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The Protective Role of *Salmonella* Typhimurium-Whole Sonicated Killed Antigen and *Syzygium aromaticum* Extract on the Histopathological Changes Against Its Infection in Rabbits

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ABSTRACT

This research aimed to evaluate the protective ability of *Syzygium aromaticum* methanolic extract against *Salmonella* Typhimurium infection in rabbits. Three groups of eight rabbits were given the following: The first group was subcutaneously given *Salmonella* Typhimurium-whole sonicated killed antigen (ST-WSKAg); the second group was orally given S. aromaticum extract as well as KWCSAg-ST subcutaneously; the third group was left as positive control group. The rabbit groups were orally challenged by 9×10^8 CFU/mL *Salmonella* Typhimurium then the animals were sacrificed 10 days postchallenge for histopathological study. Intestine, liver, kidney, and spleen were studied for pathological changes. The results showed that the second group had the least histopathological changes with mild inflammation and cellular infiltration of inflammatory cells, as well as low amount of vacuolation. The ST-WSKAg group showed multifocal aggregation of inflammatory cells and inflammation of all organs studied, as well as slight interstitial edema with few cellular infiltration and cystic tubular dilation of neighboring tubules in kidney. The positive control group showed the most extensive signs of destruction, severe inflammatory cells infiltration, as well as existence of live bacterium in tissue. In conclusion, *Syzygium aromaticum* extract showed to give a suitable protective ability against inflammation and destruction of virulent *Salmonella* Typhimurium infection in comparison to both of ST-WSKAg and positive control groups.

Keywords: *Salmonella* Typhimurium, *Syzygium aromaticum*, rabbits

INTRODUCTION

Globally, *Salmonella* enterica is a major cause of foodborne illness, with Typhimurium being one of the most common serotypes (1). Salmonellosis is one of the global causes of gastrointestinal diseases. Over 150,000 deaths are reported each year as a result of the disease, it is thought to impact up to 17 million individuals (2). While *Salmonella* serotypes connected to enteric fever cause systemic infection by surviving and multiplying in mononuclear phagocytes, serotypes linked to gastroenteritis which regulates the inflammatory and secretory response in the intestine. (3). When infected food or water is consumed, *Salmonella* enters the intestinal epithelium and cause gastrointestinal illness (4).

After ingesting contaminated food or water, a group of proteins encoded within *Salmonella* Pathogenicity Island 1 (SPI1) allowing for *Salmonella* to infect the epithelial cells that is lining the small intestine (5). The initial membrane-bound compartment or phagosome is altered by SPI2 effectors to create the "*Salmonella* containing vacuole"

(SCV). Since SCV is unable to fuse with lysosomes, *Salmonella* can evade antimicrobial substances. Macrophages can phagocytose *Salmonella* that penetrates the gut wall; and SPI2 is involved in both macrophage survival and the spread of the pathogen to organs like the liver and spleen (6). The histopathological changes of experimentally infected *Salmonella* Typhimurium in rabbits shows congested intestines and watery diarrhea, along with presence of various alterations, including polymorphonuclear cells (PMNCs) infiltration, crypt destruction, villus atrophy, and mucosal and submucosal blood vessels congestion (7).

Salmonella Typhimurium is prevalent at diverse rates, and there are hints that this bacterium is resistant to certain antibiotics in various nations (8). Antibiotics are no longer advised in the event of uncomplicated *Salmonella* gastroenteritis since they do not shorten the duration of the infection but considerably increase the number of fictitious pathogens and antibiotic-resistant strains. (9). Vaccination methods are frequently employed. There are positives and negatives to each of these tactics. Inactivated vaccinations, for instance, are safe but may have lesser immunogenicity than live attenuated vaccines (10).

In light of the emergence of multi drug resistance *Salmonella*, which have caused health and economical concerns, This research aimed to find alternative solution to the use of antimicrobial drugs, as well as, discovering the effectively of vaccines as a protective agent against *Salmonella* Typhimurium to substitute the excessive use of antibacterial drugs, and to observe weather *Syzygium aromaticum* extract can induce higher productivity, reducing inflammatory damage and protecting the tissue of the internal organs in experimentally infected rabbit. This study examined protective abilities of KWCSAg-ST and *Syzygium aromaticum* extract against the histopathological changes induced by virulent *Salmonella* Typhimurium infection in rabbits.

MATERIALS AND METHODS

Ethics

The experimental design and procedures used in this study were reviewed and approved in accordance with animal welfare ethical standards by the Scientific Committee of the Department of Microbiology, College of Veterinary Medicine, University of Baghdad, in its session held on October 5, 2021, and the Ethics Committee of the College of Veterinary Medicine, University of Baghdad, Baghdad, Iraq.

