Journal of Entomology and Zoology Studies

Available online at www.entomoljournal.com

E-ISSN: 2320-7078
P-ISSN: 2349-6800
JEZS 2017; 5(4): 1375-1380

Received: 27-05-2017
Accepted: 28-06-2017

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An attempt to prepare an effective killed vaccine with an adjuvant (green tea extract) against *Salmonella typhimurium*

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Abstract
The present research was carried out to study the efficacy of green tea extract as a potent adjuvant mixed with killed *Salmonella typhimurium* (*S.T.*) vaccine to enhance its immunization capacity during the period from September 2014 to February 2015. Three groups of mice were immunized with the mixture of *S.T.* and green tea extract, *S.T.* alone, and phosphate buffered saline. The mixture group showed the best results in cell-mediated immunity; in which a significant difference ($P < 0.005$) in the skin thickness was detected (1.05±0.21 after 24 h and 1.35±0.162mm after 48 h and also higher records in the ELISA 1.342±0.650 nm and 1.114±0.018 nm after 35 and 53 days from the beginning of immunization, respectively. The results were verified by examination of spleen and liver histological sections. The present study suggests the effectiveness of the green tea extract as a potent adjuvant mixed with killed *Salmonella typhimurium* (*S.T.*) vaccine for the enhance the immunization of mice.

Keywords: green tea extract, *Salmonella typhimurium*, mice

1. Introduction
Salmonella infection is a serious medical and veterinary problem world-wide. All *Salmonella* serotypes are members of a single species, *Salmonella enterica* [1]. More than 2500 serovars have been described of which humans are almost exclusively infected by *Salmonella enterica* subsp. Enterica serotypes Typhi, Typhimrium, and Choleraesuis worldwide [1]. Ubiquitous (non-host-adapted) *Salmonella* serovars (e.g., *Typhimurium*) cause very diverse clinical symptoms, from asymptomatic infection to serious typhoid-like syndromes in infants or certain highly susceptible animals (mice) [1, 2]. In human adults, ubiquitous *Salmonella* organisms are mostly responsible for foodborne toxic infections [2]. Antibiotic treatment of the infection has been successful in the past, but new multi-drug-resistant *Salmonella* strains are rapidly emerging [3, 4]. Vaccination is an effective tool for the prevention of *Salmonella* infection. However, efficacy of the currently available vaccines is not always enough [5]. Although live vaccines usually have better protective effects when compared with inactivated vaccines [6], the humoral response, highly inducible by inactivated vaccines, is especially important for the control of faecal shedding [7]. Therefore, further studies on the efficacy of killed vaccines are needed because of the potential hazards associated with the using of live attenuated *Salmonella* strains, such as reversion to virulence and immunosuppression[8]. Various studies have shown significant suppressive effects of green tea against many microorganisms including *S. Typhimurium* [9, 10]. The studies that deal with the using green tea extract as an adjuvant with any vaccine are rare. Hence, the present study was aimed to evaluate the using of the green tea extract with killed *S. Typhimurium* as a vaccine in order to improve its efficacy.

2. Materials and Methods
The experiment was conducted during the period from September 2014 to February 2015**.**

2.1. Bacteria
S. Typhimurium was obtained from Zoonotic Diseases Unit, Vet. Med. College/Baghdad University. The morphological and biochemical characteristics were verified according to the method of Winn *et al*. [11].

2.2. Adjutant
Green tea extract was purchased from medical herbal drugstore licensed by Health Ministry, Baghdad, Iraq.

2.3. Bacterial suspension
It was prepared by culturing the purified bacteria on blood agar plates for 24 h, and then harvesting the bacterial growth by sterile glass rod with phosphate buffered saline (PBS). The bacterial number in the suspension was calculated according to the method of Harley and Prescott [12] and compared with McFarland tubes.

