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Immunomodulatory Activity Of Some Selected Medicinal Plants On Response To *Salmonella* typhimurium In Poultry.

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ABSTRACT

Controlling food-borne pathogens is an important microbial food safety issue for human and poultry sector. *Salmonella* Typhimurium (STy) has been major causative agent of food-borne diseases and also affect poultry performance. The efficacy of three safe extracts of edible plants to reduce STy colonization in chicks were evaluated. A total of 280 one-day-old chicks were divided into fourteen groups. Rectal swabs were taken at day 3, 5, 7, 14 and 21 post-infection. Internal organs, caecal tissues content were collected for *Salmonella* enumeration and conventional PCR and real time PCR assays were carried out. The phenolics content were determined (colorimetric method of Folin-Ciocalteu). Micronutrients analyses were carried out by flame emission and atomic absorption spectrometry. *In vivo* dietary supplementation with *Morus nigra* (LMN) reduced invasion by STy, as reflected by efficient protection against infection when supplemented before and co-infection (simultaneously with infection) as compared with the albedo extract of *Citrus aurantium* (ACA). ACA protect the chicks when supplemented the extract co-infected chicks but *Rumex vesicarius* extract only stopped the bacterial shedding. To our knowledge, no studies have been reported investigating the effects of the investigated extracts against multidrug-resistant STy infection. The activity of LMN may be related to the phenolics and micronutrients.

Keywords: *Salmonella* Typhimurium, *Morus nigra*, *Rumex vesicarius, Citrus aurantium*, Conventional PCR, Real time PCR, Caecal

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INTRODUCTION

Salmonella enterica still remains as an important disease with economic impact as it may affect animal performance and may result in foodborne disease in humans through the consumption of contaminated eggs and poultry meat [1]. In recent years food safety concerns have been focused on such pathogens which also causes considerable worldwide economic loss through chicken mortality and massive outbreaks have been occurred in several parts of the world [2]. Recently in Egypt, *S. enterica* serovar Typhimurium was reported as the dominant serovar. The high prevalence of *Salmonella* infection of *S.* Typhimurium as foodborne pathogens in raw chicken meat in Nile Delta, Egypt has been reported [3]. It was detected by nine *S. enterica* serovar Typhimurium (60.0%) compared to two *S. enterica* serovar Enteritidis (13.3%) only were identified by means of serovar-specific bands using PCR [3]. Epidemic dissemination of predominant strains has been reported for *Salmonella* serovars Typhimurium. It is frequently involved in egg and egg product related food borne illness. Poultry producer suffers losses due to *Salmonella* infection of the flock including loss of birds and production time [2]. Therefore, an interest in alternative products to overcome *Salmonella* Typhimurium infection has increased, after a ban on the use of antibiotics.

Antibiotics are used in poultry section, not only for treatment or prevent diseases, but also as growth promoter [4]. As a result of their use, food can contain antibiotic-resistant bacteria and resistance genes with important public health consequences. Additionally, in the developing countries, synthetic drugs are not only expensive and insufficient for the treatment of diseases but also have deception and side effects [5]. Therefore, researchers are gradually turning their consideration to search for safer alternative products in order to develop superior drugs against the infection of microbes challenged the poultry industry [4 - 6]. Immuno-suppression, stress or medical treatment can induce changes in the composition of the microbiota of chicks. All of these factors contribute to facilitating the mucosal colonization for pathogenic *Salmonella* Typhimurium strains [1, 5]. The innate immune system plays a crucial role in the removal of pathogens that have been targeted by an adaptive immune response [7]. So, changes in diet of chicks using safe waste natural products medicinal plants with antimicrobial properties and management practices could precipitate increased shedding of pathogens. The reduction in *Salmonella* prevalence in poultry will result in a decrease in incidence of human *salmonellosis*.

MATERIALS AND METHODS

Plant materials

Leaves of *Rumex vesicarius* L (Polygonaceae), leaves of *Morus nigra* L (*Moraceae*) and the fruits of *Citrus aurantium* (Rutaceae) were collected from a private field in the village of Mansha'at Suleiman, Al-Gharbiya Governorate. They were kindly identified by Dr. *Sherif* S. El-Khanagry, Department of Flora and Phytotaxonomy Research Unit of the *Agricultural Museum*, *Ministry* of *Agriculture (*Dokki-Giza, Egypt). Voucher Specimens were kept in the Herbarium of National Research Centre, Cairo, Egypt. The leaves *R. vesicarius M. nigra* and were air-dried in the shade and reduced to powder. The fruits of *C. aurantium* were of eating quality, and without blemishes, or damage. They were washed thoroughly with double-distilled water and dried with tissue paper. The pericarp region was peeled off from the edible part using a peeler. The white, spongy albedo (non-pigmented portion) was recovered by shaving the flavedo (the pigmented portion) from the rinds.

