ORIGINAL ARTICLE

INTERNATIONAL JOURNAL OF RESEARCH IN PHARMACEUTICAL SCIENCES

Published by JK Welfare & Pharmascope Foundation Journal Home Page: www.ijrps.com

Immunomodulatory and Anti-Inflammatory Potentials of Trigona Honey in the Therapy and Prevention against Respiratory Infection in Wistar Rats

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Article History:

Received on: 18 Feb 2020
Revised on: 19 Mar 2020
Accepted on: 20 Mar 2020

Keywords:

inflammatory,
Gene expression,
Immune response
Pathogenicity,
S. aureus,
Respiratory infection

ABSTRACT

Trigona honey (TH) is well known for its therapeutic characteristics. To date, the study of Trigona honey as a prophylactic or immune booster prior to the bacterial infection of the invivo model is not well covered. This study aims to investigate anti-inflammatory and immune activities in Wistar rats infected with respiratory infection following with Trigona honey. 25 Wistar rats were assigned to five groups, negative control group, positive control group was fed TH (5 g / kg body weight) orally, the untreated group was infected with *Staphylococcus aureus* to induce respiratory infection, the treated group has been infected with *S. aureus* followed by treatment with TH at a dose of 1.5×10^8 CFU / mL and the preventive group ingested TH one week before *S. aureus* infection. Blood was obtained for biochemical analysis. Lung tissues have been collected for molecular examination. The results showed a significant decrease in serum levels of ALT, AST, urea and creatinine in the preventive and treated groups, serum IgG increased significantly (P<0.05) in the preventive and treated groups, IFN-y increased in the preventive group while decreased in the treated group, and IL-8 increased in the treated group while decreased in the preventive group. The mRNA expression of AGP is up-regulated in the positive control, preventive and treated groups. The α2-MG, TNF-α , and mRNA expressions showed lower regulation after administration of TH in preventive and treated groups. The results show the ability of TH to counteract immune and inflammatory changes in serum levels and gene expressions.

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ISSN: 0975-7538

DOI: https://doi.org/10.26452/ijrps.v11i3.2385

Production and Hosted by

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INTRODUCTION

Staphylococcus aureus has received high attention as a major human and animal pathogen that confers antibiotic resistance to respiratory infections ranging from folliculitis to labor pneumonia (Ayukekbong *et al.*, 2017). Pathological consequences of chronic lung infection as a response to persistent pathogens lead to reduced lung function combined with life-threatening conditions of septicemia and meningitis (Reddy *et al.*, 2017). In bacterial infections, pro-inflammatory mediators such as interleukins, tumor necrosis factor, and interferon are affected by bacterial defense equipment, leading to a reduction in immune responses, specifically neu-

trophils, and a large amount of circulating leukocyte in innate immune response and the first responders to a bacterial infection (Hussein *et al.*, 2012). Antibiotics are considered to be the ultimate chemotherapeutic agent for the treatment of bacterial infections. Unfortunately, they are losing their efficacy against microorganisms as a result of the development of different resistance mechanisms. Recent studies therefore focus on exploring alternative antimicrobial drugs derived from natural sources such as plant extracts and honey products for the treatment of bacterial infections (Maddocks and Jenkins, 2013; Sofowora *et al.*, 2013). Honey has been used as a potential antimicrobial agent in human infections since ancient times (Khan *et al.*, 2018). However, the elucidation of antimicrobial mechanisms of honey against bacterial infections is a hallmark of clinical therapy (Bakar *et al.*, 2017). The phytochemical properties of honey, antimicrobial, antioxidant, anticancer, anti-inflammatory, and healing remedies have been studied by different scientific groups (Rao *et al.*, 2016).

Malaysian honey, such as Trigona, Tualang and Gelam, has been researched to deduce potential phytochemical compounds for different pharmacological uses (Zainol *et al.*, 2013). Stingless bee honey as Trigona honey (TH) has been involved in several medical procedures and has been used for therapeutic purposes (Maringgal *et al.*, 2019). TH contains high levels of total phenolic content (TPC) with an average of 784.3 mg of Gallic acid equivalents / kg. TH honey has an aberrant potential antibacterial effect against *S. aureus*, *Bacillus cereus*, *Escherichia coli*, and *Pseudomonas aeruginosa* (Kek *et al.*, 2014). Interestingly, there has been no plausible evidence of resistance to honey or toxicity or side effects in combination with the administration of honey. The implications of low therapy costs and abundance of resources, as well as the hallmark of using honey as an alternative antimicrobial therapy, underline the imperative need for further investigation (Zainol *et al.*, 2013). Unfortunately, in vivo, there has been less research on sting less bee honey. As a result, this current study evaluated the effect of TH after *S. aureus* infection to determine the effect of TH as an immune activator and anti-inflammatory agent using a rat model.

