Antibacterial effect of grape seeds polyphenols against Salmonella Typhimurium infection in mice
Nidhal Raoof Mahdi and Aseel Jasim Mohammad
College of Veterinary Medicine/ University of Baghdad

Abstract
Infection with Salmonella Typhimurium causes systemic infection and gastroenteritis in human and animals. Polyphenols especially in grape seeds have immunomodulation anti-inflammatory effects and antimicrobial activities. The current study was designed to investigate the antibacterial activity of GSP on the amelioration of liver and intestinal tissues inflammation and cell apoptosis induced by S. Typhimurium in infected mice. The parameters which were used in this study, including that determination the histopathological changes in liver and intestinal paraffin embedded block tissue and cells apoptosis that confirmed by using Dead End™ Fluorometric (TUNEL System). The histopathological changes in the liver and intestinal tissues showed less changes with potent inflammatory response in the treated groups, that lead to the decreasing of pathological sings in liver and intestinal tissue and showed lower apoptotic activity.

Keywords: Grapes seed polyphenols (GSP), S. Typhimurium, histopathology, apoptosis, mice.

Introduction
Plant-derived antioxidant compounds possess free-radical scavenging activities and are highly produced in flowers, seed and leaves in response to oxidative stress and senescence degenerative processes, nutritionally important minerals and secondary metabolites like ascorbic acid, polyphenol, flavonoids and carotenoids, which are collectively called antioxidants (1). Grape polyphenols have been reported to increase resistance to a variety of...
microbial pathogens due to capacity to suppress a number of microbial virulence factors, such as reduction of host ligands adhesion, inhibition of biofilm formation, neutralization of bacterial toxins, and show synergism with antibiotics(2). The mechanisms thought to be responsible for phenolic toxicity to microorganisms include enzyme inhibition by the oxidized compounds, possibly through reaction with sulfa hydroly groups or conjugation of phenolic and protein in microorganism, especially key enzyme might be major pathway to inhibit the growth of microorganism(3). Products derived from grape polyphenol showed bactericidal effects against many of aerobic mesophilic bacteria and lactic acid bacteria and inhibited Enterobacteriaceae and the product derived from whole winery products presented bacteriostatic activity against these microorganisms (4). Salmonella enteric serovar Typhimurium Gram negative facultative intracellular bacterial pathogen capable of infecting a number of hosts and causing significant morbidity and mortality globally, some serovars have zoonotic potential (5). Salmonella Typhimurium is one of the leading causes of severe gastroenteritis in humans(6). It also causes a systemic typhoid-like disease in susceptible mice(7). Its also an important pathogen of food-producing animals including cattle, pigs and chickens. In the USA alone, there is an estimated 1.4 million cases of non-typhoidal Salmonella annually, resulting in over 1000 deaths (8). The aim of this study was to evaluate the antibacterial effect of GSP in liver and intestinal tissues in groups of mice infection with S. Typhimurium, by determine histopathological changes and apoptosis in liver and intestinal tissues.

Materials and Methods

- **Experimental animal:** Forty female white Swiss BALB/C mice, aged 6-8 weeks and weight (20-25g), were used in this study. They were housed and maintained in a conventional animal facility, with controlled conditions of temperature 20°C and 10-14 hours of light and dark respectively.

- **Grape Seed polyphenol preparation:** Grapes Seeds Polyphenols (GSP) was purchased from Dixaing Aneling Snow Lotus Herb Bio-technology co. Ltd. Its chemical's composition was examined by the producer using HPLC, that containing 95% proanthocyanidin(OPC). According to the pilot experimental study the concentration of 300mg/ mouse was used, it was prepared by suspending 3mg in 10 ml of D.W. and given to mice by gavages using stomach tube. The GSP treated group were supplements orally (by intragastric tube) a volume of 0.2 ml with a dose of 300 mg/ mouse.