Bacterial strain and culture medium

Salmonella enteric serovar Typhimurium (*S.* Typhimurium), isolated from the poultry was supplied from the Central Laboratory for Evaluation of Veterinary Biologics (CLEVB), Cairo, Egypt. The bacterial strain was suspended in *tryptic soy broth* (Nissui Pharmaceutical Co., Tokyo, Japan) and incubated at 37 °C for 20 h then the bacterial colony count method was applied [8] which was assessed to get $10^{7.0}$ colony forming units (CFU)/mL.

Chemicals

All chemicals used in the present study were of high analytical grade, products of Sigma (USA), Merck (Germany), BDH (England), Riedel de Hàen (Germany) and Fluka (Switzerland).

Extraction procedure

The albedo of C. *aurantium* (ACA) was subsequently cut into small pieces (~10 mm) with a knife. The albedo and the leaves of *M. nigra* (LMN) and *R. vesicarius* (LRV) were dried separately in an oven at 40 °C continuously until a permanent weight was reached. Each material was immersed in ethanol (70%) in 2L conical flask. The mixtures were subsequently heated (40 °C) for 15 minutes with continuous stirring using shaker, after which the aqueous alcoholic extract was cooled to room temperature. The mixture was filtered using a Buchner funnel, vacuum pump, and Whatman No. 1 filter paper. The process was repeated three times. Thereafter, the collected filtrate was evaporated of the solvent under reduced pressure, the respective ethanolic extracts were obtained. Percentage yield of ACA, LMN and LRV were 8.28, 24.3 and 13.50 %, respectively. The dried crude extracts were made from one lot of each herb and kept at 4 °C until further use. The extracts were dissolved in 50% DMSO before use [9], and kept at 4 °C until further use.

Experimental design

A total of 280 one-day-old specific pathogen-free (SPF) chicks were obtained from Nile SPF eggs, Koom Oshiem, Fayoum, Egypt. They were kept in biosafety isolators in Central Laboratory for Evaluation of Veterinary Biologics (CLEVB), Cairo, Egypt. They were divided into fourteen groups (20 chicks each) as shown in Table 1. Compliance with ethics requirements was considered. The chicks' care and experimental protocols were in compliance with guidelines of ethical standards released by Cairo University policy on animal care and use. All efforts were made to ensure ethical and humane treatment of the chicks. The **chicks** were *infected* orally with virulent 1×10^7 CFU *S.* Typhimurium [10].

Table 1: The experiment groups' categories

Group	Supplement (extract)	Supplementation time
G1a	Leaves extract of *Morus nigra* (LMN)	Pre-infection
G1b	Leaves extract of *Rumex vesicarius* (LRV)	
G1c	Albedo extract of *Citrus aurantium* (ACA)	
G2a	Leaves extract of *Morus nigra* (LMN)	Co-infection (Simultaneous with infection)
G2b	Leaves extract of *Rumex vesicarius* (LRV)	
G2c	Albedo extract of *Citrus aurantium* (ACA)	
G3a	Leaves extract of *Morus nigra* (LMN)	Post-infection
G3b	Leaves extract of *Rumex vesicarius* (LRV)	
G3c	Albedo extract of *Citrus aurantium* (ACA)	
G4	Unsupplemented but infected	
G5a	Supplemented but uninfected	
G5b		
G5c		
G6	Unsupplemented uninfected	

After the experimental infection, cloacal swabs were taken at three, five, seven, fourteen and twenty-one days post-infection for cultural platting and detection of the bacterial shedding. Two chicks from each group were sacrificed; caecal tissues were collected and stored at −80 °C for real time PCR. Caecal contents were collected and frozen at −20 °C for cPCR. To enumerate *S*. Typhimurium, samples of spleen, liver, and cecum were collected as well.

Cloacal swabs

Rectal swabs from all groups were taken after infection at day 3, 5, 7, 14 and 21 post-infection. Swabs were placed into 10 mL of tryptic soya broth and incubated for 24 h at 37 °C. At each time, 0.1 mL of broth was plated on MacConkey agar and incubated for 18 h at 37 °C [11].