2.4. Laboratory animals
A total of 26 Swiss albino white mice from genders, 6 week aged and 20-25 gm weighted were purchased from Vet. Med. College. Mice were divided as following:-

1. Two mice were used to collect blood and culture all the internal organs to confirm deprivation of mice used in this experiment from any microbial infections and absence of anti *S. Typhimurium* antibodies.
2. Six mice were used to calculate minimum lethal dose, as follows:
3. One mouse is injected with *S. Typhimurium* suspension containing 1×10^6 CFU/ ml in a dose of 0.2ml s/c.
4. One mouse is injected like above but the suspension contained 1×10^7 CFU/ ml.
5. One mouse was injected with 3×10^8 CFU/ ml suspension in a dose of 0.25 ml s/c.
6. One mouse was inoculated s/c with 0.25 ml of suspension contained 6×10^8 CFU/ ml.
7. One mouse was inoculated as above (suspension contained 6×10^8 CFU/ ml s/c), but in a dose of 0.3 ml.
8. The last one was injected s/c with a suspension of 6×10^8 CFU/ ml in a dose of 0.5 ml.

Eighteen mice were used for immunization and divided into3 groups, each containing 6 mice immunized s/c with 0.1 ml of the following:-

1. Group 1: mixture of *S.T.* & green tea extract.
2. Group 2: *S.T.* only**.**
3. Group 3: Control group immunized with PBS.
   After 14 days, all groups were given the second dose as the first immunization.

2.5. Preparation of *Salmonella* antigen used for DTH test:

1. Prepare *S. Typhimurium* suspension containing 6×10^8 CFU/ ml as mentioned previously.
2. Culture 250 ml nutrient broth with 2 ml of the above suspension at 37°C for 24 h.
3. Confirm purity by staining with Gram's stain.
4. Put the cultured media in 50 ml centrifuge tubes for centrifugation at 4000 RPM for 30 min, and then wash the sediment twice at the same speed for 15 min.
5. Add suitable amount of PBS to the sediment (pellet) and compare with McFarland tube no. 2 that contains 6×10^8 CFU/ ml.
6. Put the suspension in autoclave at 121 °C for 30 min.
7. Keep in refrigerator until use.

2.6. Killed *S.T.* vaccine
S. Typhimurium vaccine was prepared according to the method of Papezova *et al.* [13], with few modifications. It was prepared as above (the steps 1-4 are the same).
5- After centrifugation, add PBS containing 0.4% formalin to the pellet and incubate at 37°C.
6- Culture on blood agar plate was done daily to affirm killing of bacteria.
7-After bacterial killing, it was washed 3 times with PBS by centrifuge at 3000 RPM for 15 min.
8-The sediment was suspended with PBS to become equal to McFarland tube no. 2 (6×10^8 CFU/ ml) and it was denoted as *S.T.* in this study.
9- Killed vaccine (*S.T.*) was mixed in a proportion of 1:1 with the adjuvant green tea extract.

2.7. DTH
Delayed-type hypersensitivity test was performed for 32 days from the beginning of immunization. The skin test was applied in the day 18 from the second immunization dose, in which the right foot pad of each mouse was injected I.D with 0.1 ml *Salmonella* antigen using disposable insulin syringe and sterile PBS to the left foot pad in the same way as a control. Skin thicknesses were read before injection, 24 and 48 h post-injection using caliper.
After 35 days of immunization, 2 mice from each group were anesthetized with inhaled ether, and then were sacrificed. Blood samples were collected directly from the heart without anticoagulant to obtain serum for ELISA test. Pieces of organs about 1 cm^3 thickness from spleen and liver were kept in 10% Neutral Buffered Formalin for studying histological changes [14].

2.8. Protection experiment
Mice in all groups were challenged 53 days post-immunization by s/c injection of 6×10^8 CFU/ ml *S. Typhimurium* in a dose of 0.25 ml. After 10 days of challenge, all the mice were anesthetized with ether and sacrificed to collect blood samples for the ELISA. Few drops of blood and pieces of internal organs (lung, kidney, liver, and spleen) were used for bacterial culturing on blood agar and MacConkeys agar. Parts of spleen and liver were used for histopathological examination as described previously.

2.9. ELISA
Antibody titers were estimated using ELISA kit produced by Frenchcompany, SYNBIOTICS, according to the instructions of the company. Blood samples were collected from mice after 35 and 53 days of immunization using suitable tubes without anticoagulant. Blood samples were left for 24 h in the refrigerator at 4°C, and then centrifuged at 3000 RPM for 10 min to obtain serum for ELISA. ELISA reader was used to record the results as optical density (OD) for each serum sample on the wave length 450 nanometer. The value 0.153 nm and above was regarded positive, while the reading below 0.153 nm was regarded as negative, according to the instructions of the produced company.