Syzygium aromaticum Extract Preparation

The *Syzygium aromaticum* dried buds were purchased from a local herbal market in Baghdad province, Iraq and authenticated by the botanist at the Iraqi National Herbarium, Directorate for Seed Testing and Certification, Ministry of Agriculture, Abu-Ghraib, Baghdad, Iraq. The extract was prepared according to (11), with some modifications. The dried buds were ground into fine powder by a stainless-steel grinder, and then weighed. The powder was dissolved and extracted in 80% methanol, then transferred to a shaker for 4 h at room temperature to ensure adequate mixing. The material was then stored in the refrigerator overnight. The material is then filtered with cotton using a Büchner flask with the vacuum for optimal filtering. Subsequently, the spray dryer was used to dry the filtered product. The result of the drying process was a fine powder.

Salmonella Typhimurium-Whole Sonicated Killed Antigen Preparation

The isolate of *Salmonella* Typhimurium was obtained from the Department of Microbiology, College of Veterinary Medicine, University of Baghdad, Baghdad, Iraq. *Salmonella* Typhimurium-whole sonicated killed antigen (ST-WSKAg) was prepared according to (12). *Salmonella* Typhimurium was harvested by adding 3 mL of 0.6% formalized phosphate buffer saline (FPBS) to the *Salmonella Shigella* (SS) Agar plate. The growth was scraped, transferring each media to its own sterile tube. The material was then centrifuged at 3000 rpm trice. The tube was put in the fridge overnight. On the second day, the harvest was checked for viability. The process was deemed successful when there was no growth. The killed bacteria in the universal tube were thawed. The sonication was done for 30 min in an iced environment using a Probe sonicator at 250 kHz. Sonicated samples were cultured in SS Agar to determine viability, and centrifuged at 3000 rpm for 20 min The supernatant was obtained, filtered through a 0.45 µm Millipore filter, and frozen at -20 °C

Animals

Twenty-four Albino rabbits (both sexes) ranging in weight from 900 to 1800 g and in age from 6 to 12 months were obtained from the animal house of the College of Veterinary Medicine, University of Baghdad. Animals were housed under standard conditions of controlled temperatures, humidity, and photoperiods. Commercial pellets and water were provided to the animals *ad libitum* throughout the entire experiment.

Study Design

The animals were randomly allocated into three groups of eight animals each. The first group was immunized with 1 mL ST-WSKAg at a dose of 1000 µg/mL subcutaneously (S/C). The second group was orally received 1000 mg/kg *Syzygium aromaticum* extract for a week before day 1 of the experiment on alternative days and immunized S/C with 1 mL of KWCSAg-ST at a dose of 1000 µg/mL. The third group was S/C injected 1 mL of PBS and served as a positive

control group. Booster dose was given at 14th day post immunization for the first and second groups of ST-WSKAg S/C. While the 2nd group was orally given an additional dose 1000 mg/kg of *Syzygium aromaticum* extract. Rabbits were given the challenge dose of virulent *Salmonella* Typhimurium at a dose of 9×10^8 CFU/mL (13) which was prepared according to (14) at day 21 to all groups orally. After 10 days of the challenge dose, the animals were sacrificed, and portions of vital organs (liver, spleen, kidney, and intestine) were harvested for histopathological study according to (15).

RESULTS

Histopathological Changes Intestine

The 1st group showed mild mononuclear cells (MNCs) infiltration in lamina propria with hyperplasia of interstitial glandular tissue (Figure 1). The second group showed mild infiltration of inflammatory cells in the mucosal layer accompanied with slight submucosal layer edema (Figure 2). The third group revealed necrosis with sloughing of mucosal epithelial layer and necrotic debris in lumen. Other findings revealed focal villous fusion with moderate MNCs in lamina propria and submucosa, also diffused MNCs infiltration in mucosal and submucosal layers with severe degenerative changes and necrosis of adjacent glands (Figure 3).