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Enumeration of *S.* Typhimurium

Spleen, liver, and cecum samples were subjected to pre-enrichment in buffered peptone water and enrichment in tryptic soya broth and plated on MacConkey and *Salmonella shigella* agar. Samples that were positive only after enrichment were assigned a value of one and negative samples were assigned a value of zero.

Conventional PCR (cPCR)

Approximately 25 mg of caecal tissue and caecal content were transferred into one mL of tryptic soya broth. cPCR Master Mix was Emerald Amp GT PCR master mix (Takara) Code No.RR310A and DNA Molecular weight marker Gel Pilot 100 bp ladder (cat. no. 239035) supplied by QIAGEN (USA).

Real time PCR

PCR Master Mix used for real time PCR was QuantiTect catalogue no. 204443 Contains Hot Start Taq DNA Polymerase, buffer (Tris-CL, KCL,$(NH_4)_2SO_4$, 8 µM $MgCl_2$, ready-to-use molecular grade dNTPs mix (Contains dATP, dCTP, dGTP and dUTP of ultrapure quality) and fluorescent dye (ROX). Table 2 represented the oligonucleotide primers and probes used in real time PCR (Metabion, Germany). Table 3 detected the components and volume/reaction for the preparation of PCR Master Mix according to Emerald Amp GT PCR mastermix (Takara) Code No. RR310A kit. Extraction of DNA according to QIA amp DNA mini kit instructions. Table 4 showed the cycling conditions for cPCR.

Table 2: Oligonucleotide primers and probes used in real time PCR (Metabion, Germany)

Target gene	PCR	Primer sequence (5'-3')	Reference
invA	Real time	GCGTTCTGAACCTTTGGTAATAA	(a)
		CGTTCGGGCAATTCGTTA	
		5′-FAM-TGGCGGTGGGTTTTGTTGTCTTCT-TAMRA-3′	
	Conventional	GTGAAATTATCGCCACGTTCGGGCAA	(b)
		TCATCGCACCGTCAAAGGAACC	

a: (Daum et al., 2002); b: (Oliveira et al., 2003)

Table 3: Preparation of PCR Master Mix according to Emerald Amp GT PCR mastermix (Takara)

Component	Volume/reaction
Emerald Amp GT PCR mastermix (2x premix)	12.5 µL
PCR grade water	4.5 µL
Forward primer (20 pmol)	1 µL
Reverse primer (20 pmol)	1 µL
Template DNA	6 µL
Total	25 µL

Table 4: Cycling conditions for conventional PCR (cPCR)

Primary denaturation	Secondary denaturation	Annealing	Extension	No. of cycles	Final extension
94˚C 5 min.	94˚C 30 sec.	55˚C 30 sec.	72˚C 30 sec.	35 35	72˚C 7 min.

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Table 5: QuantiTect Probe RT-PCR Master Mix

Component	Volume/reaction
2x QuantiTect Probe RT-PCR Master Mix	12.5 μL
Forward primer (50 pmol)	0.5 μL
Reverse primer (50 pmol)	0.5 μL
Probe (30 pmol)	0.125 μL
RNase Free Water	5.375 μL
Template DNA	6 μL

Table 6: Cycling conditions for taqman real time PCR of *invA gene*

Stage	Temperature	Time	Cycles
Primary denaturation	94 °C	5 min.	1
Amplification a) Secondary denaturation	94 °C	30 sec.	40
b) Annealing	49 °C	30 sec. (optics on)	
C) Extension	72 °C	10 sec.	

DNA molecular weight marker and agarose gel electrophoreses

The ladder was mixed gently by pipetting up and down. Certain μL of the required ladder (6 μL) were directly loaded. Agarose gel electrophoreses with certain modification was carried out [14]. Electrophoresis grade agarose was prepared and allowed to cool then ethedium bromide was added and mixed thoroughly. The warm agarose was poured directly in gel casting apparatus with desired comb in apposition and left at room temperature for polymeri zation. The comb was then removed, and the electrophoresis tank was filled with TBE buffer. PCR product samples, negative control and positive control were loaded to the gel. The power supply was 1-5 volts/cm of the tank length. The run was stopped after about 30 min and the gel was transferred to UV cabinet. The gel was photographed by a gel documentation system and the data was analyzed. Table 5 showed the QuantiTect Probe RT-PCR Master Mix and Table 6 showed the cycling conditions for taqman real time PCR of *invA gene*.

