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Original Article

Study the Ability of *Salmonella typhimiurm* to Induce Cytokine TNFα in Mice Treated with Olive Leaves Extract and Ciprofloxacin

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Abstract

TNF-α is a type of cytokine that is produced by inflammatory cells. These inflammatory cells produce cytokines such as TNF-α, IFN- γ, IL-1, etc. Ciprofloxacin is the last drug of choice to clear the infection. Ciprofloxacin, a grace inhibitor, kills bacteria by inducing chromosome fragmentation and reactive oxygen species (ROS) in the bacterial cell. This study was designed to investigate the ability of *Salmonella typhimiurm* to induce TNF-α in cells. 30 NMRI mice from both genders were divided randomly into 3 groups (n=10) and treated as follows: The first group was intraperitoneally (I.P.) injected with a 0.1 ml/4×10^8 CFU/ ml bacterial suspension of *S. typhimiurm* as a positive control**.** The second group was injected (I.P.) with *S. typhimiurm* 0.1 ml (4×10^8 CFU/ml and then injected with 30 mg/kg/0.3 ml of CiprofloxacinCiprofloxacin intramuscular (I.M.). The olive leaf alcoholic extract was given to the third group through a gavage tube for two weeks before giving I/P 0.1 ml/4×10^8 CFU/ml *S. typhimiurm*. *After*ward, they were post-treated with 30 mg/kg/0.3 ml of olive leaf extract orally by gavage tube until the end of the experiment (30 days). The recorded data from group 1 showed heavy deposition of TNF-α marker in the spleen and liver, and examination showed dark brown cellular components. Also, in the second group, the spleen and liver tissue sections showed heavy deposition of TNF-α marker; other sections showed moderated deposition of TNF-α marker. In the third group, TNF marker was found in small amounts or not in tissue samples from the spleen and liver. Histopathological examination of infected 1st group liver and spleen tissue sections shows lobular hepatic necrosis with mononuclear cells. Aggregation manifests as granuloma lesions, particularly lymphocytes in the portal area around the bile duct and blood vessels, with the proliferation of macrophages known as kupffer cells and depletion of white pulp in spleen tissue when compared to the second and third groups, which demonstrated moderated lesions in infected and treated mice. The results showed that olive leaf extract reduces the infection of *Salmonella typhimiurm* in the pre and post-treated groups better than CiprofloxacinCiprofloxacin.

Keywords: *Salmonella typhimiurm*, TNFα, Antioxidants, Olive Leaves Extract

1. Introduction

Salmonella typhimurium are gram-negative bacteria consisting of non-spore-forming bacilli and are a member of the family *Enterobacteriaceae*. They are a significant cause of salmonellosis because their intracellular survival and replication are essential virulence determinants, and the bacteria can infect phagocytic and non-phagocytic cells (1). The primary host defense against *Salmonella* species occurs through the neutrophils, followed by mononuclear cells. These inflammatory cells produce cytokines like TNF-α, IFN-γ, IL-1, IL-2, IL-6, IL 8 (2). IL-2 is the major growth factor of T lymphocytes, and interleukin 4 (IL4) is a cytokine that induces the differentiation of naive helper T cells (The cells) to Th_2 cells (3). Olive leaf alcoholic extract raises energy levels and is found to reduce

hypertension, increase immunity and maintain the cardiovascular system (4, 5). Olive leaf alcoholic extract has an antibacterial effect on *L. monocytogenes*, *Staphylococcus aureus*, and *E. coli*, which cause food-borne diseases (6). Ciprofloxacin is the last drug of choice to clear the infection. Ciprofloxacin, a grace inhibitor, kills bacteria by inducing chromosome fragmentation and reactive oxygen species (ROS) in the bacterial cell. It is a commonly used antibiotic for urinary tract infections that interacts with bacterial topoisomerases, leading to the oxidative radical generation and bacterial cell death (7). Ciprofloxacin is medically reported (a fluoroquinolone) as an antibiotic used to treat several bacterial infections, including bone and joint infections, respiratory tract infections, intra-abdominal infections, and certain types of infectious diarrhea, typhoid fever, and urinary tract infections (8). In the present study, the effects of olive leaf extract on mouse models infected by *S. typhimurim* compared to ciprofloxacin treatment were investigated.