MATERIALS AND METHODS

Bacterial strain

Methicillin Resistant *S. aureus* (MRSA) was provided by Udder and Neonates Department, Animal Reproduction Research Institute, Egypt. The active bacterial culture was grown in the nutrient broth (NB) at 37^{o}C for 24 hours and was centrifuged at 14,000 $\times$ g for 20 minutes. The pellet was washed three times using phosphate buffer saline (PBS). Approximately 2×10^{9} CFU / mL the viable bacterial count was adjusted using PBS. In order to induce respiratory tract infection in rats, *S. aureus* was injected to the rats at a dose of 1.5×10^{8} CFU / mL per rat (AbdEl-Hafez *et al.*, 2016).

Preparation of TH sample

TH is produced by stingless bees, the study was conducted with fresh samples of TH obtained from a bee farm located in Bachok, Kelantan, Malaysia. Honey samples were stored at dark ambient temperature prior to further analysis.TH was dissolved in drinking water and orally fed to rats (5g / kg body weight). This solution was sterilized by a 0.22 μm nylon syringe filter (Pall, USA) kept dark at room temperature and fed orally to rats (Alagwu *et al.*, 2011).

Animals and Experimental Design

Animal handling was approved by the Sultan Zainal Abidin University Animal Ethics Committee. The number of animal ethical approvals is UAPREC/04/18/002. The approval was also obtained from the Office of the Ethics Committee of the Scientific Dean, Taif University, Kingdom of Saudi Arabia. Twenty-five male Wistar rats, 9 weeks old, weighing between 200 and 225 g, were purchased from King Abdul-Aziz University (Jeddah, Saudi Arabia). The rats were housed in condition (25 ± 2 ^{o}C) with 12:12-hour day-night cycle and were given free access to food and water. The positive control group received TH but was not exposed to *S. aureus* infection. Both bacterial infections and honey ingestion were not exposed to negative control groups. Intraperitoneal injection (IP) of *S. aureus* was given to the untreated group for 1.5×10^{8} CFU / mL but not recovered with TH. The treated group was injected with *S. aureus* 5×10^{8} CFU / mL in the IP and recovered with TH for 3 weeks following clinical signs of respiratory tract infection occurring within 3 days. The preventive group administered TH with drinking water for one week prior to exposure to *S. aureus* 1.5×10^{8} CFU / mL, signs and symptoms of infected rats have been observed and recorded (Al-Rubiay *et al.*, 2008; Prasad *et al.*, 2011). Rats were fasting overnight before being anesthetized using 1% isoflurane in an inhalation chamber (Al-Waili, 2004; Hadi, 2015).

Biochemical Analysis

From eye retro-orbital venous plexuses blood was collected using a capillary tube. Blood was collected in EDTA container (Fisher, USA) and then refriger-

ated for 30 minutes before centrifugation at 5 °C for 20 minutes. Supernatant containing serum was collected and stored at-20 ° C prior to biochemical analysis. Serological kits for aspartate aminotransferase (AST) and alanine aminotransferase (ALT) for liver biomarkers, urea and creatinine for renal biomarkers have been used spectrophotometrically as indicated in the manufacturer's manual (Biodiagnostic Co., Giza, Egypt). To determine immunoglobulin G, Rat interferon-gamma (IFNγ) and Interleukin 8 (IL8) we used ELISA Kit (ab157737), ELISA Kit (ab46107) of Abcam, and ELISA Kit (Sandwich ELISA) - LS-F36750 (BioSciencesBiosciences, USA), respectively, following the instruction of the manufacturer pamphlets (Twigger, 2002).