2.10. Statistical analysis
Data were subjected to analyze by using SAS software. Means were compared using paired t test. $P < 0.05$ is considered significant.

3. Results
3.1. Determination of challenge dose (minimum lethal dose):
Mice injected with *S. Typhimurium* suspension containing 1×10^6 CFU/ ml in a dose of 0.2 ml, 1×10^7 CFU/ ml in the same dose and 3×10^8 CFU/ ml in a dose of 0.25 ml, separately. While mice injected with 0.3 ml and 0.5 ml suspension containing 6×10^8 CFU/ ml succumbed to death after 48 h and 24 h, respectively. The mouse injected with

0.25 ml of 6×10^8 CFU/ ml suspension was died after 72 h and this dose was regarded as the minimal lethal dose used in the experiment.

3.2. DTH test.

Higher difference of skin thickness was noticed in the group vaccinated with the mixture of green tea extract and *S.T.* The results were 1.05 ±0.212 mm and 1.22 ±0.162 mm after 24 h and 48 h, respectively (table 1), while the group immunized with *S.T.* only showed a difference in skin thickness of 0.93 ±0.287 mm after 24 h and 1.11 ±0.368 mm after 48 h (table 2). Control group showed negative results.

3.3. ELISA.

After 35 days of immunization; 14 days from booster dose, the results were 0.635±0.059 nm for S.T. group without adjuvant, and 1.342±0.650 nmfor the mixture composed of *S.T.* and green tea extract. 53 days from the beginning of immunization. The mixture group has given the result 1.114±0.018 nm, while 0.535±0.256 nm was recorded in the *S.T.* group.

3.4. Protection experiment

In immunized animals, no important macroscopic lesions were noticed on different organs, but enlargement of spleen in mice vaccinated with the mixture (Fig. 1), whereas mice immunized with *S.T.* did not showed any macroscopical change (Fig. 2). There was no bacteria isolated from internal organs cultured on bacteriological media.

Control animals. Macroscopic changes in mice challenged with *S. Typhimurium* involved enlargement and paleness of liver with slight enlargement of spleen and petechial hemorrhage on lung surface.

After challenge of control animals with 0.5 ml of 6×10^8 CFU/ ml *S. Typhimurium*, bacteria were isolated from mice in large numbers (++++) from spleen. There were no bacteria isolated from heart, lung, and kidney after 24 h of animal death. Animals challenged with 0.25 ml of 6×10^8 CFU/ ml *S. Typhimurium* succumbed to death after 72 h of injection. The bacteria were isolated in large numbers (++++) from spleen, (+++) from blood, (++) from liver and few numbers (+) from kidney.

3.5. Pathological changes

In group 1, in which animals were immunized with *S.T.* and green tea extract, the pathological lesions in liver included mononuclear cell aggregation around the central vein and in the portal area (Fig. 3) with proliferation of kupffer cells (Fig. 4). In other animals, the liver has shown mononuclear cells aggregation in one side of congested central vein and neutrophils in their lumen, also mononuclear cells aggregation in liver parenchyma. The lesions in spleen characterized by amyloid-like substance and deposition around the white pulb, also there was mononuclear cells aggregation around the sinus in the red pulb.

In group 2, in which mice were immunized with *S.T.* only, the histopathological changes in spleen after 14 days of immunization have revealed moderate hyperplasia of white pulb and moderate mononuclear cells aggregation around the sinusoid (Fig. 5). The changes is spleen of one of the animals after challenge included moderate amyloid-like substance deposition in the parenchyma and moderate hyperplasia of mononuclear cells around sinuses (Fig. 6). The spleen of other mice has shown hyperplasia of white pulb and mononuclear cells proliferation (Fig. 7) and there was moderate white pulb hyperplasia with proliferation of megakaryocyte in another mouse. Histological changes in liver after 10 days of challenge have shown proliferation of kupffer cells with moderate mononuclear cells infiltration in liver parenchyma.

Control group immunized with PBS and challenged with virulent *S. Typhimurium*, revealed severe changes in liver (Fig. 8) included multiple area of necrosis in liver parenchyma with inflammatory cells infiltration and dilatation of sinusoids (Fig. 9). Spleen in the main lesion has characterized by depletion of white pulb with inflammatory cells infiltration in the red pulb. Also, spleen of other animals showed amyloid- like substance deposition around the depleted white pulb.

