control samples. On a particular sampling time (0, 24, 48, 72, 96 h), fish pieces were transferred individually to stomacher bags and homogenized with Phosphate-buffered saline (PBS; at pH 7.0) to make a 10-fold dilution using stomacher for 2 min. The homogenate was serially diluted with PBS and surface spread in duplicate on

XLD agar plates for the enumeration of survivors. The seeded plates were incubated at 35°C for 24 h then examined for colonies and counted. Reduction percent in salmonella cells was calculated from the equation

Reduction % = (Count of control – Count of treatment) x 100 / Count of control

3.5. Effect of thymol and nanoparticles on pH of fish

On a particular sampling time (0, 24, 48, 72, 96 h) a fish fillet were homogenized with 20 ml distilled water by blendeding for 30s. The pH of sample was measured by a digital pH-meter (Gallenhamp No.101284) standardized at pH 4 and 7 as recommended by (Sallam, 2007).

3.6. Statistical analysis

The statistical analysis was done using SPSS program for windows (version 12.0.1) according to (SPSS, 2007). The differences between groups were done by using of a Student "t"-test. Significance level was considered at P < 0.05.

RESULTS

Table 1: Minimum inhibitory concentrations (MICs) and minimum lethal concentrations (MLCs) of phenolics (mg/ml) against *S.* Typhimurium.

Phenolic compounds	MICs		MLCs
	Visual method	Spectrophotometric method	
Thymol (Th)	0.08±0.01 [a]	0.16±0.02 [b]	0.08
Gallic acid (GA)	10.00±1.49	10.00±1.55	10.00
Cinnamic acid (CA)	5.00±0.79	5.00±0.79	5.00
Benzoic acid (BA)	1.25±0.14 [a]	2.50±0.44 [b]	2.50
Coumarin	2.50±0.37	2.50±0.32	↑10.00
Curcumine	10.00±1.57 [a]	5.00±0.79 [b]	10.00

*In the same raw means with different superscript letters are significantly different (p<0.05)

Table 2: Growth inhibition percentages (GI %) produced by phenolics against *S.* Typhimurium.

Phenolics (mg/ml)	GI %
Thymol (0.16)	100.00
Thymol (0.08)	55.30
Gallic acid (10.00)	100.00
Gallic acid (5.00)	94.10
Cinnamic acid (5.00)	100.00
Cinnamic acid (2.50)	90.40
Cinnamic acid (1.25)	61.77
Benzoic acid (2.50)	100.00
Benzoic acid (1.25)	98.20
Benzoic acid (0.63)	85.90
Curcumine (5.00)	100.00
Coumarin (2.50)	100.00
Coumarin (1.25)	74.50

Table 3: Encapsulation parameters of nanoparticles.

Parameter	Percent
Encapsulation efficiency of TLCNPs	99.54
Loading capacity of TLCNPs	64.17
Yield particles of TLCNPs	96.30
Yield particles of CNPs (control)	99.45

Table 4: Minimum inhibitory concentrations (MICs) and minimum lethal concentrations (MLCs) of chitosan, CNPs and CLCNPs against *S.* Typhimurium.

Treatments	MICs (mg/ml) mean values		MLCs (mg/ml)
	Visual method	Spectrophotometric method	
Chitosan	0.63±0.07	0.63±0.09	0.63
CNPs	0.80±0.13	0.80±0.13	1.60
TLCNPs	0.80±0.16	0.80±0.19	1.60

Table 5: Growth inhibition percentages (GI%) produced by chitosan, CNPs and TLCNPs against *S.* Typhimurium

Treatments (mg/ml)	GI %
Chitosan (0.625)	100
CNPs (0.8)	100
TLCNPs (0.8)	100

Table 6: Effect of thymol minimum inhibitory concentration (MIC) and (2 MIC) on quality of fish inoculated with *S.* Typhimurium and stored at 4°C.