Gene Expression and Reverse Transcription Polymerase Chain Reaction

(RT-PCR)

The total RNA from lung tissues (100 mg per sample) was extracted according to (Soliman *et al.*, 2015b). Lung samples were flash-frozen in liquid nitrogen, followed by −80 °C in 1 mL of TRIzol (Invitrogen, USA). Homogenizer (Brinkman, USA) was used to homogenize frozen samples. After that, 0.3 mL of chloroform was added to the homogeneous samples. The homogeneous mixtures were stirred for 30 seconds after a refrigerated centrifugation of 16,500 × g for 15 minutes. The supernatant was loaded into new tubes, then a similar volume of isopropanol was added to the samples for 20 seconds, the samples were shaken to mixture homogeneity and refrigerated centrifugation for 15 minutes, with 70% ethanol, rinsed the RNA pellets, then dried and dissolved in diethylpyrocarbonate (DEPC) water.NanoDrop ND-1000 Spectrophotometer (Bio-Rad, USA) was used to examine total RNA concentrations. RNA purity levels were evaluated using the absorption ratio 260/280, with sample ratios only accepted between 1.7 and 1.9 for conversion to cDNA. 1.5 % denaturateddenaturized agarose gel was used to ensure RNA integrity, and agarose gel was stained as an intercalating agent with ethidium bromide (AbdElHafez *et al.*, 2017). Using a high capacity RNA to cDNA conversion kit (Qiagen Valencia, CA, USA), total RNA samples were converted to complementary DNA (cDNA) library.A mixture of 4 μg of total RNA and 0.5 ng of oligo dT (Qiagen, USA) in a total volume of 11 μL was mixed with sterilized DEPC water, then incubated for denaturation at 65 °C for 10 minutes in the thermal cycle (Bio-Rad T100TM). The following contents were added: 2 μL of 10X RT buffer, 2 μL of 10 mM dNTP and 100 U Moloney Murine Leukemia Virus (M-MuLV) reverse transcriptase (Novosibirsk, Russia). Added 20 μL of DEPC water to total volume. In the Bio-Rad thermal cycle, the mixture was incubated at 37 ° C for 1 hour, followed by incubation at 90 ° C for 10 minutes for inactivation of the enzyme Primers for RT-PCR analysis have been retrieved as shown in Table 1 . The final volume was 25 μl PCR, 12.5 μl PCR Master Mix, 1 μl 10 pM forward primer, 1 μl 10 pM reverse primer, 2 μl cDNA template and topped with nuclease-free water up to 25 μl. The following PCR protocol (Bio-Rad T100TM thermal cycle) was used: denaturation at 95oC for 5 minutes with one cycle followed by 29 cycles, amplification at 94oC for 1 minute with one cycle and final elongation: at 72oC for 7 minutes with an additional final extension at 72^{o}C for 7 minutes. The expression glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was used as a reference. PCR products were electrophorized using 1.5 % agarose gel (Bio Basic, Canada), ethidium bromide was used as an intercalating agent for stain agarose gel in the Trisborate–EDTA (TBE) buffer. PCR products were viewed under UV light and photos were taken using Genius 3.0 Gel Documentation System. (USA, Syngene). The band intensity was measured densitometrically using ImageJ Software Version 1.52 (http:/www.imagej.en.softonic.com/) (Ismail *et al.*, 2016).

Statistical Analysis

Data were expressed as mean ± standard mean error, one-way analysis of variance and graphing was performed using SPSS, version 20 (SPSS, IBM, Chicago, IL, USA). Values with P < 0.05 considered statistically significant.

RESULTS

Liver and kidney function test

Serum levels of liver biomarkers were fairly homogeneous as ALT and AST did not show a significant difference between positive and negative control groups. Whereas the pre-administration of administration of TH in both preventive and treated groups showed a significant decrease (P < 0.05) in liver biomarkers compared to the untreated group. The hallmark of the use of TH as a therapeutic drug has higher rates of healing and eradication in treated rats compared to preventive rats. Congruently, Urea and creatinine biomarkers for kidney showed no significant differences between the positive and negative control groups, while the use of TH in the preventive and treated groups resulted in a significant decrease (P < 0.05) in the levels of enzymes, particularly in the treated group, with reference to the untreated group (Table 2).

Table 1: Specific primers used for RT-PCR analysis

mRNA	Product size (bp)	Annealing temp (°C)	Number of cycles	Direction	Primer sequence (5′-3′)
α-2M	230	57	32	Forward	GCTCCTGTCTGTTTCCTTAGTT
				Reverse	ATTGGCCTTTCGTGGTTTAG
AGP	325	55	33	Forward	GCTCCTGTCTGTTTCCTTAGTT
				Reverse	GGCTTTTTGTTGTTTGCTTCTATTTC
TNF-α	256	58	30	Forward	CCACCACGCTCTTCTGTCTAC
				Reverse	ACCACCAGTTGGTTGTCTTTG
GAPDH	309	52	25	Forward	AGATCCACAACGGATACATT
				Reverse	TCCCTCAAGATTGTCAGCAA

PCR: Polymerase chain reaction, α-2M: alpha-2 macroglobulin, AGP: alpha-1 acid glycoprotein, TNF-α: tumor necrosis factor-alpha and GAPDH: Glyceraldehyde 3 phosphate dehydrogenase.