Table 1: The difference of thickness in foot pad of mice vaccinated with the mixture *S.T.* & green tea extract

Animal No.	Difference of Thickness (mm)	
	24 h	48 h
1	1.5	1.9
2	1.7	1.9
3	0.8	1.2
4	0.3	0.8
5	1.3	1.4
6	0.7	0.9
Mean± Standard Error	1.05± 0.21	1.35± 0.19
P	0.005	

Table 2: The difference of thickness in foot pad of mice immunized with *S.T.* only

Animal No.	Difference of Thickness (mm)	
	24 h	48 h
1	1.6	0.6
2	0.4	0.75
3	1.0	1.2
4	0.5	1.0
5	0.8	0.8
6	2.3	2.3
Mean± Standard Error	0.93± 0.287	1.11± 0.368
P	0.97	

Fig 1: Spleen of an animal immunized with S.T. & green tea.

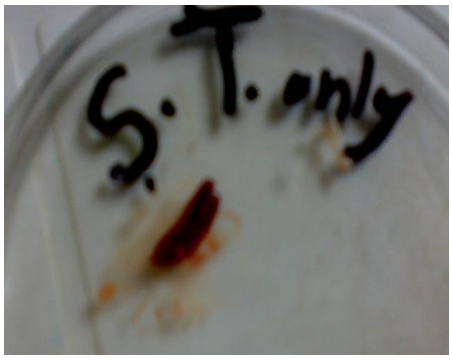

Fig 2: Spleen of a mouse immunized with S.T. only.

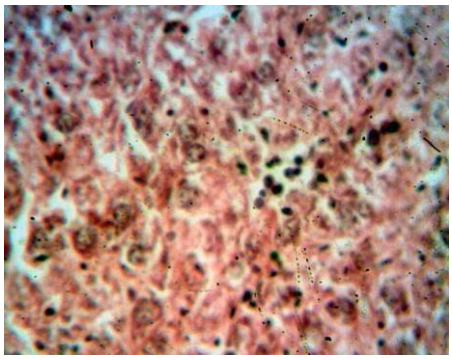

Fig 3: Histological section in the liver of an animal immunized with the mixture challenged with virulent *S. Typhimurium* shows proliferation of kupffer cells and aggregation of mononuclear cell in liver parenchyma (H & E 40×).

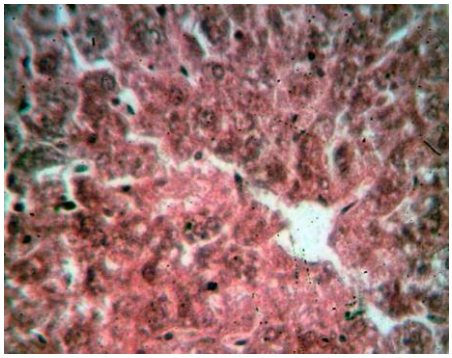

Fig 4: Histological section in the liver of an animal immunized with the mixture and challenged with virulent *S. Typhimurium* shows proliferation of kupffer cells (H & E 40×).

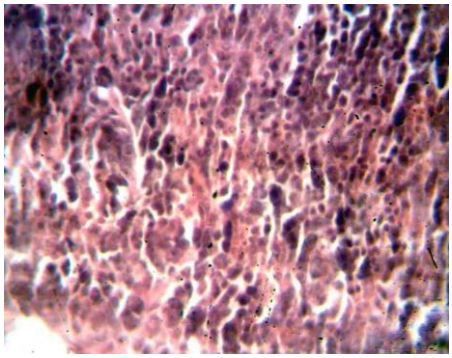

Fig 5: Histological section in the spleen of one animal immunized with S.T. only 14days post-immunization shows hyperplasia of white pulb and proliferation of mononuclear cell around the sinus formed cord-like structure (H & E 40×).

