

## Combination of Thermal-Treated Kelulut (Stingless Bee) Honey and *Phyllanthus niruri* Extract: Evaluation on Antibacterial Activity Against *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Staphylococcus epidermidis*

Muhammad Faiz Razali, Jenorico Aripin, Nadia Natasha Iderus, Nuraisha Syazwani Mohd Adnan and Noor Akhmazillah Mohd Fauzi\*

Department of Chemical Engineering Technology, Universiti Tun Hussein Onn Malaysia, Johor 84600, Malaysia

(\*Corresponding author's e-mail: akhma@uthm.edu.my)

Received: 13 September 2022, Revised: 25 October 2022, Accepted: 1 December 2022, Published: 2 June 2023

### Abstract

Kelulut honey (KH) has a broad spectrum of antibacterial effects that conceivably deteriorate upon heat treatment. However, further synergistic combinations with plant extract might be of value in improving the bactericidal capacity. Therefore, this study reports on the combination of KH with *Phyllanthus niruri* extract as both are independently known to be bactericidal. *P. niruri* extract was incorporated into 2 types of KH; untreated and thermal-treated (50, 60 and 70 °C for 5, 10, 15 and 30 min); and tested against 4 bacterial strains (*Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Staphylococcus epidermidis*) through disc diffusion method. Untreated KH in general had a bigger inhibition zone than thermal-treated. The inhibition reaction was considered sensitive (> 8 mm) against *S. aureus*, *E. coli* and *P. aeruginosa*. Whereas, the effect of thermal treatment on KH was significantly ( $p < 0.05$ ) apparent against *S. aureus*, in which the inhibition zones were mostly not sensitive (< 8 mm). In combination, the mixture of 70:30 (KH:*P. niruri*) had the highest inhibition zone as compared to other ratios. However, the interaction was only deemed to be synergistic with untreated KH, principally against *S. aureus* and *P. aeruginosa*. The results thus showed the vast inhibition spectrum of KH as affected by thermal treatment and combination with *P. niruri* extract. Thermal treatment reduced the bactericidal of KH while incorporation with *P. niruri* extract could synergistically improve the bactericidal with a ratio of 70:30.

**Keywords:** Stingless bee honey, Thermal treatment, Antibacterial, *Phyllanthus niruri*

### Introduction

Kelulut honey (KH) or internationally known as stingless bee honey is a superfood containing many beneficial bioactive compounds. These compounds have therapeutic values that are useful for medical purposes. In general, various compounds found in honey have diverse therapeutic values in which it has been shown useful in the treatment of diseases such as diabetes, wound healing, anticancer, eye diseases and fertility problem [1]. However, information regarding KH's therapeutic values in the medical database is still not vast enough [1]. Limited scientific information showed that KH is beneficial for wound healing [2], metabolic syndrome [3], can act as antifungal [4], antibacterial [5-7], antidiabetic [8,9], anti-aging [10], contained high antioxidant [11], promote fertility [12], and stem-cell development [13]. The versatility of KH as the source of therapeutic component is paramount thus insinuating the potential to be further investigated.

Among all the therapeutic potential, honey is renowned for its antimicrobial capacity. Honey is appealing as a natural antibacterial source due to the fact that its properties are contributed by multiple components like hydrogen peroxide, phenolic compounds, the high viscosity of sugar and acidity level [14]. These multiple antibacterial components working in tandem decrease the likeliness of bacteria to develop resistance [15,16]. Honey is particularly useful in wound management as it can combat wound infection by bacteria while promoting reepithelialisation and angiogenesis as well as stimulating skin and immune cells [15]. This capability of honey thus granted its infamous usage in alternative medicine and antimicrobial research. This is especially rampant with the emergence of antibiotic-resistant bacteria as listed in a global

priority list of antibiotic-resistant bacteria by World Health Organization (WHO). The 12 pathogenic bacteria were listed into 3 levels of priority; 1) Critical, 2) High and 3) Medium. These 12 bacterial species possess a serious threat to global public health and demand more new drugs and antibacterial agents to be discovered.

Compound with therapeutic value is often jeopardised during post-harvest production of honey. In conventional production, thermal treatment has always been used as a medium of processing. This process is often carried out to ease up the packing process, pasteurize and lengthen the shelf-life by reducing moisture content. Thermal treatment is particularly important for honey containing high moisture content like KH. The heat involved in this treatment is either produced from electric resistive heating or fuel combustion prior to being transferred to the food product [17]. Despite the benefits, thermal treatment compromises the existence of thermolabile components in honey, hence detrimental to the overall quality and biological properties as well as masking originality [18,19]. Heating honey at 60 °C and above has been shown to decompose vitamins and also destruct the integrity of enzymes [19]. This will consequently affect the antibacterial capacity of honey as multiple components grant it as aforementioned, which has the risk of deteriorating upon heat. However, thermal treatment is still being opted due to its technology readiness in which the equipment is easily accessible.