Table 2: Effect of TH on liver and kidneybiomarkers of Wistar rat

	Negative control	Positive control	Untreated	Preventive	Treated
ALT(U/L)	31.4± 1.9	30.15± 0.9	47.78±0.84	33.54±2.5$^{\#}$	35.3± 0.58$^{\#}$
AST (U/L)	24.44±2.3	22.88± 1.1	42.98± 3.9	19.86± 2.7$^{\#}$	22.56± 2.4$^{\#}$
Urea (mg /dl)	38.94±2.5	37.73± 1.7	43.88± 1.6	35.28±2.14$^{\#}$	32.83±7.7$^{\#}$
Creatinine(mg/dl)	0.71±0.06	0.60± 0.13	1.17± 0.09	0.92± 0.1$^{\#}$	0.78± 0.12$^{\#}$

Values are means ± standard error (SEM) for 5rats per group. Values are statistically significant at *P< 0.05comparison between negative control and positive control groups and $^{\#}$P<0.05 comparison between the untreated group with preventive and treated groups.ALT: alanine aminotransferase and AST: aspartate aminotransferase.

Table 3: Effect of TH on the immune response of Wistarrat.

	Negative Control	Positive Control	Untreated	Preventive	Treated
IgG(ng/mL)	103.5± 14	81.98± 3.7*	67.90±11.7	75.93±2.8$^{\#}$	92.18±3.3$^{\#}$
INF-γ(Pg/mL)	207.06± 4.5	118.75± 6.9*	162.0± 27	149.0± 35$^{\#}$	222.5±11$^{\#}$
IL-8 (Pg/mL)	135.02± 9.8	221.0± 5.5*	212.25±45	278.0± 63$^{\#}$	98.0± 4.5$^{\#}$

Values are means ± standard error (SEM) for 5rats per group. Values are statistically significant at *P< 0.05comparison between negative control and positive control groups and $^{\#}$P<0.05 comparison between the untreated group with preventive and treated groups. IgG: immunoglobulin G, IFNγ: interferon-gammaand IL8: interleukin 8.

Immune response for bacterial and TH challenge

Compared to the negative control group, immunoglobulin G (Twigger, 2002) levels were lower in the positive control group, while the administration of TH in the preventive and treated groups showed a significant increase (P < 0.05) in the IgG level compared to the untreated group. The levels of IFN- γ inflammatory cytokines decreased in the positive control group compared to the negative control group, while the levels increased after supplementation of TH in the treated group, while the levels decreased in the preventive group compared to the untreated group. IL-8 levels increased in the positive control group compared to the negative control group.While there was a significant decrease (P < 0.05) in the treated group, the preventive group showed the highest level compared to the untreated group (Table 3).

Preventive and therapeutic effects of TH against *S. aureus* induced alterations on AGP, α2-MG and TNF-α expression in the lung of Wistar rats.

TH up-regulated AGP mRNA expression in the positive control group compared to the negative control group. The administration of TH as a preventive agent showed a slight increase in AGP mRNA expres-