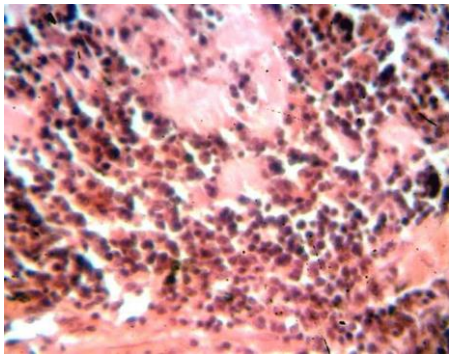

Fig 6: Histological section in spleen of an animal immunized with S.T. without adjuvant and challenged with virulent *S. Typhimurium* shows moderate myeloid- like substance deposition in the spleen parenchyma with moderate hyperplasia of mononuclear cell around the sinus (H & E 40×).

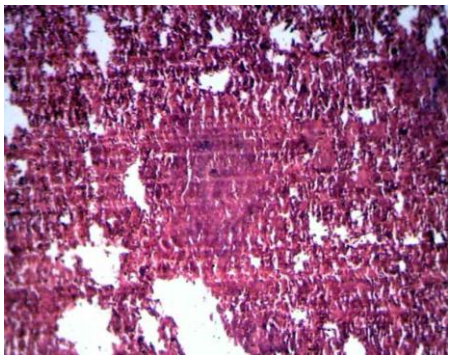

Fig 7: Histological section in the spleen of one animal immunized with S.T. only and challenged with virulent *S. Typhimurium* shows hyperplasia of white pulb and proliferation of mononuclear cell around the sinus (H & E 40×).

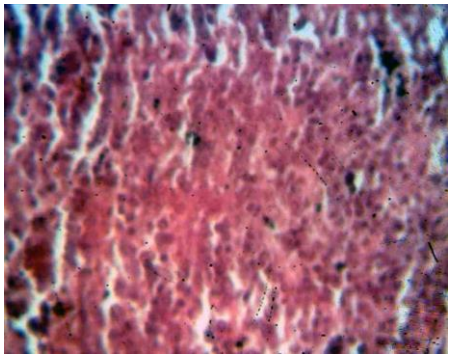

Fig 8: Histological section in the liver of one control animal infected with virulent *S. Typhimurium* shows congestion ofcentralvein and sinusoid with inflammatorycell (macrophage and neutrophil) infiltration in the interstitial (H & E 40×).

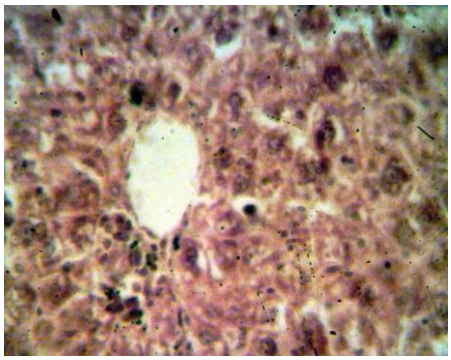

Fig 9: Histological section in the liver of control animal immunized with PBS and challenged with S.T. shows mononuclear cell aggregation in the liver parenchyma around the central vein (H & E 40×).

4. Discussion

|The increased level of IL-1, IL-6, IL-8 and *in vitro* nitric oxide production indicates that the host inflammatory and pro-inflammatory responses were clearly augmented by the killed vaccine [8]. Therefore, the present study was focused on the use of killed *S. Typhimurium* vaccine mixed with an adjuvant which was green tea extract.

Tiwari *et al*., [15] have claimed that the green tea extracts were effectively inhibited the growth of *S. Typhimurium* 1402/84. Enhanced antibacterial activity with green tea extract may be due to the higher content of catechin and some oil fractions besides water-soluble fraction [16. 17]. Tea polyphenols which are constituents of tea extracts have been shown to have antibacterial activities against human and animal disease-related bacteria and the main constituent of tea polyphenols responsible for antibacterial action is epigallocatechin gallate [18].

Our study indicated that the mixture of green tea extract & *S.T.* showed better results in the skin test in which the mean difference of the skin thickness was 1.05±0.21 mm after 24 h and 1.35±0.19 mm after 48h and the difference was significant ($P<0.05$). The Abs titer elicited by this mixture were 1.342±0.65 nm after 35 days from the beginning of immunization and those results slightly decreased to 1.114±0.018 nm after 10 days of the challenge and 53 days after the beginning of immunization.