Besides solely using honey, a combination with other natural products has also been investigated. This is partly due to the fact that the standardization of honey is difficult as the antibacterial components are heavily dependent on various factors, including geographical origin that influences the food sources, and climates that influence the physicochemical and nutritional properties of honey. In order to cater to these issues, the honey combination is being investigated as it might have a synergistic effect that is consequently beneficial in making uniformly broad and functional antibacterial sources. Various honey combination has been successfully investigated wherein the combination usually showed better performance than the individual components alone. Combination of ginger extract and Ethiopian honey [20], honey and extracts of *Herba Ocimi Basilici* [21], honey and alcoholic extract of mint and zatari, honey and ginger extract and starch [22], honey and *Garcinia kola* Heckel seed extract [23], sumra honey (*Acacia tortilis*) and ethyl alcohol extract of propolis [24], are some of the examples that had a synergistic effect as honey in combination showed greater antibacterial capacity than the individual components alone.

Honey combination with other natural sources has the potential to improve the therapeutic values as it has a possibility to counteract the detrimental effect caused by thermal treatments. “Dukung anak” or scientifically named as *Phyllanthus niruri* is an erect tropical herb characterized by having alternate, sessile and oblong leaves with an approximate height of 30 - 50 cm and 1 - 2.5 mm width. This herbal plant can be found abundantly in tropical countries like Malaysia and does not have specific needs for it to grow vastly. Often being overlooked in the modern age, *P.niruri* has actually been used in traditional medicine. It is prominent in ethnomedical records such as Ayurvedic to treat bronchitis, anaemia, skin diseases, asthma, cough, liver, kidney and urinary tract disorders [25], in traditional Chinese medicine to treat liver injury secondary to various hepatotoxic agents [26], and in Malay traditional medicine to treat kidney disorders and cough. Along with the advancement in science, the ability of *P.niruri* to treat diseases has been scientifically investigated. Many of its bioactive compounds are shown to confer various therapeutic potentials. Compounds extracted from *P.niruri* such as flavonoid has antioxidant property [27], quercitrin can exhibit anti-leishmanial, anti-nociceptive and anti-inflammatory properties [27,28], while extracted Saponins has anti-fungal properties [29]. The current data on its pharmacological properties showed promising values for it to be further utilized in medicine as well as to combine it with honey because, on its own, the potential of *P.niruri* is already outstanding.

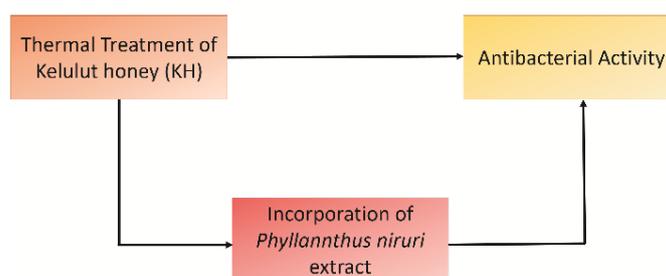
Individually, KH and *P.niruri* have impressive capacities as antimicrobials. In combination, their interaction is virtually unknown as a simple search on databases (PubMed, ScienceDirect, Google Scholar) with keywords of “Kelulut” & “*Phyllanthus niruri*” and “Stingless bee honey” & “*Phyllanthus niruri*” does not return with plausible information regarding this combination. Therefore, this study is conducted to evaluate the effect of thermal treatment on KH and its combination with *P.niruri* extract based on antibacterial activity accessed through the discs diffusion method against 4 bacteria, namely the *Staphylococcus aureus*, *Staphylococcus epidermidis* *Escherichia coli* and *Pseudomonas Aeruginosa*. The incorporation is done to 2 types of KH; untreated and thermally treated. Two samples of KH were being used as to investigate if the incorporation with *P.niruri* resulted in the same synergistic interactions.

## Materials and methods

### Conceptual framework

**Figure 1** describes the research conceptual framework. This study explored the relationship between conventional thermal treatment and the novel incorporation of *P. niruri* extract, to the antibacterial activity of Kelulut honey. Two samples of KH (untreated and thermally treated) were used and incorporation with *P. niruri* extract was carried out in specific ratios. These samples were then tested for antibacterial activity via the disc diffusion method, minimum inhibitory concentration (MIC) and synergistic evaluation. The detailed methodology was described in the following sub-chapters.

