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Effect of Grape Seed Polyphenol on immune gene expression and it's Role as Antibacterial against *Salmonella typhimurium* infection in mice exposed to sodium nitrate

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Abstract

Polyphenols especially in grape seeds have anti-inflammatory effects by immunomodulation and anti-oxidantive pathway ,as well as antimicrobial activities .The current research conducted to evaluate the immunomodulatory effect Grape Seed Polyphenol extracts (GSP) against *Salmonella* Typhimurium infection and fed sodium nitrate in mice.The parameters which were used in this study, including determention the bacterial clearance duration in liver tissue by counting the bacterial colony forming unit(CFU)/gram and detecting the gene expression of Tumer nicrosis facter (TNFα) and interleukin-10 (IL-10) in liver tissue by the using quantitative real time reverse transcription PCR (RT-qPCR)technique . The log^{10} CFU/liver count was demonstrated at days 5 ,10and 15 after challenge; the treated groups recorded low bacterial count with a significant difference ($p < 0.01$) in GSP treated groups due to the activated innate immunity was an important key to activate the protective Th1 responses through a significant decrease in the bacterial liver count after treated .The results obtained from the gene expression in mice treated with GSP , showed a lower values of TNFα mRNA in liver tissue .While IL-10 mRNA showed significant ($P < 0.05$) increase .

Keywords: Grapes Seed Polyphenols(GSP), S.Typhimurium , Immune response, quantitative real time reverse transcription PCR (RT-qPCR)

Introduction

Phenolic compounds can modulate the immune system ,these compounds are used in numerous sectors of the food industry as natural additives as well as in the cosmetic and pharmaceutical industry (Zillich ,*et al*., 2015). The mechanisms of grape seed procyanidin extractand their anti-inflammatory action remain poorly understood; however, several studies suggest thatit is related to oxygen free-radical scavenging, antilipid peroxidation, and the inhibition of inflammatory cytokine secretion as well as alterations in cell membrane receptors, intracellular signaling pathway proteins and gene expression and enzyme activity(Kris-Etherton, *et al*.,2004).

In recent years, considerable attention has been paid to the problem of nitrate due to the exhaustive use of nitrates as agricultural fertilizers which reach to humans and animals by different routes **(** Awodi ,*et al*., 2005 ;Mande ,*et al*., 2012).

Salmonella enteric serovar Typhimurium Gram negative facultative intracellular bacterial pathogen capable of infecting a number of hosts and causing significant morbidity and mortality globally. Someserovars have zoonotic potential (Crump and Mintz,2010; Crumpand Heyderman,2014).*Salmonella* infections can be difficult to treat. Salmonellosis is among the most common food-borne diseases in humans (representing 20% of all food-borne infections), and is a major public health and economical burden worldwide (Coburn *et al*., 2007).

Using of grape seed polyphenols specially proanthocyanidins as hepatoprotective agent against damage inducing agent has been reported in many studies including Hazem(2012) who used grape seed extract to prevent ethanol induced cytotoxicity in liver ,as well as Ghulam Mustafa Khan *et al* ; (2012) ,who used grape seed extract to modulate the effect of carbon tetrachloride induced changes in rat liver .

The Aim of this study was to evaluate the immunoprotactive effect of GSP in liver tissues in groups of mice with nitrate supplemented and /or S.Typhimurium infection ,by determine the bacterial clearance and gene expression of TNF-α and IL-10 in liver tissue.

Materials and Methods

1.Experimental animal:

Eighty 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.

2.Grape Seed polyphenol preparation

Grapes Seeds Polyphenols (GSP) was purchased from DixaingAneling Snow Lotus Herb Bio-technology co.Ltd. Its chemical's composition was examined by the producerusing HPLC, that containing (95%) proanthocyanidin(OPC).

According to the pilot experimental study the concentration of 300mg /mouse was used,itwas 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 a volume of 0.2 ml with a dose of 300mg/mouse.