Chemical composition

Determination of total phenolic constituent's content

The total phenolic constituent's content in the leaves extract of *M. nigra* was determined according to Folin–Ciocalteu procedure [15]. Four hundred microlitres of sample (two replicates) were taken in test tubes; 1.0 mL of Folin–Ciocalteu reagent (diluted 10-fold with distilled water) and 0.8 mL of 7.5% sodium carbonate were added. The tubes were mixed and allowed to stand for 30 min and the absorption at 765 nm was measured against a blank, which contained 400 µL of ethanol in place of sample. The total content was expressed as gallic acid equivalents in mg/g of ethanol extract.

Micro- and macroelements analysis

A mixture of acids (concentrated nitric, perchloric, and sulfuric acid, respectively, 8:1:1, v/v/v) was added to the sample (1.00 ± 0.005 g) and digested with heating [16]. Elements were determined in the digested solution by flame emission spectrometry (Na, Ca, Mg, P, and K) and atomic absorption spectrometry (Hg, Pb, Cd, Cu, Fe, Mn, Zn, Li, Se, and Co) [16].

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RESULTS

Salmonella recovery (caecal swabs)

The caecal swabs from all chicks were analyzed by cultivation with culture method for the detection of *S.* Typhimurium. Each swab was analyzed immediately after the swabbing procedure and after 24 h of incubation on *Salmonella* agar media. The culture showed that samples of group supplemented by albedo extract of sour orange; *Citrus aurantium* (ACA) pre-infection and post-infection were positive and also group supplemented by leaves extract of *Morus nigra* (LMN) post-infection in addition to groups supplemented by leaves extract of *Rumex vesicarius* (LRV) and the positive control group (infected but non-supplemented group). Table 7 illustrated the results of shedding test.

Table 7: Shedding test of experimental groups at different days post-infection

Group / Days	G1a	G1b	G1c	G2a	G2b	G2c	G3a	G3b	G3c	G4	G5a	G5b	G5c	G6
3rd	-	+	+	-	+	-	+	+	+	+	-	-	-	-
5th	-	+	-	-	-	+	-	-	-	+	-	-	-	-
7th	-	-	-	-	-	-	-	-	-	+	-	-	-	-
14th	-	-	-	-	-	-	-	-	-	+	-	-	-	-
21 d	-	-	-	-	-	-	-	-	-	+	-	-	-	-

Table 8: Cloacal swab of the conventional PCR and Real time PCR assays

Group	Supplemented extract	supplementation time	Culture result	Conventional PCR result	Real time PCR result
G1a	LMN	Pre-infection	-	+	-
G1b	LRV		-	+	+
G1c	ACA		-	+	+
G2a	LMN	Simultaneous with infection	-	+	+
G2b	LRV		-	+	-
G2c	ACA		-	+	-
G3a	LMN	Post-infection	-	+	+
G3b	LRV		-	+	+
G3c	ACA		-	+	+
G4	Non supplemented but infected		+	+	+
G5a	LMN	Supplemented but Uninfected	-	-	-
G5b	LRV		-	-	-
G5c	ACA		-	-	-
G6	Unsupplemented uninfected		-	-	-

LMN: Leaves extract of *Morus nigra*, LRV: Leaves extract of *Rumex vesicarius* and ACA: Albedo extract of *Citrus aurantium*.

Identification of the isolated *Salmonella* by cPCR using specific primers

Figure 1 showed the presence of specific PCR product at the correct expected size of the *S.* Typhimurium (284 bp) was revealed. L: 100 bp marker; Pos: positive control; Neg: negative control; Lane 1-10: samples of groups G1a, G1b, G1c, G2a, G2b, G2c, G3a, G3b, G3c and G4.

Characterization of the *Salmonella* Typhimurium by real time PCR in caecal tissue

Figure 2 showed the positive *Salmonella* Typhimurium in caecal tissue collected at 7, 14 and 21 days post infection. The curve showed three negative samples (1, 5 and 7) of groups (G1a, G2b and G2c) which are supplemented with leaves extract of *M. nigra* (LMN) pre- and post-infection and ACA post-infection respectively.

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The curve shown three negative samples (1, 5, 7) of groups (G1a, G2b, G2c) which are supplemented with leaves extract of *M. nigra* (LMN) pre- and post-infection and Albedo extract of *Citrus aurantium* (ACA) post-infection respectively. Table 8 showed the results of cloacal swab of the conventional PCR and Real time PCR assays.