In this study, killed *S.T.* vaccine without adjuvant showed lower results in the both of the humoral and cellular immunity as compared with that given with adjuvant. CMI elicited by it was 0.93±0.287mm and 1.11±0.368mm after 24 and 48 h, respectively. While, Ab titers were 0.635±0.059 nm after 35 days of immunization, and decreased slightly to 0.535±0.256 nm after 53 days from the beginning of immunization program. Papezova and colleagues, [13] have revailed that the stronger humoral response to the inactivated vaccines is not only associated with the viability status of the vaccine but also with the route of vaccine application and the presence of adjuvants in the inactivated vaccines.

The mixture of *S.T.* & green tea extract showed good results in the histological test, as characterized by mononuclear cells infiltration in both liver and spleen. Histopathological lesions were severe in control animals challenged with virulent *S. Typhimurium*. The lesions included multiple area of necrosis in liver parenchyma with inflammatory cells infiltration and dilatation of sinusoid. There was a depletion of white pulb and inflammatory cells infiltration in the red pulb of spleen besides amyloid- like substance deposition around the depleted white pulb. Genes which facilitate successful infection are clustered at particular parts of chromosomes called *Salmonella* Pathogenicity Islands (SPI). Most of the genes required by *S. Typhimurium* to invade epithelial cells are localized to SPI-1 while genes essential for the intracellular survival of *S. Typhimurium* are clustered in SPI-2 [13]. Systemic infection of mice with *S. Typhimurium* results in rapid uptake of these bacteria by mononuclear phagocytes in liver and spleen and *Salmonella*-specific Abs enhance this uptake [19]. Intracellular bacteria would not undergo lysis in the lysosomes immediately but survive for a period of time due to unknown reasons. They could provide a reservoir of antigen [20]. In liver and spleen, *S. Typhimurium* survives within mononuclear phagocytes [21] where bacteria are shielded from Abs. As a consequence, mechanisms that activate macrophages are necessary to control *S. Typhimurium*. The importance of macrophage activation is emphasized by the high susceptibility to S. *Typhimurium* of mice deficient in IFN-γ receptor or of mice in which IFN-γ or TNF-α are neutralized with specific Abs [22]. Both IFN-γ and TNF-α are crucial for macrophage activation. T cells are involved in this process either by secreting macrophage-activating cytokines like IFN-γ or by direct T cell-macrophage interactions. Accordingly, mice deficient in T cells, and especially in CD4+ T cells, suffer from chronic infection with attenuated aroA- *S.Typhimurium* strains. An important function of CD4+ T cells is to provide the assistance for the activation and differentiation of B cells [23]. In our study, green tea extract as an adjuvant showed prominent protection in mice against virulent *S. Typhimurium*, so that we advise to study its efficacy in other laboratory and farm animals for further evaluation.

5. Conclusion

The present study suggests the effectiveness of the green tea extract as a potent adjuvant mixed with killed *Salmonella typhimurium* (*S.T.*) vaccine for the enhance the immunization of mice.

6. Acknowledgements

Author wish to extend his gratitude to the Zoonotic Diseases Unit, Vet. Med. College/Baghdad University for their assistance in accomplishes this work.