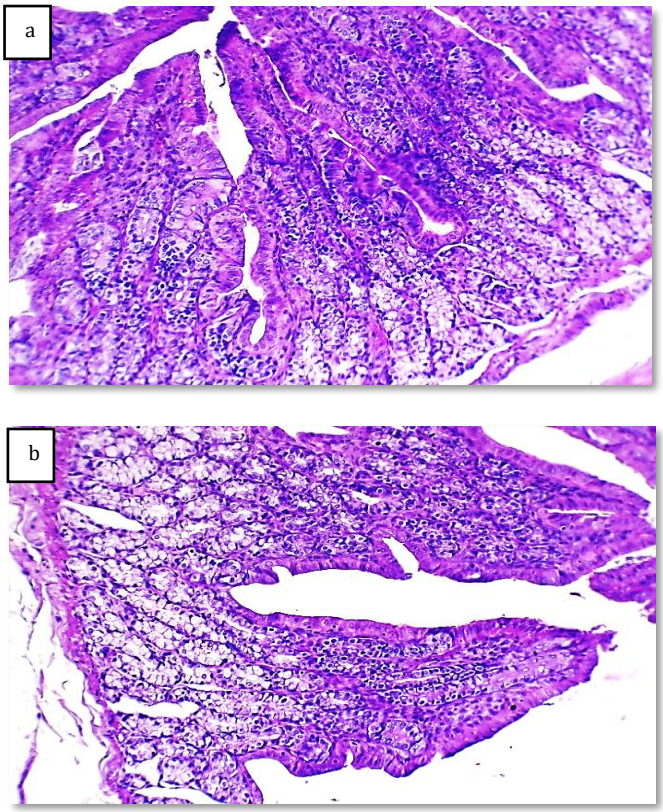

Figure 1. Representative sections in the small intestine of adult rabbits immunized with 1000 µg/mL of *Salmonella* Typhimurium-whole sonicated killed antigen (ST-WSKAg) showing a) mononuclear cells (MNCs) infiltration in lamina propria, b) hyperplasia of interstitial glandular tissue (H&E, 10×)

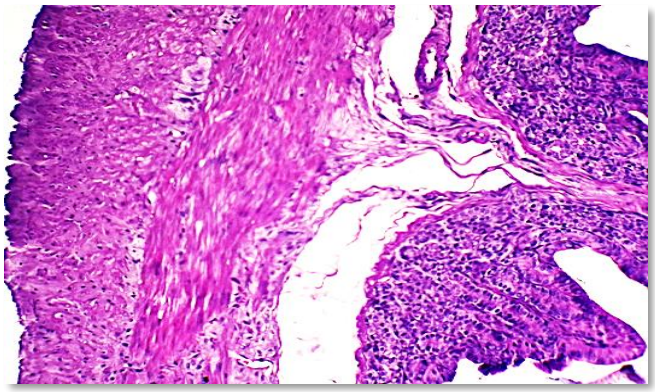

Figure 2. A representative section in the small intestine of adult rabbits administered *Syzygium aromaticum* extract at a dose of 1000 mg/kg and immunized with 1000 µg/mL of *Salmonella* Typhimurium-whole sonicated killed antigen (ST-WSKAg) showing mild cellular infiltration in mucosa with mild submucosal edema (H&E, 10×)

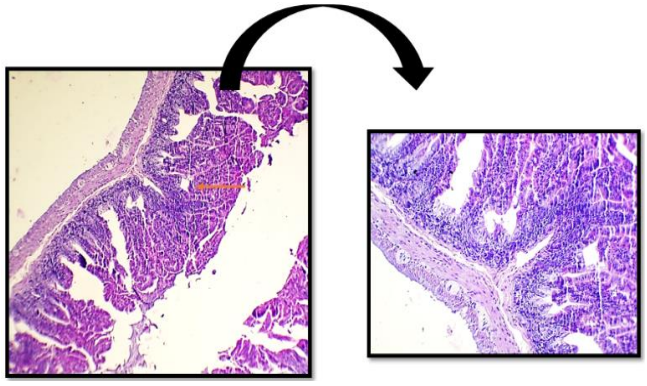

Figure 3. A representative section in the small intestine of adult rabbits of positive control group (injected 1 mL of PBS) showing focal villous fusion with moderate mononuclear cells (MNCs) infiltration in lamina properia and submucosa (H&E, 40×, 10×)

Liver

The 1st group showed multifocal MNCs aggregation mainly in portal region with evidence of central vein and sinusoids dilation, as well as intense perivascular MNCs infiltration mainly in portal region with focal necrosis of hepatocytes (Figure 4). The 2nd group showed unclear pathological alterations in the liver parenchyma with perivascular MNCs aggregation, as well as evidence of Kupffer cells proliferation (Figure 5). The 3rd group showed evidence of moderate nuclear pyknosis of periportal hepatocytes with portal vein and thrombosis with periductal MNCs infiltration with perivascular and periductal MNCs aggregation, portal vein distention with edematous fluid with ductal epithelial hyperplasia (Figure 6) accompanied with moderate neutrophilic infiltration. Granulomatous reaction in in liver parenchyma composed mainly of MNCs with slight sinusoidal MNCs infiltration, as well as portal inflammatory cells infiltration with slight venous and proliferation of Kupffer cells (Figure 7).