3.Sodium nitrate preparation

Preparation sodium nitrate(Chaina) by dissolving powder in tap water, mice were supplied with 500 mg sodium nitrate/L in drinking tap water every day through the experimental period (Mohamed and Anwar, 2010).

4. 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.Typhimurum :*

	Morphological examination	Biochemical tests
S. typhmurium	Gram stain Blood agar culture MacConky agar culture S.S Agar Nutrent agar culture	Indol test Motility test Catalase test Oxidase test

5. Primers

Three primers were used in this study including β-*actin* gene primer used as Housekeeping gene, IL-10,and TNFα gene primers that were used as target genes(Table 2). These primers were designed using NCBI- Gene Bank data base.The primers were used in quantification of gene expression using RT-qPCR techniques based SYBR Green DNA binding dye, and provided from(Bioneer,Korea) company.

Table(2) Details of primers that were used in the study:

Primer		Sequence	Amplicon
β-*actin*	**F**	GGGTGGAGCCAAACGGGTC	530bp
	R	GGAGTTGCTGTTGAAGTCGCA	
IL-10	**F**	GGACAACATACTGCTAACCGAC	256bp
	R	AAAATCACTCTTCACCTGCTCC	
TNF	**F**	TCCAGGCGGTGCCTATGT	91bp
	R	CGATCACCCCGAAGTTCAGT	

6.Experimental design

Animals were randomly divided into eight groups (10 mice/ group), they were treaded for 30 days .
1. Group 1(G1) :control negative
2. Group 2(G2):administered with GSP for 30 days
3.Group 3(G3): administered with NaNO3 for 30 days
4.Group 4(G4):administered with GSP and NaNO3
5.Group 5(G5) :injected IP with *S*. Typhimurium;
6.Group 6(G6) : treated with GSP and IP injected *S*. Typhimuriumafter 10 days of the experiment .
7.Group 7(G7): .treated with NaNO3 and IP injected with *S*. Typhimurium after 10 days of the experiment.
8.Group 8(G8): treated with GSP and NaNO3 and IP injected with *S*. Typhimuriumafter 10 days of the experiment.

This experiment was done to determine the anti-inflammatory activity of GSP in *S*.Typhimurium infected mice and fed sodium nitrate (NaNO3).The GSP was given orally from day one to the end of the experiment

The following parameter were conducted:

1-Bacterial count (CFU) in liver tissue after 5,10 and 20 days post infection (at 15,20,30 days of experiment).((Miles *et al*.,1938).

2-quantitative real-time PCR to determine the mRNA expression of the TNF-α , IL-10 in liver tissues,the samples were collected at 10,20 and 30 days of the experiment(Lehmann *et al* ., ,2001).

7.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 wereperformedto assess significant difference among means. $P < 0.05$ was considered statistically significant

Results

1. Identification of *S.*Typhimurium

The*S.*Typhimurium appeared as a small ,Gram-negative , single rod, usually motile with peritrichous flagella (Jawetz, *et al*., 2007). As well as *S* .Typhimurium colonie appeared on the selective media (S-S agar) as small rounded with black centerdue to H_2S production (Quinn *et al*, 2004).On blood agar medium it appeared as small rounded white to grayish ,non hemolytic colonies .Table (3)

Table (3): Morphological and biochemical tests of Salmonella Typhimurium

Bacteria spp.	Morphological examination		Biochemical tests	
S. Typhimurum	Gram stain	–	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

2.Bacterial count (CFU) in liver tissue:

The resuts revealed that treated group with GSP**(**G6 and G 8) showed the lowest bacterial count (increase bacterial clearance),while G 7 continued in its increased in bacterial count (Table 4)

Table (4): The effect of GSP on the Bacterial clearance.