7. References

1. Owens MD, Warren DA. *Salmonella* Infection. Emergency Medicine Infectious Diseases. emedicine.medscape.com/article/785774.2008.
2. Todar K. *Salmonella* and salmonellosis. Toder, s Online Textbook of Bacteriology, 2005.
3. Begum F, Adachi Y, Khan MSR. Immunological characterization of 37.81 kDa common immunodominant surface protein of some *Salmonella* serovars. Bangladesh Journal of Veterinary Medicine. 2008; 6(2):145-151.
4. Bacon RT, Ransom JR, Sofos JN, Kendall PA, Belk KE, Smith GC. Thermal inactivation of susceptible and multiantimicrobial-resistant *Salmonella* strains grown in the absence or presence of glucose. Applied Environmental Microbiology. 2003; 69(7):4123-4128.
5. Mastroeni P, Menager N. Development of acquired immunity to *Salmonella*. Journal of Medical Microbiology. 2003; 52:453-459.
6. Robertson JA, Lindberg AA, Hoiseth S, Stocker BA. *Salmonella typhimurium* infection in calves: protection and survival of virulent challenge bacteria after immunization with live or inactivated vaccines. Infectious Immunology. 1983; 41:742-750.
7. Desmidt M, Ducatelle R, Mast J, GoddeerisBM, Kaspers B, Haesebrouck F. Role of the humoral immune system in *Salmonella enteritidis* phage type four infection in chickens. Veterinary Immunology and Immunopathology. 1998; 63:355-367.
8. Okamura M, Lillehoj HS, Raybourne RB, Babu US, Heckert RA. Cell-mediated immune responses to a killed *Salmonella enteritidis* vaccine: lymphocyte proliferation, T-cell changes and interleukin-6 (IL-6), IL-1, IL-2, and IFN-γ production. Comparative Immunology Microbiology and Infectious Disease. 2004; 27:255-272.
9. Michalczyk M, Zawislak A. The effect of tea infusions on the proliferation of selected bacteria important for the human intestinal tract. Acta Scientiarum Polonorum Technologia Alimentaria. 2008; 7(1):59-65.
10. Jazani HN, Shahabi SH, Ali AA, Zartoshti M. Antibacterial effects of water soluble green tea extracts on multi- antibiotic resistant isolates of *Acinetobacter* sp. Pakistan Journal of Biological Science. 2007; 10(9):1477-1480.
11. Winn WC, Allen SD, Janda WM, Koneman EW, Procop GW, Schreckenberger PC *et al*. Koneman's Color Atlas and Textbook of Diagnostic Microbiology. 6 th ed. Lippincott William's & Wilkins Company, Philadelphia. 2006.
12. Harley JP, Prescott LM. Determination of bacterial numbers. In: Laboratory Exercises in Microbiology. 5th ed. The McGraw-Hill Companies. 2002, 117-120.
13. Papezova K, Havlickova H, Sisak F, Kummer V, Faldyna M, Rychlik I. Comparison of live and inactivated *Salmonella typhimurium* vaccines containing different combinations of SPI-1 and SPI-2 antigens in poultry. Veterinary Medicine. 2008; 53(6):315-323.

14. Luna LG. Manual of Histologic Staining Methods of the Armed Forces Institute of Pathology.3rd ed. McGraw-Hill, New York. 1968.
15. Tiwari RP, Bharti SK, Kaur HD, Dikshit RP, Hoondal GS. Synergistic antimicrobial activity of tea and antibiotics. Indian Journal of Medical Research. 2005; 122:80- 84.
16. Akroum S, Satta D, Laloui K. Antimicrobial, antioxidant, cytotoxic activities and phytochemical screening of some Algerian plants. European Journal of Scientific Research. 2009; 31(2):289- 295.
17. Khalaf NA, Shakya AK, AL- Othman A, EL- Agbar Z, Farah H. Antioxidant activity of some common plants. Turkish Journal of Biology. 2008; 32:51-55.
18. Su P, Henriksson A, Nilsson C, Mitchell H. Synergistic effect of green tea extract and probiotics on the pathogenic bacteria, *Staphylococcus aureus* and *Streptococcus pyogenes*. World Journal of Microbiology and Biotechnology. 2008; 24:1837-1842.
19. Saxen H. Mechanism of the protective action of anti-*Salmonella* IgM in experimental mouse salmonellosis. J General Microbiology. 1984; 130:2277.
20. Meng FP, Ding J, Yu ZC, Han QL, Guo CC, Liu N *et al*. Oral attenuated *Salmonella typhimurium* vaccine against MG7-Ag mimotope of gastric cancer. World Journal of Gastroenterology. 2005; 11(12):1833-1836.
21. Richterdahlfors A, Buchan AMJ, Finlay BB. Marine salmonellosis studied by confocal microscopy: *Salmonella typhimurium* resides intracellularly inside macrophages and exerts a cytotoxic effect on phagocytes *in vivo*. Journal of Experimental Medicine. 1997; 186:569.
22. Nauciel C, Espinasse-Maes F. Role of gamma interferon and tumor necrosis factor α in resistance to *Salmonella typhimurium*. Infectious Immunity. 1992; 60:450.
23. Mittrucker HW, Raupach B, Kohler A, Kaufmann SHE. Role of B lymphocytes in protective immunity against *Salmonella typhimurium* infection. Journal of Immunology. 2000; 164:1648-1652.