Sampling time	Conc. (mg/ml)	Quality parameters			
		S. Typhimurium (cfu/g)		pH value	
		Control	Treatment	Control	Treatment
Zero h	0.08	$6x10^4$±15811	$1x10^{4}$*±2236	5.2±0.71	5.8±0.99
	0.16		$1x10^{3}$* ±353		6.0±0.46
24 h	0.08	$6x10^4$±22360	$3x10^{4}$*±2121	5.5±0.91	5.5±0.94
	0.16		$1x10^{4}$*±1414		5.6±0.96
48 h	0.08	$6.2x10^4$±707	$5x10^{4}$*±1581	5.5±0.75	6.1±0.53
	0.16		$8x10^{3}$*±2319		6.2±1.06
72 h	0.08	$6.7x10^4$±108	$5x10^{4}$*±353	5.6±0.76	5.4±1.45
	0.16		$1x10^{4}$*±707		5.7±0.97
96 h	0.08	$7x10^5$±70710	$1x10^{5}$*±1523	6.0±1.14	5.0±0.46
	0.16		$8x10^{4}$*±1423		4.8±0.44

*= Difference between treatment and control is significant ($p < 0.05$)

Table 7: Effect of CNPs minimum inhibitory concentration (MIC) and 2 MIC) on quality of fish inoculated with *S*. Typhimurium and stored at 4ºC.

Sampling time	Conc. (mg/ml)	Quality parameters			
		S. Typhimurium (cfu/g)		pH value	
		Control	Treatment	Control	Treatment
Zero h	0.8	$6x10^4$±15811	$5x10^4$±5533	5.2±0.71	5.5±0.61
	1.6		$3x10^4$±3313		5.3±0.92
24 h	0.8	$6x10^4$±22360	$1x10^4$±110	5.5±0.91	5.1±0.56
	1.6		<100 *		5.2±0.90
48 h	0.8	$6.2x10^4$±707	<100 *	5.5±0.75	6.1±0.67
	1.6		<100 *		6.1±1.06
72 h	0.8	$6.7x10^4$±1081	$2x10^3$±221	5.6±0.76	6.0±0.66
	1.6		$2x10^3$±220		6.1±1.06
96 h	0.8	$7x10^5$ ±7071	$2x10^4$ ±3478	6.0±1.14	6.0±0.66
	1.6		$1x10^4$±1106		6.1±0.67

*= Difference between treatment and control is significant ($p < 0.05$)

Table 8: Effect of TLCNPs minimum inhibitory concentration (MIC) and (2 MIC) on quality of fish inoculated with *S*. Typhimurium and stored at 4ºC.

Sampling time	Conc. (mg/ml)	Quality parameters			
		S. Typhimurium (cfu/g)		pH value	
		Control	Treatment	Control	Treatment
Zero h	0.8	$6x10^4$±15811	$2x10^4$*±2209	5.2±0.71	4.9±0.54
	1.6		$1x10^4$*±1104		4.4±0.49
24 h	0.8	$6x10^4$±22360	$1x10^2$*±0	5.5±0.91	4.6±0.51
	1.6		<100 *		4.6±0.54
48 h	0.8	$6.2x10^4$±707	<100 *	5.5±0.75	5.4±0.63
	1.6		<100 *		5.4±0.72
72 h	0.8	$6.7x10^4$±1081	$1x10^4$*±919	5.6±0.76	5.4±0.63
	1.6		$1x10^3$*±135		5.3±0.62
96 h	0.8	$7x10^5$±70710	$1x10^4$*±1142	6.0±1.14	5.8±0.68
	1.6		$1x10^3$*±110		5.7±0.67

*= Difference between treatment and control is significant ($p < 0.05$)

Table 9: Reduction percentages produced by antimicrobials minimum inhibitory concentration (MIC) and (2MIC) in fish inoculated with *S*. Typhimurium and stored at 4ºC

Sampling times / Conc.	Thymol		CNPs		TLCNPs	
	0.08 (mg/ml)	0.16 (mg/ml)	0.8 (mg/ml)	1.6 (mg/ml)	0.8 (mg/ml)	1.6 (mg/ml)
0 h	83	98	17	50	67	83
24 h	50	83	83	100	100	100
48 h	19	87	100	100	100	100
72 h	25	85	97	97	85	99
96 h	86	89	97	99	99	100