Kidney

The 1st group showed no clear pathological changes with prominence of nuclear anisonucleosis of tubular epithelial lining (Figure 8). The 2nd group showed evidence

of vacuolation of some renal tubules with no changes in glomerular tissue (Figure. 9). The 3rd renal findings showed various forms of renal tubules degeneration characterized by fatty degeneration, cystic tubular dilation together mild intestinal inflammatory infiltration, as well as, mild cystic tubular dilation, in modular region with no signs of inflammatory reaction (Figure 10).

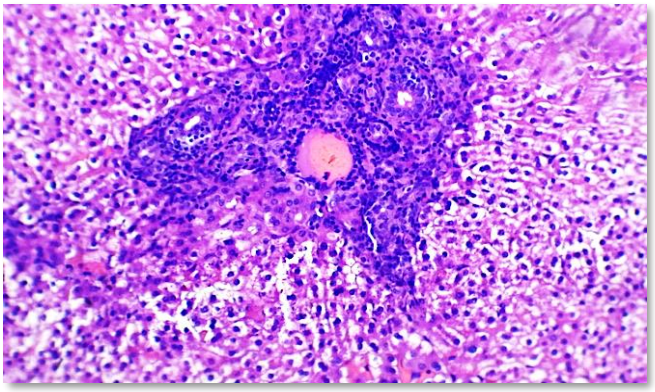

Figure 4. Section in the liver of a rabbit immunized with 1000 µg/mL of *Salmonella* Typhimurium-whole sonicated killed antigen (ST-WSKAg) showing intense perivascular MNCs infiltration mainly in portal region with slight focal necrosis(H&E, 10×)

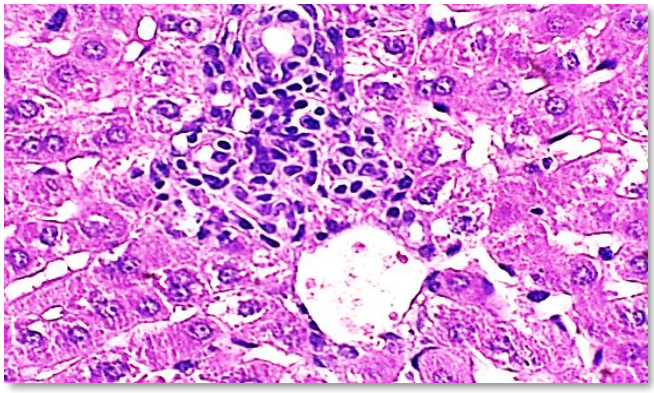

Figure 5. Section in the liver of a rabbit administered Syzygium aromaticum extract at a dose of 1000 mg/kg and immunized with 1000 µg/mL of *Salmonella* Typhimurium-whole sonicated killed antigen (ST-WSKAg) showing evidence of moderate perivascular MNCs a

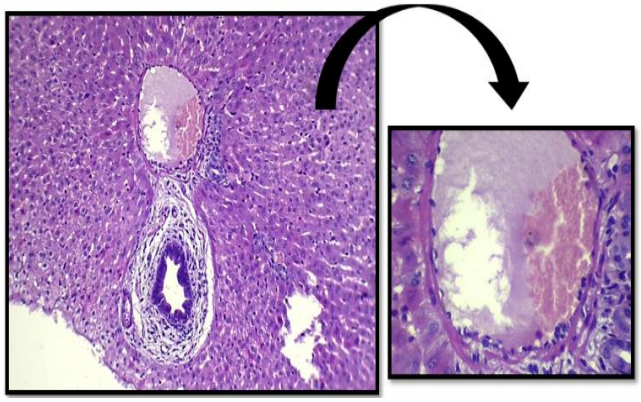

Figure 6. A representative section in the liver of adult rabbits of positive control group (injected 1 mL of PBS) showing portal vein thrombosis and periductal and perivascular MNCs infiltration (H&E, 400×, 100×)

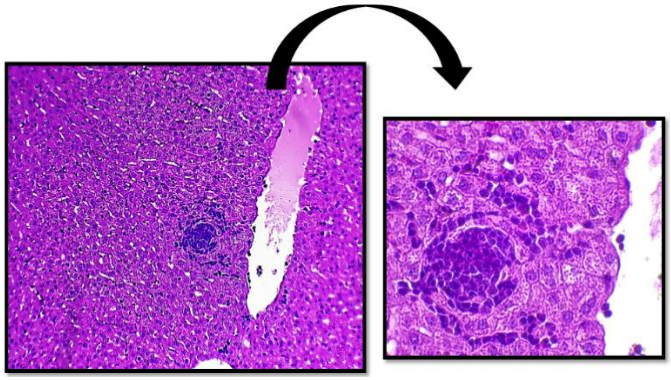

Figure 7. A representative section in the liver of adult rabbits of positive control group (injected 1 mL of PBS) showing granuloma in liver parenchyma composed mainly of MNCs with slight sinusoidal MNCs infiltration (H&E, 400×, 100×)