- **Salmonella Typhimurum:** The S. Typhmiurium isolate were obtained from the College of Veterinary Medicine/ Department of Microbiology/ University of Baghdad. Diagnosis these isolate were depended on the cultural and biochemical tests (Table 1), then the diagnosis was confirmed by using API 20 system kit.

| Table (1) Morphological and biochemical tests to S. Typhimurium |
|---------------------------------------------------------------|
| **S. Typhmurium**                                            |
| Gram stain                                                   |
| Blood agar culture                                           |
| MacConky agar culture                                        |
| S. S Agar                                                    |
| Brain Heart infusion culture                                  |
| **Biochemical tests**                                        |
| Indol test                                                   |
| Motility test                                                |
| Catalase test                                                |
| Oxidase test                                                 |
| Triple Sugar Iron (TSI)                                      |

- **Experimental design:** Animals were randomly divided into four groups (10 mice/group), they were treaded for 30 days.

1. Group 1(G1): control negative (mice were dosed 0.2ml of D.W orally for 30 days of the experiment).
2. Group 2 (G2): administered with GSP for 30 days (treated orally with 300mg/ kg B.W of GSP).
3. Group 3 (G3): injected IP with S. Typhimurium (mice were infected with 0.2 ml/mice which contain 1.5 \times 10^5 \text{ cfu/ml of S. Typhimurium} and left with out treatment).
4. Group 4 (G4): treated with GSP and interapetoneally (IP) injected S. Typhimurium after 10 days of the experiment.

This experiment was done to determine the antibacterial activity of GSP in mice infection with S. Typhimurium, by determine the histopathological changes and apoptosis activities of hepato and intestinal tissues, with GSP in S. Typhimurium infected mice. The GSP was given orally a volume of 0.2 ml with a dose of 300mg/ kg B.W from day one to the end of the experiment.

- **Samples Collection:** Each mouse was fixed on its back on anatomical dishes and disinfectant by alcohol (70%), the incision start at the end of abdominal region to the end of animal through the chest cavity and then liver and intestine were obtained in order to be used for histopathological study and tissue apoptosis.

- **Histopathological Study:** Mice from each group were anesthetized by chloroform and post mortem examination was done for all mice. Specimens were taken from all targeted organs, the tissues were kept in 10% formaldehyde solution, for fixation, and then processed routinely by using the tissue processes. Tissue sections were embedded in paraffin blocks, and sectioned by microtome at 7 micron or less and stained with hematoxylin and eosin, then examined by using light microscope (9).

- **TUNEL Apoptosis Detection Kit Dead End™ Fluorometric TUNEL System:** The Dead End™ Fluorometric TUNEL System that measures the fragmented DNA of apoptotic cells by catalytically incorporating fluorescein-12-dUTP at 3´-OH DNA ends using the Terminal Deoxynucleotidyl Transferase, Recombinant, enzyme (rTdT). rTdT forms a polymeric tail using the principle of the TUNEL (TdT-mediated dUTP Nick-End Labeling) assay. The fluorescein-12-Dutplabeled DNA can then be visualized directly by immunofluorescence microscopy. (Promega,USA)(10).

- **Statistical Analysis:** Data were analyzed using SAS (Statistical Analysis System-version 9.1). One way ANOVA, Two-way ANOVA and Least significant differences (LSD) post hoc test were performed to assess significant difference among means. P<0.05 was considered statistically significant(11).

**Results**

- **Identification of S. Typhimurium:** The S. Typhimurium appeared as a small, Gram-negative, single rod, usually motile with peritrichous flagella(12). As well as S.Typhimurium colonie appeared on the selective media (S-S agar) as small rounded with black center due to H_2S production(13). On blood agar medium it appeared as small rounded white to grayish, non hemolytic colonies Table (2).

| Bacteria spp.      | Morphological examination                      | Biochemical tests                     |
|-------------------|-----------------------------------------------|---------------------------------------|
| **S. Typhimurium**| **Gram stain** Gram-negative (single rod)     | **Indol test** -                      |
|                   | **Blood agar culture** Non hemolysis          | **Motility test** +                   |
|                   | **MacConky agar culture** yellowish colonies as it is non-lactose fermenting | **Catalase test** +                   |
|                   | **Brain heart agar** pale color colonies      | **Oxidase test** -                    |
|                   | **S.S Agar** Pale yellowish colonies with black color center | **TSI** Red Slant/yellow Bottom with H2S |