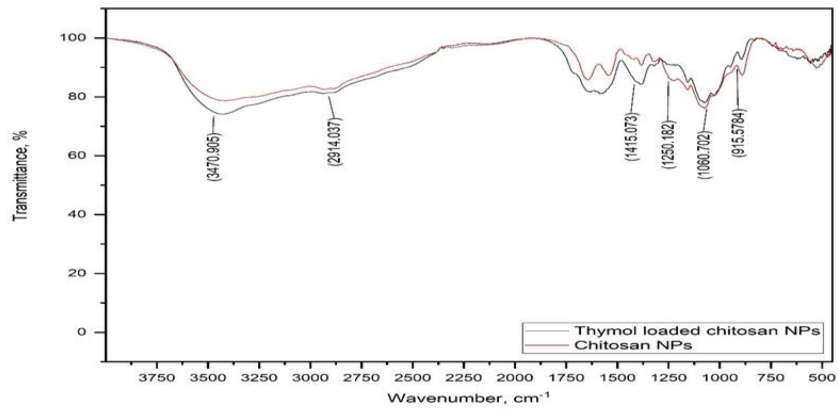

Figure 1. FTIR spectra of TLCNPs and CNPs

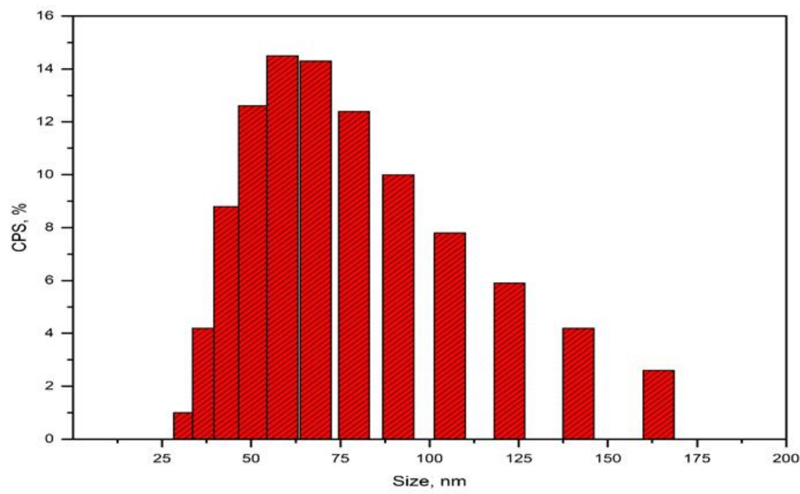

Figure 2. Particle size distribution of TLCNPs

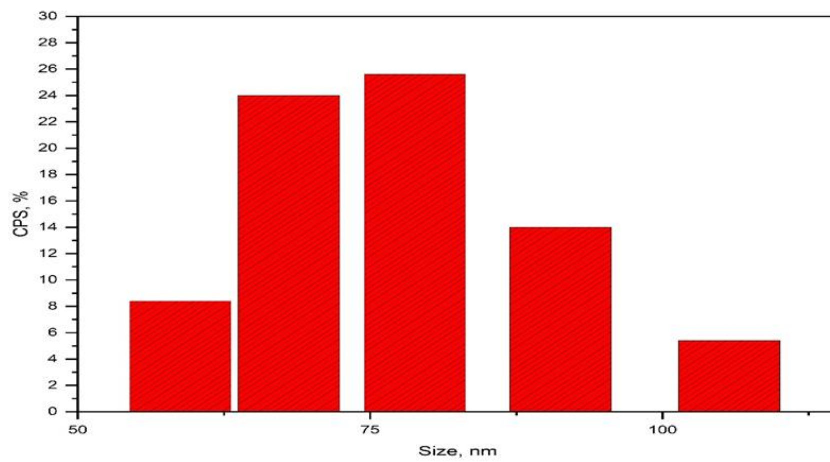

Figure 3. Particle size distribution of CNPs

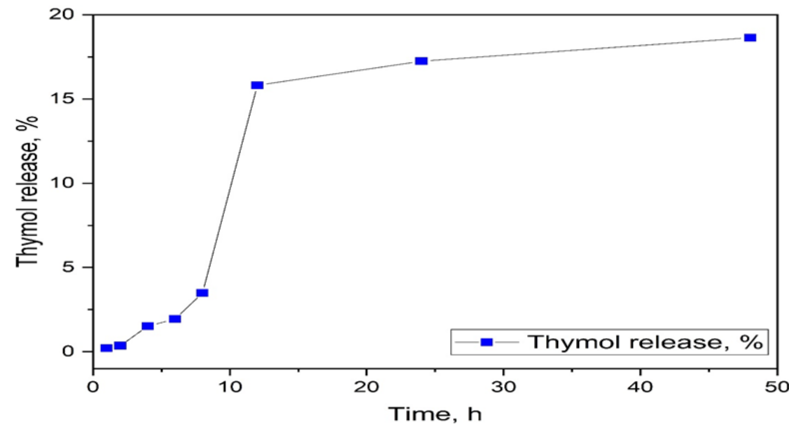

Figure 4. Thymol release profile from TLCNPs

DISCUSSION

1. Effect of phenolics against *S.* Typhimurium

Food safety is an important global concern for consumers and traders. Thus control measures are essentials to avoid the spreading of pathogens along the food chain. Phenolics such as curcumine and phenolic acids are common in the Egyptian folk medicine and traditional food additives. Phenolics mainly attack the microorganisms through enzyme inhibition by the effect of oxidized compounds, through reaction with sulfhydryl groups or through more non-specific interactions with proteins resulting in their inactivation and loss of function (Akhtar *et al.,* 2015).

The data in (Table 1) summarizes the activity of phenolics against *S.* Typhimurium. By visual assay, the MICs varied from 0.08 to 10.00 mg/ml while the corresponding values for spectrophotometric method were 0.16 to 10.00 mg/ml. The most powerful effect was obtained by thymol. It could inhibit and kill Salmonella at concentration of 0.08 mg/ml. Thymol was reported to have antiseptic action and is included in the American Food and Drug Administration (FDA) as an antibacterial and antifungal agent (Meeran *et al.,* 2017). Besides it is classified by the FDA as generally recognized as safe (GRAS), (Llana-Ruiz-Cabello *et al.,* 2015).

Thymol was reported to disintegrate the outer membrane of Gram negative bacteria (Engels *et al.,* 2009). Also, Chauhan and Kang (2014) recorded that thymol kills Salmonella by the same action and reported MIC value of 750 mg/ml. The activity of thymol against *S.* Typhimurium was also explored by studies of Silva-Angulo *et al.* (2015) and Gómez-García *et al.* (2019).