Treatment Groups	Mean log_{10} of *S.typhmurium*± SD in liver at time(days) post infection		
	5 days	15 days	20 days
G5	$1.3\times10^{3\ C}$	$9.4\times10^{3\ C}$	$9\times10^{2\ B}$
G6	$3\times10^{1\ D}$	$9.3\times10^{1\ D}$	0 D
G7	$8\times10^{3\ A}$	$9.2\times10^{4\ B}$	$2.3\times10^{3\ A}$
G8	$1.4\times10^{3\ B}$	$9.4\times10^{4\ A}$	$8\times10^{2\ C}$

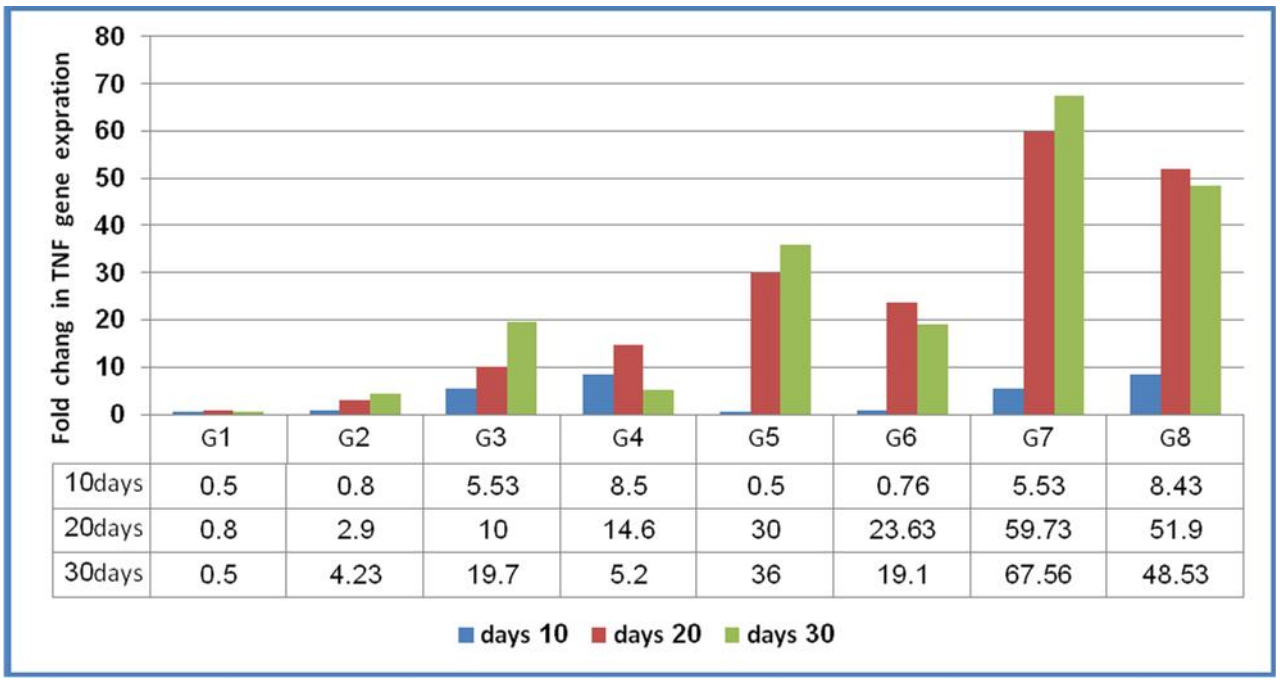
Different uppercase letter in the same column were significantlydifference ($p < 0.01$)
G5:injected IP with *S.* Typhimurium .
G6: Treated with GSP and IP injected *S.* Typhimurium after 10 days of the experiment .
G7: . Treated with NaNo3 and IP injected with S. Typhimurium after 10 days of the experiment .
G8: Treated with GSP and NaNo3 and IP injected with S. Typhimurium after 10 days of the experiment .