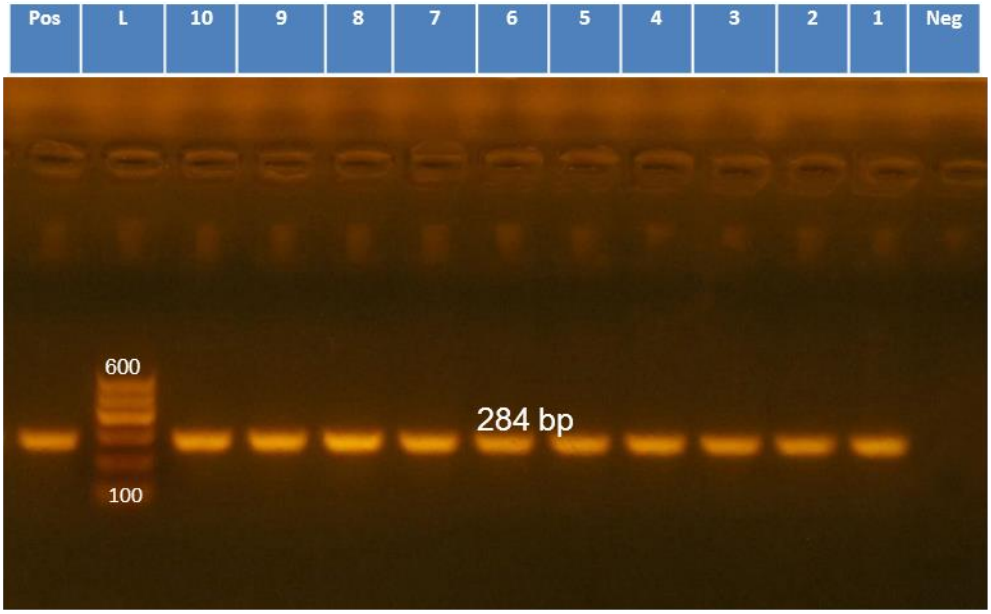

Fig 1: Electrophoresis of the amplified products for detection of the *Salmonella*

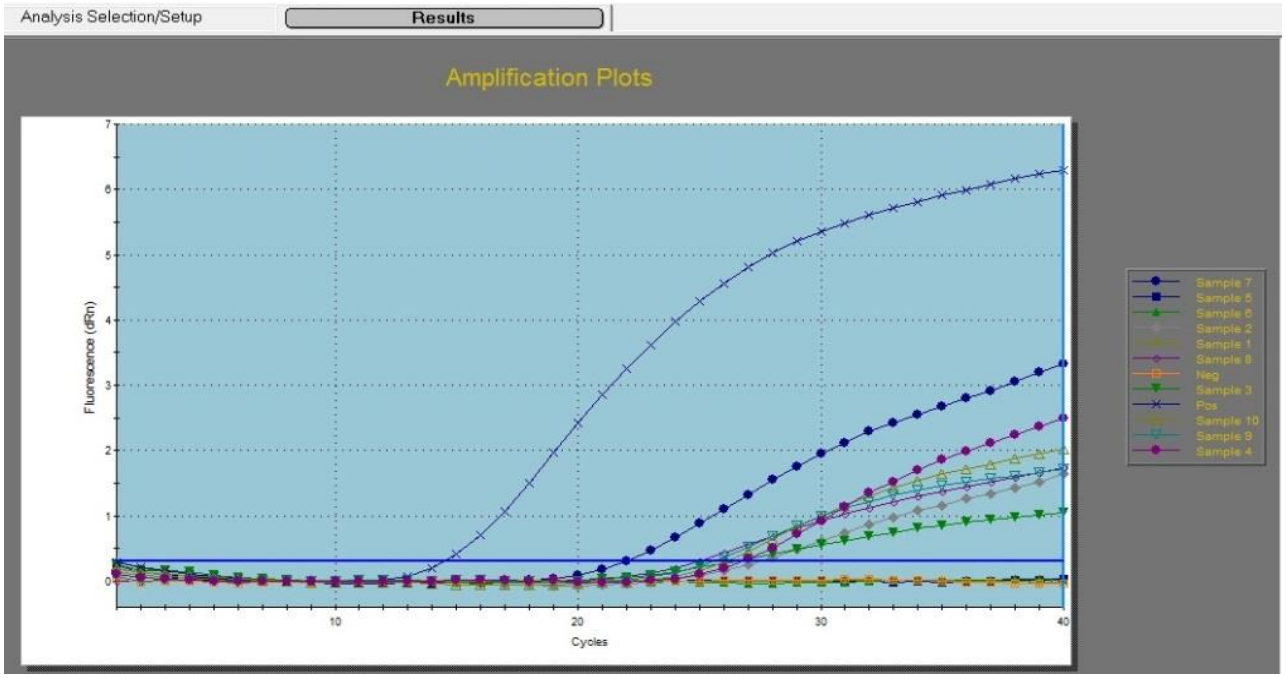

Fig 2: Positive *Salmonella* Typhimurium in caecal tissue collected at day 7, 14, 21 post-infection

Chemical composition

Total phenolics content

M. nigra showed the content of total phenolic content of leaves as gallic acid equivalents of ethanol extract (mg/g of ethanol extract) was 833.7 ± 24.6. The data performed the average values ± standard deviation (S.D.) of three samples.