Organic acids have a vital role in food preservation. They function to maintain the microbiological quality of meat and meat products (Sánchez-Ortega *et al.,* 2014) and commonly incorporated into edible coating (Cagri *et al.,* 2003). The antimicrobial activity of phenolic acids is related to their chemical structure, especially the number and position of substitution in the benzene ring, and the length of saturated chain (Cueva *et al.,* 2010). Also increasing length of the alkyl chain increases the activity (Merkl *et al.,* 2010).

Gallic acid is a phenolic with many industrial applications, such as antioxidant in food and antimicrobial agent in the drug industry (Mota *et al.,* 2010). In the current study gallic acid was inhibitor to *S.* Typhimurium with MIC of 10.00 mg/ml for both visual and spectrophotometric

methods, (Table 1). The same concentration also appeared lethal effect. The calculated inhibition % corresponding to the MIC concentration (10.00 mg/ml) was 100% while lower concentration (5.00 mg/ml) produced only growth inhibition of 94.1%, (Table 2). Gallic acid fights bacteria through affecting the integrity of the cytoplasmic membrane which leads to loss of ingredients and inhibiting activity of respiratoin (Fitzgerald *et al.,* 2004).

In a related study, treatment with gallic acid resulted reduction of 1.5 log cfu/ml of *S.* Typhimurium and *L. monocytogenes* (Ravichandran *et al.,* 2011). Meanwhile, the activity of gallic acid was found to be lowered by two- to tenfold compared to other hydroxybenzoic acids (Sánchez-Maldonado *et al.,* 2011). Furthermore, Gullon *et al.* (2016) related the activity of pomegranate juice against *S.* Typhimurium to its richness in gallic acid.

Cinnamic acid was considered by FDA as safe food additive for flavor enhancement. In the common flavor usage, it does not exceed 31 mg/l (Committee, F. W. E., 2001). In the present study, cinnamic acid showed a powerful inhibitory effect against *S.* Typhimurium. Its MICs were 5.00 mg/ml by visual and spectrophotometric methods where the inhibition was proportional to concentration (Table 1 and 2). Also it was cidal to salmonella cells at 5.00 mg/ml. A related study by Olasupo *et al.* (2003) recorded that cinnamic acid at 1g/L had antimicrobial properties against Salmonella.