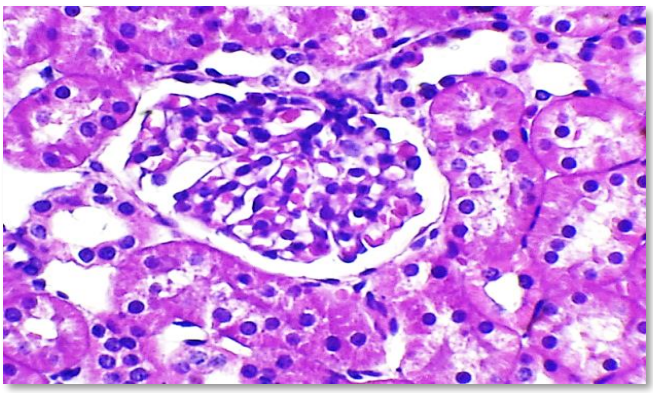

Figure 8. Section in the kidney of a rabbit immunized with 1000 µg/mL of *Salmonella* Typhimurium-whole sonicated killed antigen (ST-WSKAg) showing no clear pathological changes with prominence of nuclear anisonucleosis of tubular epithelial lining (H&E, 400×)

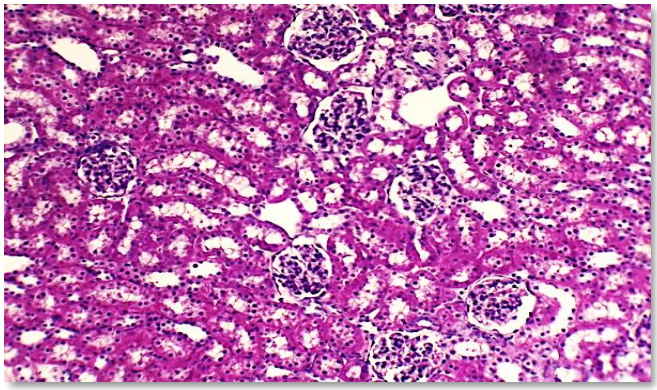

Figure 9. Section in the kidney of a rabbit administered *Syzygium aromaticum* extract at a dose of 1000 mg/kg and immunized with 1000 µg/mL of *Salmonella* Typhimurium-whole sonicated killed antigen (ST-WSKAg) showing no changes in glomerular tissue (H&E, 100×)

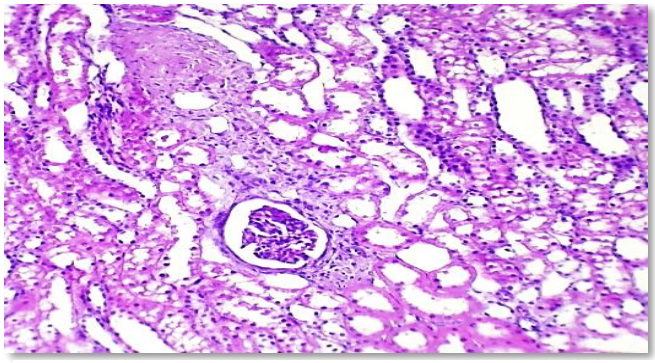

Figure 10. A representative section in the kidney of adult rabbits of positive control group (injected 1 mL of PBS) showing mild cystic tubular dilation, in modular region with no signs of inflammatory reaction (H&E, 10×)

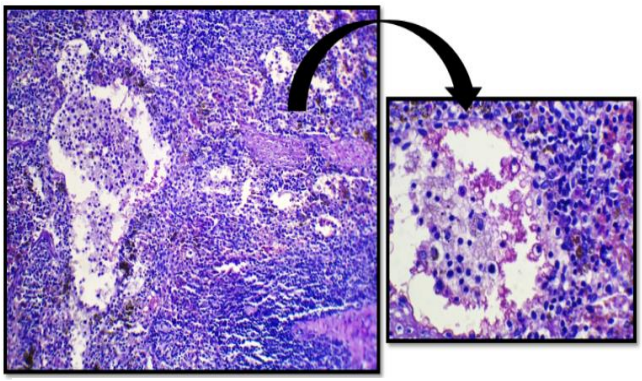

Figure 11. Section in the spleen of a rabbit immunized with 1000 µg/mL of *Salmonella* Typhimurium-whole sonicated killed antigen (ST-WSKAg) showing dilation of splenic sinuses with inflammatory cells in their lumen (H&E, 100×, 400×)