Micro- and macroelements analyses

Macroelements, calculated with reference to air-dried leaves of *M. nigra* (data not shown), were (in %): Ca (2.79), K (1.89), Mg (0.51), Na (0.60), N (1.33), P (1.19). Microelements were (in ppm): Cu (6.8), Fe (134.3), Mn (78.7), and Zn (44.2).

DISCUSSION

Salmonella is an insidious problem in the poultry industry, and this problem represents a critical food safety hazard [5, 17]. In addition, there are reports of hazard of non-typhoidal serovars Typhimurium, one of the most common serotypes causing salmonellosis in chicks [4].

In Egypt, *S.* Typhimurium is frequently involved in egg and egg product related food borne illness. Therefore, identifying a strategy for its control is important to the poultry sector. After a ban on the use of antibiotics as growth promoters in farm animals, an interest in alternative products with antibacterial or immunomodulatory properties has increased. Medicinal plants have emerged as rich and important sources of therapeutic agents with beneficial biological activities, including immunomodulation and antimicrobial [18, 19]. Therefore, many of these plants have the potential to prevent and treat several chronic diseases [20].

Various substances have been investigated for their potentially inhibitory effects on *bacterial* infection and faecal shedding; including plant-derived antimicrobials as *Curcuma longa*, *Olea europaea Scutellaria baicalensis* and *Cinnamomum verum* products. They have been reported to be used to inhibit pathogenic bacterial growth in meat and food protection [10, 17]. We therefore speculated that some plants-derived materials may inhibit *Salmonella* infection in animal model.

Several edible plants are used in Egypt since early time to control microbial infections. We conducted the current *in vivo* study to investigate the potential protective effect of supplementation with three natural product extracts as antimicrobials in a model of invasive salmonellosis.

This study was designed at evaluating the effects of three extracts from agriculture waste (leaves of *Morus nigra*, leaves of *Rumex vesicarius* and albedo of *Citrus aurantium*), used in the traditional medicine by Egyptian people for the treatment of numerous ailments, against *S. enteric* serovar Typhimurium infection in chicks. Supplementation with these extracts, is tested either effect before, after and with *Salmonella* infection. Additional interventions include feed modification and supplementation with different plant extracts with antibacterial or immunomodulatory effects.

Plant extracts as feed supplements have been repeatedly tested in chicks [21]. Hummayd (*Rumex vesicarius* L.) leaves are edible weeds, eaten fresh or cooked [22]. It was considered a dietary complementary plant, since it is a rich source of bioactive phytochemicals [23, 24]. They have been traditionally used in Egypt as medicinal herb used in treatment of digestive problems, anti-inflammatory as well as antischistosomal, and antimicrobial activities. The species of *vesicarius* is the most abundant of the nine known *Rumex* species growing wild in Egypt [25]. Fruits of *Citrus aurantium* leave behind a substantial amount of peels and also large amount of *these wastes* were obtained as by-products from food processing industry. The rinds (peels) can present similar or even higher contents of valuable compounds, in particular phenolic secondary metabolites as flavonoids, which impart nutraceutical properties to fruit residues [26].

Many bioactive secondary metabolites found mostly in the peel tissues as the flavonoids (such as eriodictyol, isosakuranetin, hesperetin, naringenin, and their respective glycosides) and limonoids, a group of

highly oxygenated terpenoids. Phenolics and flavonoids are very important biologically active constituents, since they are considered to be immunostimulant, antioxidant and antimicrobial agents [7, 23, 27].