Benzoic acid is relatively nontoxic (CIREPBN, 2001) and was not recorded to accumulate in the body. Following ingestion, it is rapidly absorbed from the gastrointestinal tract and metabolized to nhippuric acid in the liver (HSDB, 1997). The ideal levels of application of benzoic acid as a preservative in food ranged 0.05–0.1% (GSFA, 2007). In the present study, benzoic acid exhibited a marked antibacterial activity against *S.* Typhimurium where its MICs were 1.25 mg/ml by visual method and 2.50 mg/ml by spectrophotometric method. The MLC to salmonella cells was 2.50 mg/ml (Tables 1). By comparison, Ravichandran *et al.* (2011) reported that the MIC of benzoic acid against *S.* Typhimurium and *L. monocytogenes* was 5,000 µg/ml for each. Moreover, Alves *et al.* (2013) recorded that 2, 4-Dihydroxybenzoic acid appeared a broad spectrum antibacterial activity.

The antibacterial activity of benzoic acid has been attributed to its ability to act on cell wall, penetrate and inhbit enzymatic functions (Luck and Jager, 1997).The pKa and the lipophilicity are the main solubility determinants of phenolic acids in bacterial membranes (Campos *et al.,* 2009). Their lipophilicity are influenced by pH which governs the charge of the carboxyl group and also by substitutions of hydroxyl and methoxy groups on the ring (Sánchez-Maldonado *et al.,* 2011). Thus both hydroxybenzoic acids and hydroxycinnamic acids are considered weak organic acids but differ in the properties of lipophilicity and activities.

Curcumine is nontoxic, bioactive agent of turmeric that has been utilized in traditional medicine (Jahromi *et al.,* 2014). In current study, curcumine demonstrated both inhibitory and lethal effects when tested against *S.* Typhimurium. By visual method, its MIC was 10.00 mg/ml while by spectrophotometric method was 5.00 mg/ml (Table 1) with a growth inhibition varying with the concentration (Table 2). In a related study, Singh *et al.* (2010) declared that curcumine is a potent molecule in the treatment of bacterial infections. Also Rai *et al.* (2008) and Bhawana *et al.* (2011) suggested that curcumine is considered as an important antibacterial.

Coumarins were recorded to have species-dependent metabolism. In human bodies, coumarin derivatives were reported to excreted in urine without adverse health

effects (Venugopala *et al.,* 2013). The findings of the present study showed that at a concentration of 2.50 mg/ml as a MIC, coumarin inhibited the growth of *S.* Typhimurium by 100 % while the MLC was 10.00 mg/ml (Tables 1, 2). In this respect, Lou *et al.* (2012) recorded MIC of 80 µg/ml of p-coumaric acid against *E. coli* and *S.* Typhimurium. Their study explained that, like other phenols, that compound changes the permeability of the cell membrane and can bind DNA and impair cell function. In a related research work, 2, 4-dihydroxybenzoic, p-coumaric acids and cinnamic acid derivatives were the compounds that showed powerful broad spectrum of antimicrobial activity (Alves *et al.,* 2013).

The present study shared Alves *et al.* (2013) explanation that the observed difference in MICs with other studies may be related to the use of strains with different susceptibility profiles. Moreover, different studies dealt with different methodologies of antibacterial activity assessment, and organisms revealed variation in the sensitivity to phenolics (Sánchez-Maldonado *et al.,* 2011). Also, comparing the effect of natural antimicrobials, is often difficult, due to the use of different approaches endpoints determination (Balouiri *et al.,* 2016).

Broth dilution is approved by CLSI for testing aerobic growing bacteria (CLSI, 2012). Broth micro and macro-dilution are considered the most basic anti- microbial activity testing methods. Compared to micro-dilution method, the main disadvantages of the macro-dilution method are manual undertaking, and the comparatively large amount of antimicrobial solutions and space required (Jorgensen and Ferraro, 2009). So, reproducibility and little volume of reagents (CLSI, 2012) besides, ease determination of MIC endpoint by viewing devices (readers) are major advantages of the micro-dilution method. These advantages made EUCAST (2003) recommended spectrophotometric method in reading MIC endpoint as modifications to CLSI guide lines (Arikan, 2007).