3.Detection of Gene expression of TNF-α and IL-10 by using RT- qPCR

3.1.Comparism of Gene expression of TNFα in liver tissues of infected mice with *S.*Typhimerium and non infected :

The results (Fig 1)of the TNFα mRNA gene expression in liver tissue were measured at 10,20 and 30 days of the experiment of infected group with S.typhimerium(G5) the results showed that significantly higher fold ($P < 0.05$) in gene as compared to the control group(G1) , also the result in G6 (infected after 10 days of administerted polyphenols) showed significantly higher fold ($P < 0.05$) in gene expression than the G2 (the administerted with polyphenols).

On the other hands , the results of G7 (mice administrated nitrate and infected with *S.*Typhimerium) showed TNF α gene expression significantly increased ($P < 0.05$) as compared to the G3 (nitrate group) ,while the results showed that the TNFα gene expression was highly significantly increased ($P < 0.05$) G8(mice administrated polyphenol and nitrate and infected with *S.*Typhimerium) as compared G4 (mice administrated polyphenols and nitrate **).**



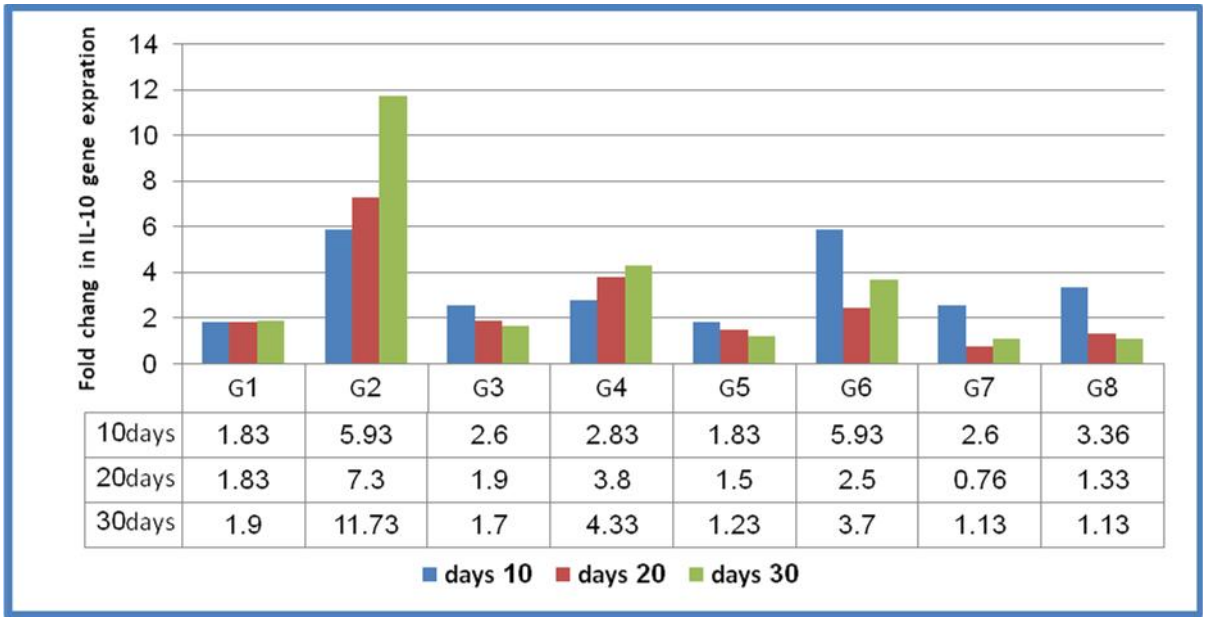
	G1	G2	G3	G4	G5	G6	G7	G8
10days	0.5	0.8	5.53	8.5	0.5	0.76	5.53	8.43
20days	0.8	2.9	10	14.6	30	23.63	59.73	51.9
30days	0.5	4.23	19.7	5.2	36	19.1	67.56	48.53

Figure(1): The relative TNFα gene expression of infected mice with S.Typhimerium and non infected for 10, 20 and 30 days post infection .Data are shown as the fold change in mRNA level in mice of G1, G2, G3,G4 ,G5, G6 ,G6 ,G7 and G8 by Q RT-PCR .