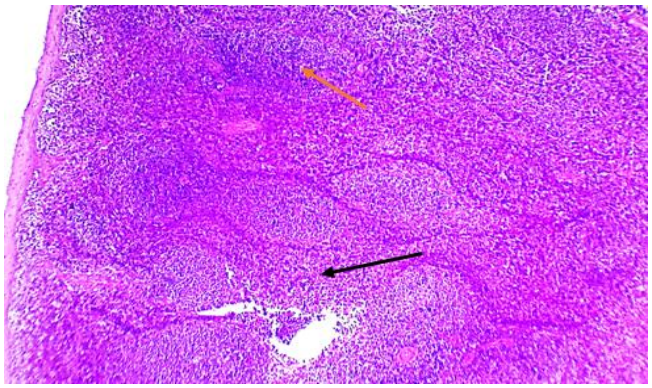

Figure 12. Section in the spleen of a rabbit administered Syzygium aromaticum extract at a dose of 1000 mg/kg and immunized with 1000 µg/mL of *Salmonella* Typhimurium-whole sonicated killed antigen (ST-WSKAg) showing mild lymphoid hyperplasia of white pul

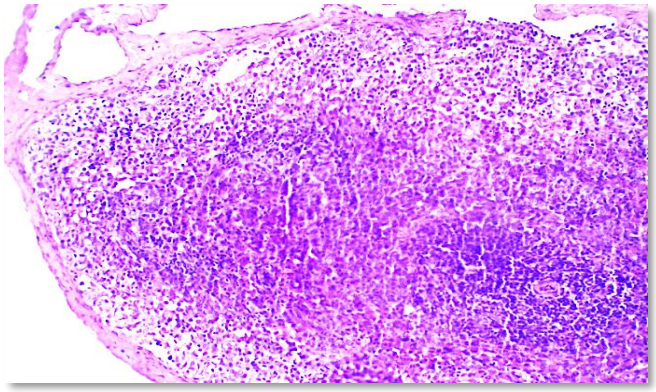

Figure 13. A representative section in the spleen of adult rabbits of positive control group (injected 1 mL of PBS) showing necrotic findings of lymphoid tissue associated with subscapular inflammatory infiltration (H&E, 100×)

Spleen

The 1st group shows paracortical lymphoid hyperplasia of splenic follicle with hemosiderosis in red pulp. Some of the splenic sinuses were dilated and filled with inflammatory cells and RBCs (Figure 11). The 2nd group showed lymphoid hyperplasia of white pulp with slight MNCs infiltration in splenic sinus, as well as slight red pulp congestion and slight trabecular fibroplasia (Figure 12). The 3rd group also showed moderate lymphoid depletion of splenic white pulp follicle. Furthermore, evidence of necrotic findings of lymphoid tissue with absence of germinal center associated with subcapsular inflammatory cells infiltration. Moderate red pulp congestion accompanied with sinus MNCs infiltration, moderate lymphoid depletion, red pulp congestion, and multifocal MNCs infiltration composed mainly of macrophages in reticular tissue with slight hemorrhage (Figure 13).

DISCUSSION

This research aimed to detect the capabilities of the killed antigen of *Salmonella* Typhimurium protection against infection of *Salmonella* Typhimurium and the protective ability of the herbal immunomodulatory *Syzygium aromaticum* extract.

The histopathological results of this research suggested that the vaccination against *Salmonella* Typhimurium gave a certain degree of protection against the pathogen. The 1st group revealed less pathological findings than that of the positive control, while the 2nd group was given *Salmonella* Typhimurium antigen with the *Syzygium aromaticum* extract showed least pathological changes and more protection against the viable challenge dose, more than the 1st group. This showed that S. aromaticum extract has a certain anti-inflammatory and protective ability than that of the antigen alone. The positive control group showed a heavy range of pathological changes, while the most inflammatory response was noticed in the 1st group with the killed antigen of *Salmonella* Typhimurium. It was also noticed that the least number of pathological changes was in the 2nd group as there was high degree of protection against the pathogen.