The results in (Tables1 and 4) revealed that MIC values by spectrophotometric method were significantly different compared to visual method in some antimicrobial assays and nearly agreed in most others. In this respect, Devienne and Raddi (2002) compared spectrophotometric to visual method in reading MIC for natural antimicrobials and recorded 100 % correlation of the methods. That was true also for Lindqvist (2006) who recorded that determination of MIC by measuring turbidity matched with count method. While Nguyen and Yu (1999) concluded that visual reading is not as accurate as spectrophotometric for MIC end points.

2. Effect of chitosan against *S.* Typhimurium

Chitosan is a high molecular weight cationic polysaccharide, resulted from the deacetylation of chitin (De Reuck *et al.,* 2009). Due to its biocompatibility and biodegradability, chitosan attracted attention as a natural additive to food. When chitosan comes in contact with susceptible microorganism, and as a positively charged polymer interact with bacterial membrane possessing a negative charge. As a result, low-molecular weight materials, nucleic acids, and proteins, are leached out (Alishahi and Aïder, 2012).

Chitosan was used in the present study as nano deliver to thymol. Its activity against *S.* Typhimurium was investigated in the preliminary work. Chitosan was inhibitor to *S.* Typhimurium at 0.63 mg/ml by visual and spectrophotometric methods (Table 4) with growth inhibition 100 % (Table 5). Also the MLC was 0.63 mg/ml. In comparison, Menconi *et al.* (2014) mentioned that chitosan at a concentration of 0.2 % significantly declined the count of recovered *S.* Typhimurium compared with

control. Also Kong *et al.* (2010) recorded that both chitosan and its derivatives are more potent antibacterial agents when tested against Gram negative bacteria. Meanwhile, Fernandez-Sainz *et al.* (2010) summarized the factors influence the effectiveness of chitosan as: the physicochemical properties (molecular weight, degree of acetylation), pH of the solution and the tested microorganism. These factors also may explain difference of results for different studies.

3. Formation of polyphenolic nanocapsules

Many of the phenolic compounds have the properties of poor water solubility, low stability and the little bioavailability (Gupta *et al.,* 2016). Nanoencapsulation can aid to overcome such problems (Conte *et al.,* 2016). That type of technology was tried in the present study with thymol as it appeared the highest antibacterial activity against *S.* Typhimurium. It was chosen for encapsulation to study its efficiency under controlled release. The polymeric encapsulating agent used in this study was chitosan.

3.1. Encapsulation efficiency percentage (EE %)

The amount of bioactive compounds could be entrapped within the nanoparticles is a marker of encapsulation efficiency (Vashisth *et al.,* 2015). It is advantageous for the encapsulation efficiency to be as near to 100 % as possible. Where high encapsulation efficiency leads to better targeted delivery. The summarized data in Table 3 cleared that 99.54 % of the total thymol was encapsulated with loading capacity of 64.17 %. The yield particles of TLCNPs was 96.3 %, while yield particles of CNPs (control) was 99.45 %.

3.2. Fourier Transmission Infrared spectroscopy (FTIR)

The results of FTIR study (Figure 1) showed for TLCNPs at 3470, 2914, 1415, 1250, 1060, and 915, 816 cm^{-1} represent the hydrogen-bonded O-H stretch band, CH stretching vibration corresponding to aldehyde compound, NH3, OH group in CH3 back bone, carbon ring in cyclic compound and OH group in phenol rvealing the presence and formation of hydrogen bonds with aliphatic compounds, primary amines and primary alcohols. The intense broad band peak at 3430 cm^{-1} was characterized for the hydroxyl functional group in alcohol and phenol compounds.

3.3. Zeta Sizer analysis

The zeta potential of the TLCNPs and CNPs were found to be 54.80 mV, and 34.50 mV, respectively. The particle size distribution of the TLCNPs and CNPs were shown in (Figures 2, 3) respectively.

3. 4. Thymol release

Figure 4 shows the release profile of thymol from CNPs at pH 7.4. It was found that a slight release of thymol until 8 h to be 3.50 %. Then, a slight increase in the release was shown being 15.81 % after 12 h. In addition, the release was achieved 18.64 % from 12 h to 48 h which indicating the stability of thymol in CNPs.