G1: control negative
G2: administered GSP orally
G3: administered NaNo3 orally
G4: administered with GSP and NaNo3 orally
G5:injected IP with *S.* Typhimurium after 10 days of the experiment.
G6 administered GSP orally and I.P injected *S.* Typhimurium after 10 days of the experiment .
G7: : administered NaNo3 orally and IP injected with S. Typhimurium
G8: administered with GSP and NaNo3 orally and IP injected with S. Typhimurium

3.2.Comparism of Gene expression of IL-10 in liver tissues of infected mice with *S.*Typhimerium and non infected

The results (Fig. 2) of IL -10 mRNA gene expression in liver tissue were measured at 10, 20 and 30 days of the experiment .In G5 (infected group) showed significantly lower fold (P <0.05) in gene expression as compared to the control group (G1) ,as well as ,the results of G6 was highly significantly difference (P < 0.05) in gene expression than the G2 , while the results of IL -10 mRNA of the gene expression in liver tissue in G7 showed significantly lower fold (P <0.05) in gene expression than G3,in addition G8 showed significantly lower fold (P <0.05) in gene expression than G4 .



Figure(2) The relative IL-10 gene expression of infected mice with S.Typhimerium and non infected for 10, 20 and 30 days post infection .Data are shown as the fold change in mRNA level in mice of G1, G2, G3,G4 ,G5, G6 ,G6 ,G7 and G8 by Q RT-PCR .

G1: control negative
G2: administered GSP orally
G3: administered NaNo3 orally
G4: administered with GSP and NaNo3 orally
G5:injected IP with *S.* Typhimurium after 10 days of the experiment.
G6 administered GSP orally and I.P injected *S.* Typhimurium after 10 days of the experiment .
G7: : administered NaNo3 orally and IP injected with S. Typhimurium
G8: administered with GSP and NaNo3 orally and IP injected with S. Typhimurium

Discussion

Many of the virulence factors of *S. Typhimurium* are encoded by genes organized on SPIs that have been acquired by horizontal transfer and colonization , that factors leads to actin cytoskeletal rearrangements ,membrane ruffling and dameg interinal organs (Vernikos, *et al.,* 2006). *S.*Typhimurium was shown to improve colonization of deep organs (spleen, liver) in mice (Fuentes,*et al*.,2008), These observations in agreement with ,Silva.,*et al* (2012) ,who showed *Salmonella* serovars cuases systemic infection of this serovar in mice

The high bacterial count (Table:4) found in the infected group with *S.* Typhimurium (G5) , indicated that bacterial strains were highly virulence and able to reduce host defense mechanism . *Salmonella* was intracellular replication which lead to the colonization of the bacteria in almost organs in the body and cuases increase bacterial count ,these evidence supported by Richter-Dahlfors,*et al* (1997), Who reported that *S.* Typhimuriumwere facultative intracellular pathogen that reside maninly in macrophage ,where it replicates wthin specialize vacuoles ,these observation in agreement with Grant ,*et al* .,(2008), who found acute infection in susceptible mice determined that hematogenous spread 48 hours post-infection resulted in *S.* Typhimurium mixing between the spleen and liver,in addition to host immune responses contribute to *Salmonella* clearance (Griffin , *et al*.,2011 ;Broz ,*et al.,* 2012) .

The resuts revealed ,thatG6 showed the lowest bacterial countcould be due to antibacterial action of polyphenol and immunostimulatory effect on immune system which active immune cell,therefore polyphenols could lower bacterial count by inhibition colonaztion of bacteria and protect the treated mice against invaded pathogens, ,these suggestion in agreement with many study ,that showed the polyphenol antimicrobial activity may be related to cause localized disintegration of bacterial outermembrane, leaking of cytoplasm and irregular shape (Lacombe ,*et al*., 2010).Also Monagas,(2010) ,who found that phinolic compound act asantibacterial due to these effects can be their chelating properties on iron, an important oligoelement for heme-utilizing bacteria