2. Materials and Methods

2.1. Experimental Design

100 Albino mice (NMRI) of both genders, aged 7-8 weeks and weighing about 20–25 g, were used in this study. They were kept at the animal house for 2 months at the University of Baghdad, College of Veterinary Medicine, Department of Pathology, in controlled conditions at a temperature of 20 ± 5ºC. The mice were housed in a plastic cage containing hardwood, fed pellets, and given water ad libitum.

2.2. Preparation of Olive Leaf Alcoholic Extract

Olive leaves were collected locally from Baghdad. Then, they were weighed, washed, and air-dried at room temperature (24±2°C) for 4 weeks to remove moisture. The dried leaves were weighted again and ground into a fine powder. 50g was taken and macerated in 95% ethanol at room temperature for 48 hours, forming a green-brown solution. The mixture was filtered in a rotary evaporator at 200RPM at 50°C for 30 minutes to suppurate the ethanol from the olive leaf extract (9).

2.3. Detected TNF*α* by Immunohistochemistry

The level of TNF-a in the blood samples was detected using ELISA kits, according to the manufacturer's procedures (10).

2.3.1. Culture and Maintenance of *Salmonella typhimurium*

Salmonella typhimurium was cultured on S.S.S.S. agar by sterilely transporting a single pure colony and incubating at 37 C° for 24 hours until total growth of colonies, then subcultured on nutrient agar at 37 C° for 24 hours to activate the bacteria. Bacterial activation was accomplished by injecting a bacterial suspension dose of 0.2 or 0.3 I.P.I.P. into mice and then waiting 24 hours. The mouse died during this duration; the bacteria was activated. If the mouse did not die, we killed the mouse and took it from the internal organ, and cultured it on the selective medium S.S.S.S. agar to identify the bacteria, harvested after 24 hrs. At 37 C°, sub-cultured it on nutrient broth, preparing for the harvest.

2.3.2. Antimicrobial Susceptibility Testing

Salmonella isolates were tested for antibiotic resistance using the standard disc diffusion assay according to the method previously described by Al-Hamadani and Al-Shahery (11).

2.4. Determination of the Infected Dose of *Salmonella typhimurium*

The counting was done using the method previously described by Miles, Misra (12). The animal was infected with 0.1 ml / 4×10^8 CFU/ml intraperitoneally according to the method previously described by Al-Saadi (13).

2.5. Histopathological Examination

All animals were examined post-mortem, and all macroscopic appearances were recorded to detect any abnormal gross changes in the internal organs (14).

2.6. Statistical Analysis and Scoring Apoptosis Marker

The Statistical Analysis System-SAS (2012). The scoring of path-immune reactions on cellular membranes was calculated in different percentages depending on the presence or not of demarcation on cellular values, and these were done according to Al-

Aamery Muna (15). $P \leq 0.05$ was considered statistically significant (15).

3. Results

3.1. Antimicrobial Susceptibility Testing

Antimicrobial resistance was common against *S. typhimurium*. The antibiotic sensitivity results showed complete resistance to Gentamycin, Erythromycin, and Penicillin and intermediate resistance to Ampicillin, Chloramphenicol, and streptomycin, while the most effective antibiotic was CiprofloxacinCiprofloxacin.

3.2. Detected TNFα by Immunohistochemistry

The first group in the study was injected with bacterial suspension at 0.1 ml/4×10^8 CFU / mL I/P dose of *S. typhimurium* as a positive control. Spleen's tissue appears to increase hypercellularity with heavy deposition of apoptotic markers around lymphocytes. Sections of the liver show hepatocyte nuclei absent of other cells surrounded with dark brown pigmentation due to TNFα deposition.

The second group was injected with bacterial suspension at 0.1 ml / 4×10^8 CFU / ml I/P dose of *S. typhimurium* and treated with Ciprofloxacin I/M injection 30mg/kg / 0.3 ml. Results determine that the lung, spleen, and liver showed rich color. Other sections showed moderated deposition of apoptosis marker that refers to infection reduction after treatment.

Group three was injected with *S. typhimurium* 0.1 ml / 4×10^8 CFU/ ml I/ P and treated orally with 30mg/kg / 0.3ml of olive leaf alcoholic extract. As shown in figures 1 and 2, lung, spleen, brain, and liver sections showed lower apoptosis marker deposition after treatment with olive leaf alcoholic extract.