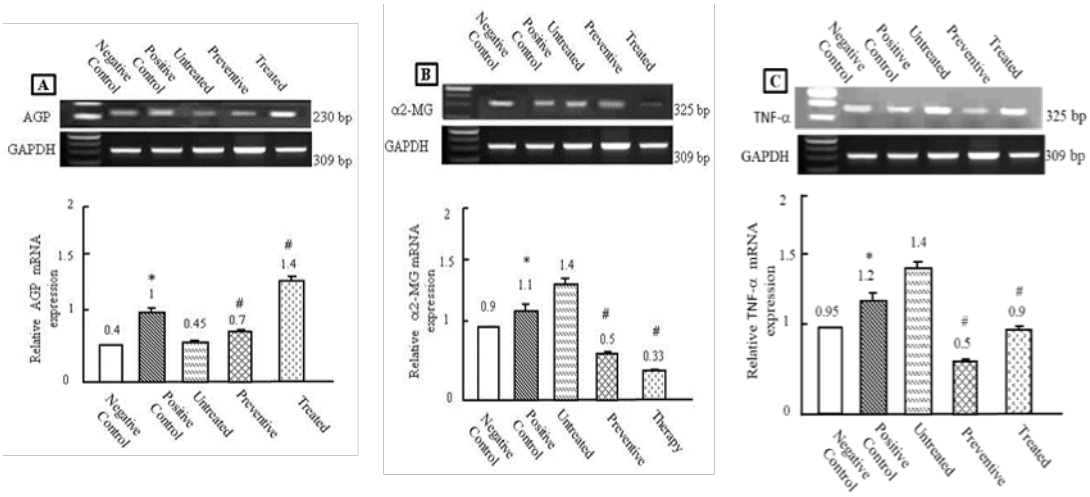

Figure 1: Semi-quantitative RT-PCR analysis of AGP (A),α2-MG(B) andTNF-α(C) mRNA expressions and their corresponding GAPDH in the lung and possibleprotection by TH. *P< 0.05 comparison between negative control andpositive control groups. $^{\#}$P < 0.05 comparison between theuntreated group with preventive and treated groups. AGP: alpha-1 acid glycoprotein,α-2M: alpha-2 macroglobulin and TNF-α: tumor necrosis factor-alpha.

sion, while the use of TH as a therapeutic agent normalized and up-regulated AGP levels compared to the untreated group, as shown in Figure 1A.

TH induced up-regulation in the mRNA expression of α2-MG in the positive control group in the lung tissue compared to the negative control group. Whereas, the use of TH as a preventive and therapeutic medication -induced suppression in the context of α2-MG mRNA expression. The effect was clearer as a therapeutic medication than a preventive agent, as shown in Figure 1B.

The positive control group showed up-regulation in the mRNA expression of TNF-a in the lung tissue compared to the negative control group. Administration of TH as a preventive and therapeutic medication-induced suppression in the TNF-a mRNA expression in the lung. The effect was more clearly defined as preventive than therapeutic compared to the untreated group as shown in Figure 1C.

DISCUSSION

The current study showed pathophysiological disability caused by respiratory infection which was protected by prior administration of TH in the lung tissue and biomarkers of liver and kidney in of the rat. Liver and kidney biomarkers AST, ALT, urea and creatinine play a key role in the diagnosis and control of chronic kidney disease and liver disease (Crăciun-Ciorbă *et al.*, 2019). The study showed a significant increase in AST, ALT, urea and creatinine in untreated rats in proportion to the destructive state in rat tissue. The administration of TH improved the level of liver and kidney enzymes and restored normal levels confirming the preventive and therapeutic role of TH against renal and hepatic patho-toxicity induced by *S. aureus* infection. Congruently, previous studies have shown a relieving effect of honey in the treatment of lead-induced stress in rats (Al-Waili, 2003; Halawa *et al.*, 2009). In addition, the admission of Malaysian Tualang honey showed significantly lower levels of AST, ALT, urea and creatinine in infected rats following treatment with honey for repeated exposure to paraquat (Tang *et al.*, 2017).

TH has facilitated in the renewal process of regeneration of hepatic and renal cells, specifically cytokine expression levels have increased, as stated in our study. Furthermore, in the previous study, the daily intake of honey increased the blood levels of antioxidants and decreased the levels of liver enzymes and reduced urea and creatinine in the blood of healthy individuals (Al-Waili, 2003).

Inflammation is the primary defense line of the body against infections. It is also a crucial part of the normal tissue repair mechanism (Ørstavik, 2019). During bacterial infection an immunosuppressant state is applied from the bacteria to mask the spreading of the causative agent invivo and in response immune system secrets cytokines of IL-8, IFN-γ, and IgG antibodies to neutralize invaders (Karin and Clevers, 2016). In this study, the levels of immune response markers such as INF-y, IL-8 and IgG antibodies showed a significant decrease in serum levels in untreated rats, whereas TH boosted the immune system by increasing IgG, INF-γ and IL-8 levels in

the preventive and treated groups, this elevation is proportional to the increase the phagocytic activity in animals through activating the innate and adaptive mechanism (Hegazi *et al.*, 2015). It is noticeable that TH had a clear effect on the immune response in healthy rats due to decreased IgG and IFN-γ and increased IL-8. Phytochemical studies reported immune sensitization activity for stimulating monocytes to release cytokines such as IL-8 (McLoone *et al.*, 2016; Popa *et al.*, 2012). Increased levels of IgG and IFN-γ are generally an indication of an individual's immune status for certain pathogens, TH has shown a decrease in IgG and IFN-y levels in healthy rats within the normal range, pointing to TH as a non-self-substance with no immunological or irritating properties, and TH has an anti-inflammatory potential suppression effect and has an anti-inflammatory potential and it has a variety of positive nutritional and health effects (Molan, 2001).