On the other hand G 7 continued in its increased in bacterial count might be due to nitrate caused dejected host defense immunity ,that led to distribution of bacteria in the tissue ,in addition to immunotoxic effect of nitrate that helped bacterial invasion ,damage tissue and inhibited immune response and led to decreased bacterial clearance in all systemic organ , these result were in agreement with Pannala ,*et al.*, (2003).who found that ingested nitrate is converted by microflora in the gastrointestinal tract to more toxic nitrit .In addintion that nitrate is a source of nitric oxide (NO) and other reactive oxygen as well as nitrogen species such as hydrogen peroxide (H2O2), peroxynitrite (ONOO-) and superoxide anion (O2-), that causes damage cellular membrane through lipid peroxidation (LOP),as a result of high concentration unsaturated fatty acids of phospholipids(Hassan,*et al*., 2009) . Thus, weakness of the cellular membrane as well as a disturbance of membrane transport that led to damage tissue and impairs liver functions (Ogur ,*et al*., 2005). Thus these compounds caused decrased host defenes and increased bacterial count .

Morever mice in group fed diet supplement with polyphenols and treated with NaNO3 post infection with *S.*Typhimuirum (G8) ,showed moderated bacterial count and showed less count than G7 ,these result could be due to immune stimulation effect of polyphenols in addition to bactericidal effect against S.Typhimurium , that act as antibacterial as well as enhance immune response that led to active immune cell and increased bacterial clearans ,these observation agreed with Karou., *et al* (2005) , who indicated that their derivatives in grape seed as flavonoids ,stilbenes and phenolic acids in grape were responsibe for antimicrobial activity ,thus polyphenol have microbicidal activities against a huge number of pathogenic bacteria .Also Viveros ., *et al* (2011) who showed that polyphenols are able to cause a shift in the microbial population in the intestinal tract of rats and broilers .

Specific T cell subsets could be stimulated to produce specific cytokines after their interaction with different natural or synthetic molecules and cytokines (Fan , *et al* .,1998). Their specific cytokines initiate and orientate the immune response. On this basis, they are divided into proinflammatory (e.g., IL-17, INF-γ, TNF-α) and anti-inflammatory (e.g., IL-4, IL-10, TGF-β) cytokines

The TNFα gene expression results of liver tissue of infected mice with *S.* Typhimurium **(G5)** showed that TNF–α mRNA was significantly higher fold of expression ($P < 0.05$) (Fig :1) ,whereas the concentration of IL-10 significantly lower fold ($P < 0.05$) (Fig :2) as compared to **G1**, that might be due to *S.*Typhimurium has bacterial surface component LPS which is a potent immunostimulatory molecule that led to initial pro-inflammatory cytokines to be released in response to the invading microbial pathogens and that plays a crucial role in the induction of inflammation . the results in agreement with Ohsaki,*et al* (2006) ; Du , *et al* (2010) ,who showed administration of lipopolysaccharide (LPS) to animals (in vivo-)induced acute systemic inflammation that causesto induced pro-inflammatory cytokines(TNFα).

The results of gene expression of liver tissue of G6**(** mice fed diet supplement with polyphenols post infection with S.Typhimuirum) showed that there were significantly ($P < 0.05$) lower fold of TNFα mRNA and significantly higher ($P < 0.05$) fold IL-10 , these indicate that the antibacterial effect of polyphenols , as well as it enhance the innate immune system by modulated the inflammatory response in the model of inflammation by enhancing systemic production of the anti-inflammatory cytokine,these results indicated that polyphenols act as anti-inflammatory and that inhibition of NF-kB activation,this was consistent with result of Wang and Mazza (2002) ; Kinneer , *et al* (2003), who demonstrated that phenolic compounds for blocking LPS-induced production of TNFα by macrophages could be the inhibition of NF-kB activation . The antibacterial effect of polyphenol in the present study considered as protective response of tissues against

pathogen invasions by activating innate immune cell and inducing cytokine and chemokines prodaction through suppresses the activation of inflammatory transcription factor NF-κB(Nishiumi, *etal* .,2012)