Rumex species are characterized by the presence of different classes of flavonoids (flavones flavanols and flavanones) and other metabolites such as carotenoids, tocopherols and ascorbic acid are also present [22]. *R. vesicarius* was characterized by abundances of flavonoids and was known to have an antimicrobial activity [24]. The direct effect of plant flavonoids against orally administered pathogens such as *Salmonella* Gallinarum was reported [28]. Our previous work showed the isolation of many bioactive flavonoids including the flavanones such as hesperetin, naringenin from *Citrus* species [26, 29]. The effect of these compounds on immunity and intestinal morphometry in lipopolysacharide-challenged chicks has been reported [30]. Dietary supplementation of these flavonoids compounds on poultry performance and immune system is reported [7, 18, 21].

The mulberry foliage has remained the primary food for silkworms for centuries. Its leaves have also been used as animal feed for livestock [31]. Black mulberry (*Morus nigra* L.) is a traditional herb known not only for its nutritional qualities but also for its traditional use in natural medicine as it has a high content of active therapeutic compounds [32]. The antimicrobial activities of different mulberries (*Morus* spp.) against Gram negative bacteria but not against the Gram positive were reported and their extracts showed special activity against *Salmonella* [33]. *Morus* herbs have traditional and current uses as fodder, food, cosmetics, and medicine. They have been used for the treatment of cold, flu, diabetes, bronchial calmative, cardiac diseases and nephritis. Our previous work demonstrated that *M. alba* and *M. rubra* inhibit proliferation and metabolic deteriorations as well as they showed free radical scavenging activity [34, 35]. Traditionally, berry are said to provide safe and effective treatments against several diseases including Antimicrobial activity on *Salmonella* spp. [36].

The results of the present study showed that supplementation by LMN extract was proven to reduced invasion by *S.* Typhimurium, as reflected by efficient prophylactic effect against infection when supplemented before and co-infection (simultaneously with infection). The results of this study revealed also the activity of ACA when supplemented simultaneously with infection of *S.* Typhimurium that is most common source of food borne diseases. Biologically active components of black mulberry are mostly secondary metabolites, such as terpenoids, glycosides and phenolics [36].

The determination of phenolics content according to the colorimetric method of Folin-Ciocalteu revealed the content of LMN extract (833.7 mg gallic acid equivalent/100 g dry weight). *Morus* species is a multi-functional plant with promising phenolic phytochemicals. Phenolics, including anthocyanins, flavanols, flavones, triterpenes, and stilbenes are found in *Morus* species in appropriate amount. Morus' phenolics represent a diverse group of compounds such as hydroxyl cinnamates, catechins, flavonols, flavanols, stilbenoids and tannic acids. Kubena et al. [5] reported that the effect of dietary content of tannic acids on the numbers of *Salmonella* cecal culture-positive chicks or in the numbers of *S.* Typhimurium in the cecal contents. Many *in vitro* and *in vivo* reports have verified the activity of phenolics against several types of intestinal pathogens with particular interest in *S.* Typhimurium [5, 36], which are major disease-causing or food-borne bacteria in poultry. Reduction in total bacterial load, suppression of pathogens, thinning of the mucosal layer, and direct modulation of the immune system may be the possible mechanisms exerted by these phenolics [37]. Oligomeric condensed tannins (proanthocyanidins) isolated from cranberry have shown a wide range of bioactive features including anti-microbial, anti-infective, and anti-adhesive properties against a number of disease-causing organisms [38]. The invasion of HeLa cells by *S.* Typhimurium was significantly reduced.

Our results suggest that the phenolics phytochemicals especially the proanthocyanidins–rich extract treatment may inhibited *Salmonella* invasion. This is likely primarily because of the perturbation of the host cell cytoskeleton by proanthocyanidins rather than an effect on bacterial virulence itself. In our current study, other *Morus'* phenolics such as flavonoids (quercetin and kaempferol derivatives), phenolic acids (hydroxybenzoic and hydroxycinnamic acids) may exerted the immunostimulatory effect of the LMR and ACA extracts. The plant based medicine are having important role as a prophylactic and therapeutic aid especially when it is accessible, easily prepared and safely administrated with low cost. In this study, the anti-*Salmonella* effects of three plant extracts were recorded. All of these extract could be used in poultry sector safely.

The results of this *study* indicate that *supplementation* chicks orally administered by drinking water with *Morus* extract (LMN) resulted in reduced invasion by *S.* Typhimurium, as reflected by efficient protection against infection when supplemented before and simultaneously with infection as compared with the ACA thus may be used in poultry production to effectively increase chicken performance. The suggested antibacterial mechanism was probably due to the damaged cellular proteins by *M. nigra* extract[39]. The activity may be attributed to the natural compositions that can inhibit food-borne pathogens growth especially the phenolic acids as syringic and chlorogenic. Additionally, a study of Kuete et al. [40] reported that the crude extract and compounds (moracins; R, M and Q, cycloartocarpesin and 3β-acetoxyurs-12-en-11-one) isolated from the stem bark of *Morus* species showed bacteriostatic activity against the growth of Gram negative microbes. Some *Morus* species is characterized by the presence of ellagitannins and organic acids. These phenolics could increase the electric conductivity of bacterial cell suspensions causing cellular leaking of electrolytes [33, 39] and showed bacteriostatic activity against the growth of pathogens.