One of the key proteins in acute-phase reactions is alpha-1 acid glycoprotein (AGP) synthesized primarily in hepatocytes and shows variation in its levels as drug response and biomarker for certain inflammatory bowel disease (IBD) and renal disease (Soliman *et al.*, 2015a). One of the functions of AGP is to mediate pharmacokinetics to reduce toxicity and tissue infections through certain drugs, such as drug-drug interactions (Soliman *et al.*, 2014). AGP mRNA expression has been suppressed in respiratory infections, whereas, TH normalized and upregulated AGP levels in infected and healthy rats, AGP is controlled by a combination of leading regulatory mediators such as glucocorticoids and a cytokine network involving mainly interleukin-1y beta, TNF alpha, and interleukin-6 related cytokines. During our study, it has been shown that honey not only affects the composition of AGP but also affects regulatory mediators such as TNF alpha, which controls the composition of AGP (Fournier *et al.*, 2000).

α-2M protein inhibits fibrinolysis which, in turn downregulates plasmin synthesis. In addition, α-2 M acts as a protein carrier for various cytokines and growth factors, and its secretion levels are decreased throughout acute liver inflammation (Canova *et al.*, 2015). The main mediator for inflammatory reactions is TNF-α from inflammatory cytokines (Robinson *et al.*, 2016). In the current study, both α-2M and TNF-α mRNA expression levels are increased in infection while TH induced suppression of mRNA expression in both preventive and treated groups is likely to be caused by an anti-plasma mechanism that inhibits plasmin synthesis that occurred when bacterial infection and toxin secretion and increases the transport of growth factors that facilitate regeneration of liver cells (Soliman *et al.*, 2015a). These results are consistent with a previous study on gelam honey, which demonstrated a decrease in inflammatory mediators such as TNF-α and COX-2 by alleviating the nuclear factor kappa-light-chain-enhancer (NF-κB) translocation to the nucleus and therefore inhibiting the activation of the NF- κB pathway, and plays a major role in the cause of pathogenesis inflammation (Shamshuddin and Zohdi, 2018). The rationale for this is that the administration of TH has lowered the regulation of α -2M to control the degree of inflammation, and there is a clear correlation between the bacterial infection and the acute-phase protein response, as well contributes to the procoagulant state particularly by enhancing the inhibition of protein.

In addition, TH upregulates the α-2M and TNF-α mRNA expression in healthy rats, which indicates TH's ability to induce immunomodulators such as TNF-α and α-2 M (Syam *et al.*, 2016). In the previous study, honey has been shown to stimulate the production of monocyte cytokines. For this time, honey appears to have specific mechanisms for triggering anti-inflammatory agents, and monocyte growth factors have not been clarified. Honey may also have an impact on various cells, especially endothelial cells and fibroblasts, wound healing and the contribution of honey to it needs clarification (Tonks, 2003).

TH has high anti-inflammatory and antibacterial properties which contribute to reducing bacterial infection resulting in α-2M and TNF-α mRNA down regulation. The studies encouraged potential uses of honey as an attainable preventive agent against chronic disease development; honey's flexibility to scavenge free radicals and to protect against lipid peroxidation may contribute to the prevention/reduction of some inflammatory diseases (Alvarez-Suarez *et al.*, 2013).

CONCLUSION

Results showed that treatment with TH by stimulating immunity and altering gene expression of intermediate strings, cytokines, and acute stage proteins can significantly reduce the pathological effects combined with bacterial infections caused by Staphylococcus aureus. In addition to this, it improves the protective effect exerted on the kidney and liver.

ACKNOWLEDGEMENT

We are thankful for Sultan Zain Al Abidin University to provide the workspace and the infrastructure. We appreciate all affiliates of the College of Health Sciences and Microbiology for their efforts and commitment.

Conflict of Interest

None.

Funding Support

None.

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