Salmonella enters the intestines without being detected by the host's defenses. The bacteria interact with non-phagocytic cells like the epithelial cells of the intestinal mucosa, stick to them with adhesive structures, called fimbriae, that encourage binding and invade these cells to cause gastroenteritis (16). Serotypes of *Salmonella* linked to gastroenteritis orchestrate an inflammatory and secretory response in the intestine, whereas serotypes linked to enteric fever generated systemic infection by surviving and replicating in mononuclear phagocytes (3). Through the Peyer's patches, (clusters of lymphoid tissue in the lowest section of the small intestine), *Salmonella* Typhimurium invaded the body, and when the pathogen is engulfed by phagocytes, it stayed viable and transferred to systemic tissue (17). Another research examined histopathological changes in infected rabbits. Pathological signs, such as areas of neutrophilic cell infiltration in the ileum and lymphoid necrosis suggestive of acute or subacute enteritis were found in histopathological study. The liver showed necrosis, congestion, and petechial hemorrhages, areas of necrosis and neutrophilic infiltration. Spleen displayed areas of lymphoid congestion and depletion, acute to subacute enteritis and hepatitis caused by septicemia/ bacteremia linked to the

experimental S. enterica infection (18). And the latter is similar to the findings of the positive control group in which severe destruction and inflammatory reaction was noted in the studied organs and there was low protective ability of the body.

The results also agreed with the findings of another research which showed that mice injected with killed antigen had pathological signs of early infection when injected with challenge dose, there were polymorphonuclear leukocytes in the reticuloendothelial system organs. The main lesions were in the vaccinated mice, in contrast to the control mice, tended to be discrete and self-limiting. After the first week of infection, they started to develop into granulomas. Three weeks after the infection, tissue regeneration and recovery were visible (19). This is similar to our result in which similar protective ability of the antigen have been noted, with the existence of many inflammatory signs. Research has shown that the use of multiple immunostimmulatory agents/adjuvant can improve the killed antigen of *Salmonella* Typhimurium significantly (20)

The dried flower bud of *Syzygium aromaticum*, also known as clove, contains a significant amount of eugenol, an anti-inflammatory and antioxidant substance that can preserve the liver from damage which it was demonstrated that eugenol had a preventive effect on liver cirrhosis. According to the study, eugenol reduced oxidative stress and inhibiting hepatic cell growth, which may be considered the mechanism of protection against liver cirrhosis (21). It was examined the ethanol-induced gastric damage in rats prevented by *Syzygium aromaticum* water extract (SAWE), Its effects appeared to be connected to antioxidant activity, increased Prostaglandin E2 (PGE2) synthesis, reduction of inflammatory cell infiltration, and loss of gastric mucosal epithelial cells, and may help preventing gastrointestinal illness linked to oxidative stress. (22) Agrees with our findings in which eugenol has a protective ability.

The effectiveness of clove against the pathogenesis of alcoholic liver disorders, including fatty liver, hepatic inflammation, necrosis, and fibrosis, has been researched. There is a significant decrease in the related liver pathology due to Clovinol's effectiveness in reducing alcohol-induced oxidative stress and inflammatory alterations. This is supported by all biochemical and molecular indicators. It was discovered that Clovinol might be a functional element for liver health (23). Another study referred to exposed rats to CCl4-induced hepatotoxicity was protected by clove oil, which possibly due to the oil's antioxidant qualities. (24). The above was in agreement with our research as the extract of S. aromaticum had a strong anti-inflammatory and protective ability against stress, inflammation, and destructive effects of the virulent *Salmonella* Typhimurium.

One study observed the effects of treatment of *Salmonella aromaticum* against *Salmonella* Typhimurium experimentally to examine the activity of peritoneal macrophages, mice were separated into 4 groups: treatment group which was exposed to S. Typhimurium; a control group that did not expose to any treatment. Leaf extract from S. aromaticum was used as the treatment group. Cloves containing eugenol molecules showed to promote macrophage production and proliferation, and as a lymphocyte proliferation modulator. The enhanced activity of mice macrophages induced by clover leaf extract is due to the stimulation by chemical in the compound extract. (25) and these results were in agreement of our results in which S. aromaticum showed to give a protection against the bacterium.

Another research has discussed the importance of immunological modulation and the capability of a good immune response in limiting the histopathological changes. As the researchers have suggested that the humoral immune response through antibodies response can give a greater protection against *Salmonella* (26). Another study noted the importance of the cytokines of the humoral response of IL-4 and IL-5; in which the effectiveness of synergic immunization was evaluated. ELISA and the tube agglutination tests were used for its analyzing. Four groups of twenty rabbits were formed at random, with the first group receiving the WCSAg-*Salmonella* enteritidis immunization. Second group immunized by (WCS Ag S. Typhimurium). 3rd group immunized by CWCSAg-(*Salmonella* Typhimurium and *Salmonella* enteritidis), Ag n compound and the fourth left as control group which injected with PBS. There were greater significant differences at the first month in the group immunized with CWS Ag (*Salmonella* Typhimurium and *Salmonella* enteritidis) comparing with other immunized groups. (27)

Another research has shown that clove had ability to modulate and increase release of IL-4, IL-10, and other cytokines, as well as enhancing the expansion of B cells. (28). This suggested that there was strong immunological and anti-inflammatory reaction in the 2nd group which resulted in the protective ability.

