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Preventive and Therapeutic Effects of Malaysian *Trigona* Honey Against Pathogenicity Induced by *Staphylococcus aureus* Infection in Wistar rats: Biological and Molecular Study

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Abstract: Background: *The increase of drug-resistant bacteria against the existing antibiotics inspire investigators to look for various natural remedies and investigated their potential based on scientific value. Malaysian Trigona honey (MTH) has a therapeutic application for the wounds and chronic infections. Study of bacterial infection with an animal model following stingless bee honey ingestion is less and needs further investigation. Therefore, we aim to investigate the effectiveness of MTH in Wistar rats infected by Staphylococcus aureus infection at cellular, molecular study.* ***Materials and methods:*** *Twenty five Wistar rats divided into five groups according to different experiments, negative control group was fed with water, positive control group was fed MTH orally (5 g/kg body weight), untreated group was infected by S. aureus without MTH feeding, therapeutic group was infected by S. aureus followed by treatment with MTH and preventive group was administrated MTH one week before S. aureus infection. Blood from orbital sinus was obtained after 3 weeks of MTH feeding for biochemical analysis. Lung tissues were collected for molecular analysis and total bacterial count.* ***Results:*** *Total colony forming unit (CFU) of lung showed significantly decreased ($P < 0.05$) in preventive and therapy groups compared with untreated group. Serum levels of GPx, SOD, and CAT in positive control group were significantly increased compared with negative control group, and in the untreated group were decreased ($P< 0.05$). While in the preventive and therapy groups serum levels were increased ($P< 0.05$) compare with untreated group. Compared to the negative control group mRNA expressions of SOD, GST, and GPx in the positive control group were significantly upregulated ($P<0.05$). Meanwhile, in untreated group was significantly downregulated ($P<0.05$). The expression of mRNA in preventive and therapy groups were significantly upregulated ($P<0.05$) compared with untreated group.* ***Conclusion:*** *Our results clarify the potential of MTH to decrease the total colony forming unit of S. aureus. Moreover, MTH was potential as an antioxidant in suppressed the genes that involved in antioxidant activity.*

Key words: *Antioxidants; Cytokines, Gene expression, Pathogenicity; S.aureus infection*

I. INTRODUCTION

Respiratory infection is one of the main diseases of admission to hospitals, especially adults and causes death all over the world. The infection of the respiratory system by bacteria results in significant morbidities (Mshelia, 2018). The infection is also increasing free radical levels especially reactive oxygen species (ROS) that break down cellular structure. This is known

as oxidative stress. (Dubovskiy et al., 2008). The seriousness of ROS and free radicals is that they contribute significantly to cell dysfunctions, infectious diseases, and aging. Therefore, the body needs a diet rich in antioxidants that can safeguard cells from damaging and may prevent the development of acute diseases. (Vallianou et al., 2014). Various sorts of honey have been explored for their antimicrobial, antidiabetic, anticancer, anti-inflammatory, and antioxidant properties (Rao et al., 2016). In traditional medicine, honey is a treatment of choice for respiratory infection and coughs symptoms. Honey inexpensive, facilely available, and secure substance for children (Ayazi et al., 2017). MTH is also known as made 'Kelulut' is stingless bee honey from Malaysia. It has excellent taste and odor (Saiful Yazan et al., 2016). MTH has been reported to be helpful for medical and therapeutic purposes (Maringgal et al., 2019). Previous study demonstrated that MTH composed of different flavonoid and phenolic compounds (Tuksitha et al., 2018). The antioxidant activity of MTH displayed superior potential in comparison with another type of honey in vitro (Bakar et al., 2017). This current study investigated the potential of MTH as an antioxidant in vivo.

II. MATERIALS AND METHODS

Bacterial strain

S. aureus was provided by the Department of Udder and Neonates, The Animal Reproduction Research Institute, Egypt. The working bacterial culture was grown in nutrient broth (NB) at 37ºC for 24 hours and was centrifuged at 14,000 × g for 20 minutes. Using phosphate buffer saline (PBS) the pellet was washed three times. Then, to approximately 2×10^9 CFU/mL The viable bacterial count was adjusted by using PBS (Abd-El-Hafez et al., 2016). To induce respiratory tract infection in the rats, the rats were injected IP with *S. aureus* in a dose 1.5×10^8 CFU/ mL per rat.