Results showed that scoring apoptosis markers within tissues in the infected group (1st group) appears mainly at severity value (76-100%) score3 (Table 1).

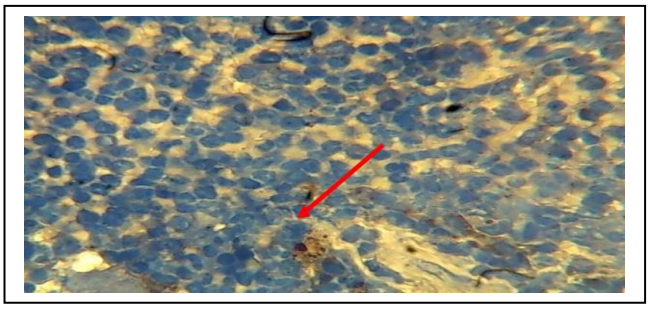

Figure 1. The mouse's spleen injected with *S. typhimurium* 0.1 ml / 4×10^8 CFU/ ml and treated with 30mg/kg / 0.3ml of Olive leaf alcoholic extract orally showed fewer dark-brown discoloration (Red Arrow) as a result of apoptosis marker. Dap Stain immunohistochemistry staining, 100X

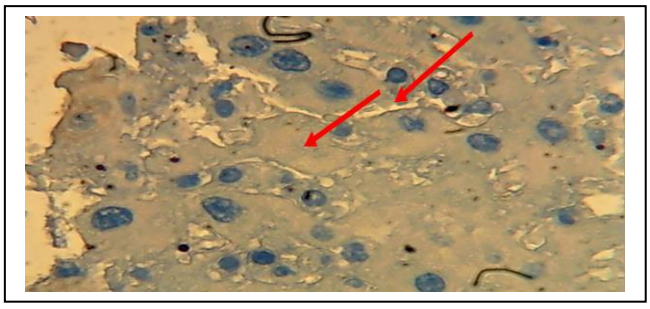

Figure 2. The mouse's liver injected with *S. typhimurium* 0.1 ml / 4×10^8 CFU/ ml and treated with 30mg/kg / 0.3ml of Olive leaf alcoholic extract orally shows fewer deposition of apoptosis markers (red arrow). Dap Stain immunohistochemistry staining, 100×

Table 1. Results of scoring after application of Tunel procedure and immunohistochemistry staining on tissues specimens

Group/number	Treatment	Mouse/Score 1 (0-25%)	Mouse/Score 2 (26-75%)	Mouse/Score 3 (76-100%)
1st /10	0.1ml IP *Salmonella typhimurium* (control Positive)	1(10%)	3(30%)	6(60%)
2nd /10	0.1ml IP *Salmonella typhimurium*+0.3ml Ciprofloxacin IM	8(50%)	1(30%)	1(20%)
3rd /10	0.1ml I.P.I.P. *Salmonella typhimurium*+0.3 Olive leaf extracts orally	1(45%)	8(40%)	1(15%)

3.3. Histopathological Examination of First Group

The liver section of the animals injected with 0.1/4× 10^8 CFU/ml (I/P) of *S. typhimurium* shows Kupffer cells and mononuclear cells aggregation in early granuloma enclosed by swelling and degenerated hepatocytes. Also, dilated and congested blood vessels were detected. On the other hand, the spleen section of mice injected with 0.1/4× 10^8 CFU/ml (I/P) of *S. typhimurium* shows depletion of a white pub appearing within the atrophic lymphoid follicle.

3.4. Histopathological Examination of Second Group

The liver section of the animals injected with 0.1/4×10^8 CFU/ml *S. typhimurium* (I/P) and treated with 30 mg/kg/0.3 ml Ciprofloxacin (I/M) showed no necrosis and fewer inflammatory reactions unless at the portal area, as well as around a still dilated central vein with no congestion. The spleen section of the animals was injected with 0.1/4×10^8 CFU/ml (I/P) of *S. typhimurium* and treated with 30mg/kg/0.3 ml of Ciprofloxacin (I/M) showed hyperplasia for lymphoid follicles in order to return to normal.