In our study, phenolic phytochemicals, including antimicrobial compounds such as kuwanon G, 1-deoxynojirimycin, mulberrofuran G, albanol B, and prenylated flavonoids may be attributed to the protective effect of black berry leaves against *S.* Typhimurium infection and enhance immunity [41]. Recently [31], *M. alba* has significantly stimulated the uptake of bacteria into peritoneal macrophages. This plant showed protective and immune-enhancing ability against infectious disease and increase phagocytosis, neutrophils, monocytes, and cytokines in the infected mice with bacteria. Our current study reports the content of microelements in remarkable quantities in *M. nigra* leaves (134.3, 78.7, 44.2 ppm for Fe, Mn and Zn respectively. Also, the presence of macroelements as Ca (2.79%) and K (1.89%) were recorded. The supplemented group with LMN rich in macronutrient and minerals (Ca, P, K, Cu, Na, Mg, Fe, Mn, and Zn) composition may affect the immune system. Macronutrients play significant roles during the entire plant life such as plant metabolism as well as protecting plants from various abiotic and biotic stresses.

Dietary interventions were reported to have good impact on the immune system and/or *Salmonella* clearance in chickens[42]. Zinc is an important nutrient in animal metabolism. In poultry, zinc serves not only as a nutrient but can also be used as a dietary supplement to manipulate the reproductive system of the bird. In our current study, the presence of zinc and Ca in the GIT of chicks might sufficiently limit *Salmonella* growth to decrease the potential for colonization and invasion [1]. It is hypothetically possible that the presence of additional dietary zinc and calcium in the gastrointestinal tract (GIT) of chicks might lie with the beneficial interaction of zinc with the immune system [1].

Inorganic mineral elements such as potassium, zinc, calcium, etc. play important roles in poultry performance and may make chicken less susceptible to pathogen infection. Clearly, if synergism occurs between direct limitation of *Salmonella* growth by nutrient intake from Zn in the GIT and a more responsive immune system, chicks under a zinc-feeding regime should be more resistant to sustained *Salmonella* infection. In addition to increased nutrient intake from micro- and macro-elements diets that stimulates the immune system and the enhanced immune response leads to a less susceptible bird to *Salmonella* invasion even if colonization in the GIT occurs [1].

In our present work, the impact of these valuable micro- and macro-elements on growth performance and *Salmonella* counts in experimentally challenged chicks may be suggested. Also, the use of leaves of *M. Nigra*, a good source of macro- and microelements, may ensure protection against mineral deficiencies and increase the immunity. The nutritional values and medicinal potential of such safe natural products are of considerable importance as they help to pinpoint traditional natural resources of poor population in developing countries. The assessment of elements (macro- and micro-) analysis and phenolic phytochemical composition of the selected potent agriculture waste of *M. nigra* may provide potential insights into their applications in poultry functional foods and nutraceuticals development.

Recently [32], a study has suggested that a functional food mixture (SFG) that is composed of four major ingredients, including *M. alba* extract, has no significant mutagenic or toxic properties, and the no observed adverse effect. This may help design better poultry nutrition to lower *Salmonella* infection in chicks and, therefore, reduce human Salmonellosis [42]. Furthermore, a better understanding on the impact of phenolic and elements on immunity of poultry will allow a better use of these products for economically effective and sustainable poultry production.

The use of these available natural products as alternative to vaccines and antibiotics with the advantage of being safe to the public health, cost-effective, and friendly to the environment together with antimicrobial and immune-stimulant activity could be considered as a viable option. So, this paper put forward new insight for the utilization of safe waste of plant-base products to counteract *S.* Typhimurium infection as still many of today's drugs are plant-derived natural products or their derivatives.

CONCLUSION

The current study revealed that the newly made dietary supplements produced from known safe natural products seemed to have valuable health effects on poultry and human. Supplementation with the investigated plants show promise as simple approach for reducing invasive Salmonellosis. These results corroborated well with the reported data suggested the importance of safe natural products rich in minerals and phenolics.

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