Preparation of MTH sample

Fresh MTH samples were obtained from a bee farm located in Bachok, Kelantan, Malaysia. The honey samples were stored at ambient temperature in a dark before further analysis. MTH was orally administrated to rat by dissolving the honey (5g/ kg body weight) in filter water. This solution was sterilized by 0 .22 mm filtration (Whatman, USA) and then stored in room temperature or immediately kept in the sterile bottle and allowed the tested rats to orally consume (AL-Saadi, 2013; Nikaein et al., 2014).

Animals and Experimental Design

Animal handling was approved by the Animal Ethics Committee of University Sultan Zainal Abidin. The animal ethical approval number is UAPREC/04/18/002. Approval was also obtained from the Ethics Committee Office of The Scientific Dean, Taif University, Kingdom of Saudi Arabia. Twenty-five male Wistar, 9 weeks old, weighing between 200 to 225 g were bought from the King Abdul-Aziz University (Jeddah, Saudi Arabia). The Rats were housed under condition (25±2 ºC) with 12:12 hours day-night cycle and obtained free access to diet and water. Rats have received MTH but did not expose to *S. aureus* infection were assigned as a positive control group (n=5). Rats in the negative control group were not exposed to both bacterial infection and honey ingestion (n=5). Rats have received an intraperitoneal injection (IP) of *S. aureus* for 1.5×10^8 CFU/mL but were not recovered with MTH were then assigned as an untreated group (n=5). Rats in the therapeutic group (n=5) were injected intraperitoneally with *S. aureus* (1.5×10^8 CFU/mL) and were recovered with MTH for 3 weeks after appearing the clinical signs for respiratory tract infection that appeared within three days. Rats were administrated MTH by drinking water for one week before exposure to *S. aureus* (1.5×10^8 CFU/mL), were assigned as a preventive group (n=5). Signs and symptoms of infected rats were observed and recorded (Al-Rubiay et al., 2008; Prasad et al., 2011). Rats were fasting overnight before

anesthetized using 1% isoflurane in an inhalation chamber. Blood samples and lungs were collected (Al-Waili, 2004; Hadi, 2015).

***Staph aureus* counting**

Lung tissues were weighing under sterile condition then were chopped into small pieces and cultivated in NB at 37°C for 24 hours. From 10^{-1} to 10^{-10} serial dilution was performed using NB then cultured on mannitol salt agar (MSA). After 24-hours incubation, The number of colonies was counted to get CFU/ mL/gram (Coulibaly et al., 2018; Dong et al., 2013).

Serum Biochemical Analysis

From eye retro-orbital venous plexuses blood was collected using capillary tube. Blood was collected in EDTA container (Fisher, USA) then was refrigerated for 30 minutes before centrifugation at 4°C for 15 minutes. Supernatant that containing serum was collected and stored at -20°C before biochemical measurements. Catalase (CAT), Glutathione peroxidase (GPx), and superoxide dismutase (SOD), were measured as antioxidant enzymes (Aebi, 1984; Nishikimi et al., 1972).

Gene expression analysis

mRNA extraction

The total RNA from lung tissues (100 mg per sample) was extracted. In liquid nitrogen, Lung samples were flash-frozen and thereafter at −80°C in 1 mL TRIzol (Invitrogen, USA). Using homogenizer (Brinkman, USA) the frozen samples were homogenized. After that, 0.3 mL chloroform was added to the homogenate. For 30 seconds the mixtures were stirred following by refrigerated centrifugation 16,500 × g for 15 minutes. The supernatant was loaded to new tubes, then a similar volume of isopropanol was added to the samples, the samples were shaken for 20 seconds and refrigerated centrifugation 16,500 × g for 15 minutes. with 70% ethanol, the RNA pellets were washed, then dried up and dissolved in diethylpyrocarbonate (DEPC) water. Total RNA concentrations were examined by using NanoDrop ND-1000 Spectrophotometer (Bio-Rad, USA). Using the absorption ratio 260/280, RNA purity levels were evaluated, with sample ratios only accepted between 1.7 and 1.9 for conversion to cDNA. The integrity of RNA was assured in 1.5% denaturated agarose gel stained with ethidium bromide (Soliman et al., 2015).