**Table (2) Morphological and biochemical tests of Salmonella Typhimurium**
Histopathological Changes: Compares of histopathological changes in liver and intestinal tissues of infected mice with *S. Typhimerium* with non infected: The histopathological changes in liver tissue of infected group with *S. Typhimerium* (G3) as compared to the control group(G1) (Fig.1), at 30 days of the experiment showed inflammatory cell infiltration in dilated central venis and large necrotic area replacement with RBC and cellular debris (Fig. 2,A). Furthermore, granulomatous lesion as well as present typhoid nodul colony (Fig. 2,B) in addition histolytic granuloma with advance hepatic vaculation (Fig. 2,C).

Fig. (1) Section in the liver of control mouse (G1) shows normal structures (H & E stain 40X)

Fig. (2, A) Section in the liver of mice at 30 days of the experiment (20 days post infection with Salmonella) (G3) infection with Salmonella) shows hemorrhage (    ) With inflammatory cells infiltration necrotic area filled with RBCs, and cellular debris (    ) (H & E stain 10x & 40x)
Fig. (2, B) Section in the liver of mice at 30 days of the experiment (20 days post infection with Salmonella) (G3) shows typhoid nodules colonies with fibrin deposition (→) (H & E stain 10x & 40x).

Fig. (2, C) Section in the liver of mice at 30 days of the experiment (20 days post infection with Salmonella) (G3) showed histolytic granuloma (→) with advance hepatic vaculation and central vein congested (← (H & E stain 10x & 40x).
The histopathological changes in intestinal tissue of infected group with *S. Typhimurium* (G3) as compared to the control group (G1) (Fig.3), at 30 days of experiment, the intestine showed atrophy of villi, vacuolation and inflammatory cell infiltration associated with epithelial cell and necrotic as well as shorting in villi (Fig. 4).

![Image of small intestine showing normal structures](image1)

**Fig. (3)** Section in the small intestine of control mice (G1) showed normal structures (H & E stain 10X)

![Image of intestine showing villous atrophy and inflammatory cells](image2)

**Fig. (4)** Section in the intestine of mice 30 days of the experiment (20 days post infection with Salmonella) (G3) shows villous atrophy (-----) with nictotic epithelial cells associated with multifocal MNCs infiltration (-----)(H &E10X&44 stain 10 x &40x).

The histopathological changes in liver tissue of the infected group (G4) with *S. Typhimurium* and administrated with polyphenols (infected after 10 days of polyphenols administrated), at 30 days of experiments, the liver showed multifocal cellullar aggregation consist of polymorphonuclear leukocytes (PMN) and mononuclear celles (MNCs) with modurated vacuolar degeneration of hepatic tissue (Fig. 5), while the main microscopical finding in polyphenols administrated group (G2) showed perivascular MNCs aggregation mainly in portal area consist of MNC atrophy with evidence vacuolation in surrounding hepatic tissue (Fig. 6).
Fig. (5) Section in the liver of mice at 30 days post administration polyphenol and infected with Salmonella G4 shows MNC with moderated vacular degeneration also shows multifocal MNC infiltration (→) with moderated vacular degeneration of hepatic tissue (→ H & E stain 10x & 40x).

Fig. (6) Section in the liver of mice at 30 days post administration polyphenol (G2) shows prevascular inflammatory cells aggregation (→) (H & E stain 40X).
The histopathological changes in intestinal tissue of G4 at 30 days of the experiment showed mild atrophy of some intestinal villi that lining with in columner epithilial with slight cellular infiltration and vascular congestion that result in lamina peroper (LP) dilition (Fig. 7), as compared to the polyphenols group (G2) showed no clear lesions of treated groups at the 30 days of experiment (Fig. 8).

Fig. (7) Section in the intestine of mice at 30 days post administration polyphenol and infected with Salmonella (G4) shows near to normal stractuer (H & E stain 40 &10X).

Fig. (8) Section in the intestin of mice at 30 days post adminstration polyphenol groups (G2) showed no clear lesions (H & E stain 40X).
Detection of apoptosis using Dead End™ Fluorometric TUNEL System: Comparism of apoptotic cells detection in liver and intestinal tissues of infected mice with S.Typhimerium and non infected: The results of the apoptosis of infected group with S.Typhimerium (G3) at 30 days of the experiment showed that the TUNEL-positive cells were highly detected and distinguished in the liver and intestinal tissue. (Fig. 9,10) as compared to the control group (G1) (Fig. 11, 12).