The results of present study of G7 **(** mice fed diet supplement with NaNO3 post infection with S.Typhimuirum) showed that that significantly (P < 0.05) higher fold of TNF **α** gene expression and significantly (P <0.05) lower fold IL-10 gene expression as compared to **G3(** mice fed diet supplement with NaNO3), this could be due to the virulence of S.Typhimurium that used in the study in addition to immunotoxic and genotoxic effects of nitrate in immune cell ,that led to decreased host defensemechanism and increase bacterial invasion , which occurred to release many endogenous antioxidant enzymes,this indicated that nitrate may be releaseROS and endogenously transformed into nitrite which in turn can react with amines and amides to produce nitrosamines and free radicals **(** Singhal *et al*., 2001; Manassaram *et al*., 2006**).**ROS have been recognized as contributing to vascular dysfunction, through mechanisms including endothelial dysfunction, vascular smooth muscle cell growth, lipid peroxidation, and inflammation .(Touyz ,*et al*., 2004)**,** these result in agreement withSindler,et al., (,2011)who reported that dietary nitrite supplementation was shown to modulate age-related inflammatory cytokines in mice .The upregulation of the inflammatory response is the consequence of a remodeling of the innate and acquired immune system with a chronic inflammatory cytokine production (Baylis ,2013) .

Also, the present result of **G8 (** mice fed diet supplement with polyphenols and NaNO3 post infection with S.Typhimuirum), showed that mRNA expression of TNF reduced significantly (P <0.05) ,while IL-10enhance significantly (P < 0.05) as compared to that G7(mice treated with NaNO3 post infection with *S.*Typhimrium), these results might be due to the direct effect of the polyphenols on various cells of the immune system, as well as bactericidal effect against S.Typhimurum which lead to identical action against bacteria through immunomodulator and antimicrobial activity (Xia, *et al* ., 2010), in addition polyphenol could lower immunotoxic effect of nitrate due to antioxidant and immunostimulating action of polyphenols .In this study, GSP decreased the expressions of TNF-*α*, these results suggested the protective effect of GSP on immunological liver inflammation by inhibiting the proinflammatory cytokines expression.This was in agreement with the Loke *et al* ., 2010; Noll *et al* .,(2013) , who found the anti-inflammatory and antioxidant activity of polyphenpls(flavan-3-ol) in vivo that modulation capacity throughout the atherogenic process, in addition Morrison *et al* ;(2014) who showed that polyphenols compound may contribute to mitigate inflammation.

The present study indicated that mice in G4 (treated with GSP and fed NaNO3) that the levels of TNF-α were significantly (P <0.05) lower fold ,while IL-10 significantly (P < 0.05)higher fold, the proanthocyanidin treatment regulated the levels of these inflammatory mediators and protected the liver tissue against NaNO3 that induced oxidative injury through the generation of free radicals , as well as the toxic agents induced biological changes in tissue and body fluid of the host cell(Mourad *et al*. ,2005).

The results of treated group with GSP indicated that GSP are potent immunostimulant which initially trigger the immunobiological function of macrophage. The activation of macrophage consists of several inter-connected process, including increased chemokines, chemotaxis, migration of macrophage to particles to be phagocytosed and degranulation leading increased gene expression of adhesive molecules on liver tissue .Many research investigations have demonstrated that the grape polyphenols possess bioactivity that have been shown to scavenge oxygen and nitrogen derived free radicals, modulating antioxidant enzymes and cellular redox transcription factors (Magrone,*et al* ., 2010; .Marzulli, *et al* 2014; Watson *et al* .,2014) .

Conclusions

Accordingly, this study was designedGSP at dose of 300mg/mouse increase S.Typhimurium bacterial clearance from liver tissue . As well as GSP act as an immunomodulator decrease the TNFα and increase IL-10 production to improve the inflammatory caused by nitrate and S.Typhimurium infection in liver tissues.

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