4. Effect of CNPs and TLCNPs against *S.* Typhimurium

Besides serving as a carrier for controlling release of the active compound, binding the core with phenolic compounds also allows for the protection from adverse effects of light, heat, and oxygen (Soto-Chilaca *et al.,* 2016). In present study, the MIC of CNPs against *S.* Typhimurium was 0.8 mg/ml by visual and spectrophotometric methods with growth inhibition 100 % while the MLC was 1.6 mg/ml (Tables 4, 5). In a related study using representative strains of Gram negative and Gram positive bacteria, CNPs showed antibacterial effect in concentrations not less than 0.3 % (Ghaderi-Ghahfarokhi *et al.,* 2017). Also it is recorded that CNPs were more efficient than chitosan solution at enhancing drug activity (Ma *et al.,* 2005). By the regard, the larger surface area of nanoparticles resulted better distribution and

potency of packaged phenolic molecules (Redhead *et al.,* 2001). Also Abdou *et al.* (2012) recorded that chitosan nanoparticles had higher antimicrobial effect than chitosan.

TLCNPs exhibited nearly the same effect of chitosan CNPs activity against *S.* Typhimurium where its MIC was 0.8 mg/ml by visual and spectrophotometric methods with growth inhibition 100 % while MLC was 1.6 mg/ml (Tables 4, 5). Loading phenolics on nano-deliver function in protection and control of release. In this respect, Lapidot *et al.* (2002) reported that due to interaction of phenolics with medium components their potency lost with time. Meanwhile, Ravichandran *et al.* (2011) recorded that by packaging in nanoparticles, phenolics could be protected from the components of media and thus retaining their potency. Also the chitosan shells could protect the enclosed bioactive compounds from natural degradation such as hydrolysis (Kim *et al.,* 2008).

Chitosan- phytochemical conjugates (CPCCs) were found to increase the osmotic pressure, induce disruption and shrinkage of the bacterial membrane and reduce its permeability to intracellular components (Eom *et al.,* 2015). Another explanation was mentioned by Kong *et al.* (2010) where CPCCs form a barrier on the bacterial surface and prevent passing of nutrients.

5. Effect of thymol and nanoparticles on survival of *S.* Typhimurium and pH of treated fish fillets

Edible coatings are food grade suspensions which upon drying cover the food surface with clear thin layer (Sánchez-Ortega et al., 2014). These coatings can act as carriers of substances to inhibit pathogenic microorganisms. That type of processing was tried in present study using thymol solution. Thymol is a natural phenolic compound present in the essential oil fraction of Thymus plants (Juven *et al.,* 1994). It is permitted by USFDA (2014) as additive to food for human consumption.

The data summarized in (Table 6 and 9) revealed that just after dipping in coating solution containing the MIC of thymol (0.08 mg/ml), the count of *S.* Typhimurium survivors in fish fillets was significantly ($p<0.05$) reduced (83% reduction) compared to control. By application of 2MIC, the reduction increased to 98%. During refrigerator storage and by MIC, the effect of thymol showed fluctuation in activity where after 24h the reduction declined to 50%. The declined reduction continued during the two successive days to reach 25 % by end of 72 h but still significant. Then reduction increased again to reach 86% by end of 96h. Using 2MIC and during refrigerated storage the effect was significant with slight fluctuation where reduction was within the range of 83-89%, (Table9).

Thymol has been shown to exhibit antibacterial activity including food pathogens (Delgado *et al.,* 2004). That effect was attributed to impairing the cytoplasmic membrane through the destruction of the lipid bilayer in (Lambert *et al.,* 2001) which results an efflux of ions and ATP with proton motive force dissipation and eventually cell death (Guevara *et al.,* 2015).

For flesh foods, pH is an important quality index. It is one of the most important factors in affecting microbial growth and deterioration of foods (Anvari *et al.,* 2012). Meanwhile, ES 3494/ (2005) recommended a value of 6.5 as a maximum level of pH for cold stored fish. By the two applied concentrations, thymol coating resulted slightly alkalizing effect to fish fillets compared to control. That effect was not significant and the pH value still within recommended values, (Table 9).