Fig. (9) Section of intestinal tissue from G3 shows that the TUNEL-positive cells were appearing in the section (10x)
Fig. (10) Section of intestinal tissue from G3 shows that the TUNEL-positive cells were appearing in the section (10x)
Fig. (11) Section of liver tissue from G1 shows that the TUNEL-positive cells were no detected (10X)
Fig. (12) Section of liver tissue from G1 shows that the TUNEL-positive cells were no detected (10X)
The results of the apoptosis in liver and intestinal tissue of the infected group with *S.Typhimerium* and administrated polyphenols (G4) (infected after 10 days of polyphenols administrated) at 30 days of experiment, showed that the TUNEL-positive cells were a little more distinguished in the liver and intestinal tissue (Fig. 13,14), as compared to the polyphenols administrated group (G2). Fig. (15, 16).
Discussion

Inflammation is a biological process initiated by the immune system in response to tissue injury caused by microbial infection and other noxious stimuli. Acute inflammatory response is characterized by vasodilation, leakage of the vasculature, and infiltration of leukocytes into the site of infection to destroy invading pathogens and is followed by a rapid resolution phase and repair of the damaged tissue (14). The present study showed severe pathological changes in examined organs mainly liver of infected group with S.Typhimurium (G3) (Fig. 2), as compared to the control negative (G1) (Fig. 1), this results indicated that the S.Typhimurium was highly virulente, as well as the liver were the maine target organs for bacterial infection, the result were in agreement with Agbor, et al (15), who explained that Salmonella, resides after internalization in vacuoles that undergo acidification but do not act as lysosomes. As well as Salmonella have effectors secreted by the second Type Three Secretion System (T3SS)2 of Salmonella play important roles in the remodeling of these Salmonella containing vacuoles(SCV), several effectors secreted across the vacular membrane remodel locally the actin cytoskeleton, allowing the polymerization of an actin basket surrounding these vacuoles and regulating bacterial virulence(16). In this study (G3) the granulomatouse lesion was characteristic lesion in liver and intestinal tissue of infected mice with S.Typhimurium. This result indicated that S.Typhimurium considered as intracellular pathogens that causes localize infection through granulomatous reaction associated with development of cell mediated immunity (CMI) response (17), the same observation was mentionned by Shukla, et al., (14), who reported the inflammatory reactions, marked by infiltration of polymorphonuclear leukocytes (PMN) and macrophages, charactarised by large granulomatous lesions consisting from aggregation of active macrophages and lymphocytes, compared to the control negative, these necrotic foci were infiltrated with small numbers of polymorphonuclear leukocytes (PMNs). The destructive effect of S.Typhimurium on intestinal tissue of G3, as inflammatory cell infiltration and atrophy of villi (Fig. 3), was indicated that the microorganism invade the intestinal epithelia to the lamina propria and then mucosal glands and stimulate the resident macrophage to produce pro-inflammatory cytokines such as(IFN-γ) that attracted the neutrophils to the site of infection causing tissue damage and necrosis as a results of by products, and this in consistent with Kuehn, et al., (18), who observed that infections by S. Typhimurium is dependent on a T helper cell type 1 (Th1), CMI, which is characterized by production of interferon (IFNγ) and TNFα (19), and by the subsequent activation of macrophages, as well as Ono, et al., (20), who suggestion that this CMI by the host that serves to limit the infection to the intestinal mucosa or gut-associated lymphoid tissue (GALT) and to prevent distribution to the liver and spleen. In the present study the mild to moderate pathological lesions were seen in the mice treated with polyphenols post infected with S.Typhimurium (G4), as compared to the polyphenols admenstrated group (G2), this result could be due to the antimicrobial effects of polyphenolic compounds against bacteria, this result was agreement with Berrin, et al., (21) who demonstrated that polyphenols compounds act as antibacterial due to their ability to complex with extracellular and soluble proteins and to complex with bacterial cell walls, as well as Plumed-Ferrer, et al., (22), who found that dietary supplementation of polyphenol act as antibacterial effect against Gram-negative bacteria, due to ability to destabilization of the outer membrane of Gram-negative microorganisms, also interactions with the cell membrane might be one of the specific mechanisms behind the antibacterial action. The
pathological result of G4 (Fig. 5) showed less lesion, due to protective effect of polyphenols these results indicated that polyphenol activated the bactericidal activity of immune cell in addition to decrease production of proinflammatory cytokine, this observation was in agreement with Hassan-Khabbar, et al.,(23) and Sebai, et al., (24), they indicated that polyphenol compound that protected the internal organs from oxidative stress induced by LPS, and ischemia/ reperfusion injury, that lead to reduce lesion. The pathological result in the intestinal tissues of G4 (Fig. 6) were in agreement with Johnston, et al.,(25), who explained that polyphenols able to changed villus height: crypt depth ratio, histological sections from duodenum and jejunum were analyzed as these parts of the small intestine are the main sites of absorption of nutrients and histological changes can be estimated in these parts of the small intestine, also Viveros, et al., (26), who demonstrated that polyphenols improved gain feed ratio and an increased villus height crypt depth ratio in jejunum in broilers fed a diet supplemented with polyphenol-rich grape pomace extract. Most histopathological changes in mice (G2) treated with GSP (Fig. 7), showed perivascular MNCs aggregation mainly in portal area in liver tissue, might be due to immunostimulation effect of polyphenols that led to active immune response, this suggestion was in agreement with Li, et al., (27), who explained that polyphenols effect related with reducing granulocyte infiltration and decreasing the production of proinflammatory cytokine IL-1β in the colon of rats, in addition to its antioxidant effects.