Another research was used to determine whether certain feed additives could lower the rate of *Salmonella* Typhimurium infection in broilers. At one week old, 40 broiler chicks were divided into 4 treatments, each with 10 chicks: T1: the healthy control. T2: Iraqi probiotic plus *Salmonella* Typhimurium infection. T3: Poultry grow 250 + *Salmonella* Typhimurium infection. T4: Only cases of *Salmonella* Typhimurium infection. The birds were experimentally infected orally with *Salmonella* Typhimurium a day after consuming feed additives. The proportion of mortality and quantity of *Salmonella* Typhimurium expelled in the feces show that the Iraqi probiotic did not lessen the severity of infection with this bacterium, in comparison, poultry grow significantly increases body weight and feed conversion ratio. (29) In comparison with the current research, the clove extract has

given a positive protection against the infectious *Salmonella* Typhimurium.

Another study studied three concentrations of the plant (eugenol extract) were prepared to be treatments, T1, T2, and T3, with percentages of 100%, 50%, and 25%, respectively. Four different pathogenic bacterial strains (Staphylococcus auras, Listeria monocyogenes, *Salmonella* Typhimurium, and Escherichia coli) were obtained and isolated from contaminated food. The best therapy was T1, which evaluated for eugenol qualitative and quantitative activity that was determined by utilizing the disc diffusion method to measure antimicrobial activity (HPLC). The results demonstrated that the inhibitory zones increased with increasing quantities of eugenol extract. The findings strongly support the use of natural resources, such as some plants or plant parts, to address issues caused by bacterial activity that result in food poisoning (30)

In conclusion, *Syzygium aromaticum* has a great ability to protect against tissue damage and can be used as a great adjuvant/ immunomodulatory against virulent pathogens, to give strong protective ability and anti-inflammatory effects.

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N/A.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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دور الحماية لمستضد جرثومة السالمونيلا ولخلاصة نبات القرنفل على التغييرات المرضية النسيجية ضد جرثومة السالمونيلا تايفموريم في الأرانب

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الخلاصة

هدف هذا البحث إلى تقييم القدرة الوقائية لمستخلص الميثانول لنبات القرنفل ضد جرثومة السالمونيلا التيفيموريوم في الأرانب. تمت التجربة على ثلاث مجموعات مكونة من ثمانية أرانب. المجموعة الأولى كانت لدراسة تأثيرات المستضد النسيجية، واعطيت المستضد تحت الجلد. المجموعة الثانية كانت لدراسة تأثيرات المستضد مع مستخلص نبات القرنفل كعامل مساعد ومنظم مناعي فأعطيت مستضد جرثومة السالمونيلا ((KWCSAg-ST تحت الجلد مع مستخلص النبات فمويا وكذلك المجموعة الثالثة مجموعة السيطرة الموجبة. أعطيت كافة المجموعات جرعة تحدي قدرها (x108 CFU/ml 9) لجرثومة السالمونيلا تايفموريم فمويا، وتم ارسالها بعد 10 أيام بعد التحدي لدراسة التأثيرات المرضية ، وتمت دراسة الأمعاء والكبد والكلى والطحال. أظهرت النتائج أن المستضد ومجموعة المستخلص كانت أقل تغيرات نسيجية مرضية مع التهاب خفيف وانتشار في الخلايا الالتهابية ، وأظهرت مجموعة المستضد تراكمًا متعدد البؤر للخلايا الالتهابية والتهابًا لجميع الأعضاء التي تمت دراستها ، كما أبرزت وذمة خلالية طفيفة مع ارتشاح خلوي قليل وتمدد أنبوبي كيسي للأنابيب الكلوية. وأظهرت مجموعة السيطرة الموجبة أكثر العلامات المرضية انتشارًا مع تجمع شديد للخلايا الالتهابية، وكذلك وجود تراكم بكتيري في الأنسجة. يستنتج من هذا ان مستخلص نبات القرنفل مع المستضد في المجموعة الثانية قد اعطى قدرة وقائية مناسبة وحماية من الالتهاب والتدمير ضد عدوى جرثومة السالمونيلا تايفمويوم اكثر من الستضد بمفرده ومن مجموعة السيطرة الموجبة.

الكلمات المفتاحية: السالمونيلا التيفيموريوم، القرنفل، الأرانب، زلال البيض