Conversion of RNA to cDNA

Reverse transcription of RNA was performed with Oligo dT (Qiagen Valencia, CA, USA). A mixture of 4 µg of total RNA and 0.5 ng oligo dT primer in a whole volume of 11 µL was mixed with sterilized DEPC water, then, incubated at 65°C for 10 minutes in the thermal cycle (Bio-Rad T100™) for denaturation. after that, 2 µL of 10X RT buffer, 2 µL of 10 mM dNTPs, and 100 U Moloney Murine Leukemia Virus (M-MuLV) reverse transcriptase (Novosibirsk, Russia) were added. To reach the required total volume, via DEPC water the total volume was completed up to 20 µL. Then the mixture was again incubated in Bio-Rad thermal cycle at 37°C for 1 hour and at 90°C for 10 minutes for enzyme inactivation.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Primers for RT-PCR analysis were retrieved from previous studies as shown in Table 1. In a final volume of 25 µl the PCR was conducted, it was containing, 12.5 µl of PCR Master Mix, 1µl of 10 pM of forward primer, 1µl of 10 pM of reverse primer, 2µl of cDNA template and topped up with nuclease-free water to 25µl. The following PCR protocol (Bio-Rad T100TM thermal

cycle) was used: denaturation at 95 ^{o}C for 5 minutes one cycle followed by 29 cycles, amplification at 94 ^{o}C for 1 minute one cycle and a final elongation annealing: at 72 ^{o}C for 7 minutes with an extra final expansion at 72°C for 7 minutes. The expression of glyceraldehyde-3- phosphate dehydrogenase (GAPDH) mRNA was used as a reference gene. PCR products were electrophorized on 1.5% agarose gel (Bio Basic, Canada) stained with ethidium bromide in Trisborate–EDTA buffer before visualized under ultraviolet light gel using Genius 3.0 gel documentation system (Syngene, USA). The bands intensities were measured densitometrically using ImageJ Software Version 1.47 (http: //www.imagej. en.softonic.com/) (Ismail et al., 2016).

Table (1): Specific primers used for RT-PCR analysis in this study

mRNA	Product size (bp)	Annealing temp(°C)	Number of cycles	Direction	Primer sequence (5′-3′)
SOD	410	55	29	Forward	AGGATTAACTGAAGGCGAGCAT
				Reverse	TCTACAGTTAGCAGGCCAGCAG
GPx	406	57	30	Forward	AAGGTGCTGCTCATTGAGAATG
				Reverse	AAGGTGCTGCTCATTGAGAATG
GST	575	55	30	Forward	GCTGGAGTGGAGTTTGAAGAA
				Reverse	GTCCTGACCACGTCAACATAG
GAPDH	309	52	25	Forward	AGATCCACAACGGATACATT
				Reverse	TCCCTCAAGATTGTCAGCAA

PCR: Polymerase chain reaction, SOD: Superoxide dismutase, GPx: glutathione peroxidase GST: Glutathione-S-reductase, GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

Statistical Analysis

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

III. RESULTS

Antibacterial effect

Table 2 shows that MTH was able to reduce significantly ($P < 0.05$) colony formed by *S. aureus* in preventive and therapy groups after 24 hours incubation compared to untreated group.

Table 2: CFU in preventive and therapy groups following MTH treatment

	Untreated	**Preventive**	**Therapy**
Mean	$131.6 \times 10^7 \pm 6.24 \times 10^7$	$23.64 \times 10^4 \pm 4.70 \times 10^4$	$39.66 \times 10^7 \pm 8.97 \times 10^7$
P-value	-	$P < 0.05$	$P < 0.05$

Values are means ± standard error mean.

Antioxidant activity assessments

To explore the benefits of MTH as an antioxidant supplement, the negative control group and positive control group were compared. The levels of GPx, SOD, and CAT were significant increased ($P< 0.05$) in positive control group. In addition, to

determine the effectiveness of MTH as an antioxidant in bacterial infection, the preventive and therapy groups were compared to the untreated group. We found that all antioxidant enzymes were increased as shown in Table 3.

Table 3: The measurement of antioxidant enzymes in lung following exposure to MTH

	Negative control	**Positive control**	**Untreated**	**Preventive**	**Therapy**
GPx (mU/mL)	0.67± 0.2	0.73± 0.12*	0.56±.02	0.81± 0.5$^{\#}$	0.80± 0.09$^{\#}$
SOD (U/mL)	2.57± 0.16	4.04± 0.23*	2.49± 0.24	4.81± 1.5$^{\#}$	4.22± 0.4$^{\#}$
CAT(U/L)	726± 29	786.0± 48*	680.25± 44	744.5± 30$^{\#}$	782.5± 32$^{\#}$

Values are means ± standard error mean (SEM) for 5 rats per each group. Values are statistically significant at *P < 0.05 comparison between negative control and positive control groups and $^{\#}P$< 0.05 comparison between untreated group with preventive and therapy groups. SOD: superoxide dismutase, CAT: catalase, and GPx: Glutathione Peroxidase.

Preventive and therapeutic effects of MTH against *S. aureus* have suppressed on SOD, GST and GPx mRNA expressions in the lung of Wistar rats.