- Detection of apoptosis using DeadEnd™ Fluorometric TUNEL System:

Apoptosis is a form of programmed cell death, that highly organized and genetically controlled type of cell death, essential during embryonic development to ensure proper organogenesis (28). The results of apoptosis study revealed high number of apoptotic cell of infected group (G3) in liver and intestinal tissue as compared with control group (G1), these due to S.Typhimurium were able to invade intestinal epithelial cells to intracellular replication and survive, that led to colonization to from the Salmonella-containing vacuoles (SCV) to the cytoplasm of macrophage cells(29). In addition, bactericidal antimicrobials can induce cell death by stimulating the production of reactive oxygen species, principally O2−, which induces oxidative damage (30). Superoxide dismutase are responsible for the destruction of these superoxide anion radicals. In addition to their detoxifying function, bacterial superoxide dismutase have also been shown to be important virulence factors (31). Slight to moderated distinguished of apoptotic cell was present (G4), these result could be due to that polyphenol had antibacterial effect against S.Typhimurium that led to lower bacterial colonization by interaction with negatively charged microbial cell surface, that led to the direct antimicrobial activity by direct interaction, these suggestions were in agreement with Paulo et al., (32), who found the antibacterial activity of grape phenolics compound attributing to the antimicrobial effect and bacteriostatic action by changes in cell morphology and DNA content. Therefore, the cell cycle is affected by grape phenolics, these evidence in agreement with hispathological study, that showed polyphenol activate immune stimulation through increased levels of immune cell as of PMN and MNCs (G4), might be due to stimulated effect of polyphenols against S.Typhimurium. The current finding showed no apoptotic cell in G2, that might be due to polyphenol activated immune response and regulation many biological systems, these suggestions were in agreement with Hudson et al., (33), who reported that grape polyphenol regulation survival pathways of cell apoptosis was the phosphatidyl inositoln 3-kinase-Akt and mitogen-activated protein kinase (MAP) survival pathways. The extracts reduced Akt transcription, and enhanced proteasome degradation. Conclusions, Accordingly, this study was designed GSP at dose
of 300 mg/mouse act as antibacterial and protective effects, this may be due to improved the immune response in *S. Typhimurium* infected mice, decreased free radicals, reduced tissues damage, as well as polyphenols minimized apoptosis in the liver and intestinal tissues in groups of mice infection with *S. Typhimurium*.

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