3.5. Histopathological Examination of Third Group

The liver section of a mouse injected with 0.1/4×10^8 CFU/ml (I/P) of *S. typhimurium and* treated pre-infection with 30mg/kg /0.3 ml of olive leaf alcoholic extract orally shows hepatocyte swelling due to degeneration with apoptotic bodies appearing in dilatation sinusoidal space with an inflammatory response represented by heavy infiltration of MNCs.

4. Discussion

In this study, the result of histopathological examination showed severe pathological lesions in the examined organs of animals infected with *S .typhimurium* compared with the control group. These results indicate that the organism was highly virulent and overcame the host defense mechanisms disseminated to all internal organs. This idea was consistent with Pardo-Esté, Hidalgo (16)**,** who explained that *S. typhimurium* is an intracellular bacterium that overcomes host immune system barriers for successful infection. The bacterium colonizes the proximal small intestine, penetrates the epithelial layer, and is engulfed by macrophages and neutrophils. Intracellular *S. typhimurium* encounters highly toxic reactive oxygen species, including hydrogen peroxide and hypochlorous acid. His finding was consistent with the findings of Djenane, Gómez (17), who found that Salmonella invasion induced macrophage apoptosis as part of the infection process, which may allow it to avoid detection by the innate immune system. *Salmonella* invasion had been inducing the apoptosis of macrophages as part of the infection process, which may allow it to avoid detection by the innate immune system. Macrophages of cytochrome c protein level in the cytoplasm could be involved in caspase-3 activation. Salmonella-induced apoptosis of macrophages is known to be accompanied by the activation of caspase-3 and an increase in the inflammatory cytokines TNF_2, IL-6, and IL-1 (18). The results of the current study revealed that the animals infected by *S. typhimurium* and treated with CiprofloxacinCiprofloxacin expressed lower levels of an apoptotic marker than those infected by *S. typhimurium.* This result is consistent with Anuforom, Wallace (19), who reported that CiprofloxacinCiprofloxacin enhanced the response of immune cells and their interaction with bacteria, increasing bacterial adhesion to macrophages and increasing cytokine production. Increased expression of IL-1 fosters apoptosis of Salmonella-infected macrophages and clearance by neutrophils. IL-1β induces leukocyte infiltration and activation of macrophages, releasing Salmonella to be ingested by neutrophils (20). Neutrophils play a role in inducing a cell-mediated immune response by producing IFN-γ, which attracts activated macrophages and other immune cells to the site of infection (21). Our study found that animals infected by *S. typhimurium* and treated with olive. The current study showed that the animals infected

by *S. typhimurium* and treated with olive leaf alcoholic extract expressed lower levels of apoptotic marker deposition than those in infected animals with *S. typhimurium.* A study by Özcan and Matthäus (4) reported that olive leaves are rich in phenolic compounds and are antioxidant-rich compounds that prevent the effects of oxidative metabolism due to their ability to scavenge H_2O2. GSH is abundant in olive leaf and plays a vital role in controlling mitochondrial transportation, which is influenced by oxidation of the protein thiol group of inner mitochondria, resulting in mitochondrial permeability transition to facilitate the release of cytochrome C from mitochondria, which activates the capiases cascade, leading to cell apoptosis (22).

Olive leaf alcoholic extract reduces the infection of *S. typhimurium* in the pretreatment and post-treatment groups better than CiprofloxacinCiprofloxacin. Also, olive leaf extract produces more efficacy as an antioxidant by increasing glutathione levels and reducing oxidative stress. From the experiments, we can conclude that *S. typhimurium* induces oxidative stress, its infections induce necrotic granulation reactions and severe cellular apoptotic events in the liver and spleen of infected groups.

Authors' Contribution

Study concept and design: D. H. D. A.
Acquisition of data: D. H. D. A. and A. T. A.
Analysis and interpretation of data: A. T. A.
Drafting of the manuscript: L. Q. J.
Critical revision of the manuscript for important intellectual content: R. H. M. A.
Statistical analysis: D. H. D. A.
Administrative, technical, and material support: R. H. M. A.

Ethics

The study was approved by the Animal Research Committee at Al –Esraa College University, Baghdad, Iraq.

Conflict of Interest

The authors declare that they have no conflict of interest.

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