Rats in positive control group showed upregulation in SOD mRNA expression compared to negative control group. MTH normalized and upregulated SOD mRNA expression in the preventive and therapy groups compared to untreated group in lung, densitometry analysis revealed that using MTH as a protective medication is more beneficial than the therapeutic effect (Figure 1 A).

The positive control group showed significant upregulation (P<0.05) in GST mRNA expression compared to negative control group. *S. aureus* in untreated groups verified downregulation in mRNA expression of GST. On magnification of densitometry analysis that MTH showed a significant preventive and therapeutic effect on the lung of rats injected *S. aureus* compared to untreated group. MTH showed a more therapeutic effect on GST expression compared to the preventive effect (Figure 1 B).

MTH administration in positive control group showed significant upregulation (P<0.05) in GPx mRNA expression compared to negative control group, while in untreated groups showed downregulation in mRNA expression of GPx, Lung of rats injected with *S. aureus* showed upregulation in mRNA expression of GPx in preventive and therapy group compared with untreated group, also MTH showed therapeutic effect on GPx expression more than preventive effect (Figure 1 C).

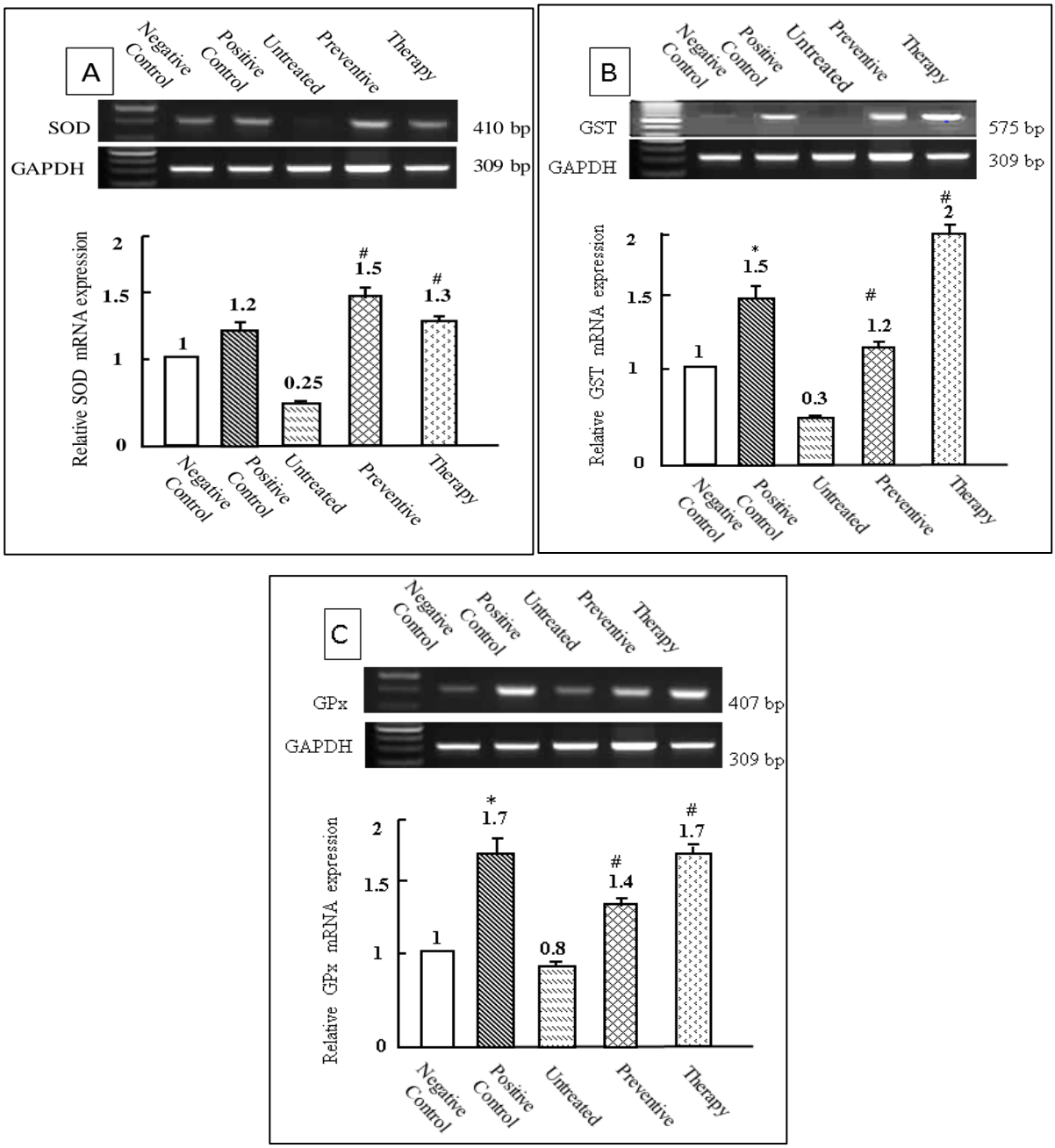

Figure 1: Semiquantitative RT-PCR analysis of SOD (A), GST (B) and GPx (C), mRNA expressions and their corresponding to GAPDH in the lung following exposure to MTH. **P* < 0.05 versus the negative control group. Values are statistically significant at **P* < 0.05 comparison negative control and positive control groups and $^{\#}P$< 0.05 comparison between untreated group with preventive and therapy groups

IV. DISCUSSION

One Gram-positive strain, *S. aureus* was selected for this study. Bacterial infections caused by the genus *S. aureus* are a significant threat to both humans and animals. Moreover, it spreads pneumonia (Wang et al., 2019). In this study, MTH showed a higher antibacterial effect in the preventives and therapy groups. This result is agreement with the previous study that reported MTH has qualitatively antibacterial potency in agar well diffusion assay against *S. aureus* and the honey useful medically and therapeutically (Zainol et al., 2013). Peroxide and non-peroxide components are the main cause of anti-bacterial activity in honey. MTH contains antibacterial activity that does not contain peroxide, and this may lead to the presence of many organic antibacterial agents (Zainol et al., 2013). In addition is characterized by the presence of compounds inhibitory activities like