Chitosan is representing one of biopolymers that are safe for human consumption .Besides it has several effective delivery methods (Hintz *et al.,* 2015). Its films are

advantageous as they are semipermeable, durable, long-lasting and inexpensive. In the current study, coating of fillets with solution containing particles within nanoscale was tried with chitosan. CNPs showed potential reduction against *S.* Typhimurium. Application of MIC (0.8 mg/ml) produced potential delayed effect while the 2MIC showed potential on both immediate and delayed effect. Salmonella cells were reduced by 17% after immediate coating with MIC of CNPs compared to a significant reduction (50%) by 2MIC trial (Table7, 9). The CNPs activity continued increasing and the effect was maximized (100% reduction) at 48h storage for MIC application and during 24-48h for 2MIC trial. Then effect for both trials still nearly constant and significant till the end of storage time (96h). The degree of activity of nano-antimicrobials was found to depend on outer structural arrangement of the bacterial wall where the amount of time needed for nanoparticles penetration varied accordingly (Mazumder *et al.,* 2013).

By application of CNPs coating, treated fillets appeared slight alkalinity at some sampling periods but not significantly affected, (Tables 7). The same observation was reported by Mohan *et al.* (2012) by application of 1 and 2% chitosan coatings to frozen-stored sardines.

By Nanoencapsulation of phenolics unpleasant taste and aroma can be masked, release can be controlled and solubility of lipophilic compounds can be improved (Pisoschi *et al.,* 2017). In current study, application of TLCNPs increased the safety of fish fillets in concern with salmonella. From (Tables 8, 9), the effect of coating of fillets with TLCNPs produced immediate reduction in salmonella cells by 67 and 83% for MIC (0.8 mg/ml) and 2MIC trials, respectively. By proceeding of time at refrigerator storage, the activity of TLCNPs against salmonella was maximized by end of 24h and still potential within the three successive days. These findings agreed with those of Ravichandran *et al.* (2011) who reported that nanoparticles can act as a successful delivery system for phenolic compounds and enhancing their antimicrobial efficacy. In related studies, loading phenolic compounds to polymeric nanoparticles resulted advantageous effects included controlled release (Li *et al.,* 2012), improved solubility (Wu *et al.,* 2012), and equal or more efficient antimicrobial activity (Iannitelli *et al.,* 2011).

As index of the physical properties of treated fillets, the pH of fillets coated with TLCNPs appeared within the range of fresh fillets recommended by ES 3494/ (2005) regulations till end of 4 days of refrigerated storage, (Table 8).

The findings of this study suggest the potential use of thymol for inactivation of *S.* Typhimurium in food. The newly developed technology in which thymol is encapsulated in chitosan nanodeliver represents a significant step in the direction of producing nanocapsules with prolonged antimicrobial activity. Such polymeric nanocapsules have the potential and efficiency in improving food safety.

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الفينولات- نانو كمضادات للسالمونيلا تيفيميوريوم

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صممت هذة الدراسة لتقييم نشاط ستة مركبات فينولية. النشاط ضد ميكروب السالمونيلا تيفيميوريوم تم تقييمة بالطريقة العينية (تخفيف الانابيب) وطريقة مقياس الطيف الضوئى. كان نشاط تللك المركبات كالتالى الثيمول>حمض البنزويك>الكومارين> حمض السيناميك >الكركومين >حمض الجالك. اظهر الثيمول تاثيرا قاتلا للميكروب عند تركيز 0.08 mg /ml . فيما عدا الكركومين فان باقى الفينولات اظهرت تاثيرا قاتلا للميكروب بتركيزات فى المدىmg /ml 10.00 – 1.25. وجد أن هناك فروق معنوية لقيم أقل تثير متبط بطريقة مقياس الطيف الضوئى مفارنة بطريقة تخفيف الأنابيب عند تقييم بعض مضادات الميكروبات ولم توجد فروق معنوية بين الطريقتين عند تقييم البعض الاخر.

بمعاملة شرائح الأسماك بحلول الثيمول او الشيتوزان النانوى إنخفضت أعداد السالمونيلا تيفيميوريوم معنويا. كما نتج عن تطبيق تقنية النانو لتحميل الثيمول على الشيتوزان النانوى التحكم فى انطلاق الثيمول مما أدى إلى استمرار الفاعلية معنوية أثناء حفظ الأسماك بالتبريد دون تاثر خواصها الطبيعبة ممثلة فى الأس الهيدروجينى.تظهر الدراسة فاعلية الفينولات فى تطويرتقنية الأغشية المستخدمة لحفظ الأغذية والتاثير الواعد لكبسول الثيمول شيتوزان نانو فى درء خطر ميكروب السالمونيلا والتحكم فية.