naringenin against *streptococci* and methicillin-resistant *S.aureus* (Jibril et al., 2019). Antioxidants are molecules that work safely with ROS and help eliminate chain reaction before biomolecules are damaged. Herbs and spices have been identified as sources of various phytochemicals, and have a role in antioxidant defense like clove, cinnamon, turmeric and Cocoa (Griffiths et al., 2016). The liver has a range of endogenous antioxidant defenses are square measure shaped by enzymes like GST, SOD, and CAT (Anderson et al., 1999; Arteaga et al., 2017). Our study is the first to show that MTH may provide some protection and therapy to the respiratory infection. The preventive and therapy groups showed a significant increase in total SOD, CAT and GPx activity than the untreated group. From these results show that MTH supplementation reflected the increase in oxidative stress-induced that mean this honey has varied compounds with antioxidant potential. Our finding consistent with the previous study which showed a significant decrease in the signs of oxidative stress, with a marked increase in the activities of antioxidant enzymes SOD and GPx for tualang honey ins exposed rats (Azman et al., 2019). In another study, kelulut honey supplementation prevented oxidative damage in diabetic mice by increased antioxidant enzyme SOD and Glutathione (Budin et al., 2017). The antioxidant enzymes' potential is stabilizing and damaging free radicals before getting to the cell and attacking their cellular components. Its importance lies by reducing the energy of the free radicals or by giving up some of their electrons for them. like SOD, GST,CAT and GPx, the primary biological role of these enzymes to safeguard the organism from oxidative injury (Mohamed et al., 2011). In this study the untreated groups showed significantly downregulated in mRNA expression of SOD, GST and GPX. This finding confirmed the results in the prior study showed the antioxidant potential of *Hetero Trigona itama* and *Genio Trigona thoracica* stingless bee honey in Malaysia by determined the antioxidant activity spectrophotometrically of honey samples. High antioxidant activities for both *G. thoracica* and *H. itama* honey may be useful for use in dietary and pharmaceutical supplements (Bakar et al., 2017). This study showed that MTH increase the bioavailability of SOD, GST and GPX probably by decreasing cell damage of the lung, The results obtained show that MTH helps reduce oxidative stress by eliminating free radicals such as peroxenitrite (ONOOH), O2 and non-free radicals such as nitrogen oxide (NO) (Erejuwa et al., 2012).

V. CONCLUSION

This is the first comprehensive study at cellular and molecular levels in rats infected by *S. aureus* after ingestion with MTH. Our results demonstrated that MTH has the potential to effectively inhibit *S. aureus* by decreased the bacterial colony in the lung tissues. MTH increased the antioxidant activity of enzymes in blood serum tests. Therefore, the ingestion of MTH for rats showed significant upregulation in gene expression and this confirms the ability of MTH to contribute in improvement to increase the levels of endogenous antioxidants and to scavenge free radicals.

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VI. REFERENCES

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