### Research Conceptual Framework



**Figure 1** Conceptual framework.

### Honey samples preparation

Untreated Kelulut honey was freshly collected from the local breeder (Nyaleh Trigona Farm, Melaka, Malaysia) and it was stored in a glass bottle at about 10 °C until the experiment is done.

### Thermal treatment

Thermal treatments were performed at 50, 60 and 70 °C for 5, 10, 20 and 30 min using a thermostatic water bath (KNK, Korea). The temperatures chosen are close to the recommended temperature by National Honey Board (2013). For the treatment, samples were submerged in the distilled water bath. The actual temperatures were obtained by measuring the thermometer readings (which are located in the center of the water bath) during the process. After each treatment, all samples were immediately placed in ice-cooled water before analysis. All honey samples were from the same honey batch and the process was carried out in triplicate.

### *Phyllanthus niruri* preparation

*Phyllanthus niruri* plant was freshly collected from Chaah, Johor, Malaysia. The plant was identified and confirmed as species of *Phyllanthus niruri* based on its morphology. The whole part of *Phyllanthus niruri* was cleaned under running tap water to remove dust particles [30]. Clean plants were then dried using the oven at a temperature of 40 °C for 48 h. Dried *P. niruri* was then ground into the powder form to provide a greater surface area during the extraction process. The respective powder is stored as stock in air-tight containers before further use.

### *Phyllanthus niruri* extraction

*Phyllanthus niruri* was extracted via the traditional method with distilled water as solvent. This method was used to avoid the usage of harsh solvent and a previous study has indicated that different extraction method does not yield a significant difference in the level of total phenolic compound [31]. Five g of powdered *Phyllanthus niruri* was diluted with 100 mL of distilled water in a beaker. The solution mixture was then heated up on a hot plate while the magnetic stirrer ensures a continuous and homogeneously mixed mixture during the extraction process. After 30 min of boiling, the solution mixture was allowed to cool down at room temperature before being centrifuged at 10,000 rpm and 26 °C. The supernatant produced was the extracted sample.

### **Incorporation of *Phyllanthus niruri* extract into Kelulut honey**

Crude aqueous extract of *P.niruri* was incorporated with KH in a ratio of 70:30, 50:50 and 30:70 (KH:*P.niruri* extract v/v). The mixture was freshly mixed and use on the same day as the antibacterial experiment was conducted.

### **Bacterial strains**

Bacterial strains chosen for this work were commonly associated with wound infection. Four bacteria strains were used, 2 of those were gram-positive bacteria; *Staphylococcus aureus* ATCC 5538 and *Staphylococcus epidermidis* ATCC 51625; and another 2 were gram-negative bacteria; *Pseudomonas aeruginosa* derived from ATCC 9027 and *Escherichia coli* ATCC 8739. All 4 bacterial strains were standard isolates and maintained in nutrient agar (Himedia) at 4 °C throughout the study.

### **Standard inoculums**

Standard inoculum suspensions used for antibacterial testing (disc diffusion) were prepared by transferring a single colony from pure colonies maintained in nutrient agar into 10 mL of sterile nutrient broth. The bacterial suspensions were then visually adjusted to 0.5 McFarland turbidity standard ( $1.5 \times 10^8$  CFU/mL) and ready to be inoculated for susceptibility test. All bacterial suspensions were freshly made and used within 30 min after being prepared [32].

### **Susceptibility test (disc diffusion method)**

The disc diffusion method was done according to Zakaria *et al.* [33], with slight modification. Six mm sterile paper discs were impregnated with sample solution prepared (20  $\mu$ L) and dried for 15 min before usage. The 100  $\mu$ L of standard inoculum was suspended on Muller Hinton agar and evenly spread out using a sterile spreader. The inoculated plate was then left to dry for 15 min. The impregnated disc was then placed onto the agar using sterile forceps, along with the Streptomycin disc as the positive control and the disc impregnated with sterile water as the negative control. All plates were incubated at 37 °C for 24 h. The antibacterial activity was evaluated by measuring the diameter of the zone of growth inhibition. The experiment was triplicated for each bacterium. The results were recorded as mean  $\pm$  standard deviation.

### **Minimum inhibitory concentration (MIC)**

Serial dilution was employed on the test samples (untreated, thermally treated, combination and *P.niruri* extract) using sterile nutrient broth so that the concentration of the solution was between 1 MIC to 1/64 MIC. Each test tube was inoculated with 0.1 mL bacterial suspension prepared, homogenized and incubated at a temperature of 37 °C for 24 h. Test tubes that had the lowest concentration which exhibited no bacterial growth were determined as the minimum inhibitory concentration [34].

### **Determination of synergistic**

Synergism was determined according to Noori *et al.* [35], with slight modification. The synergistic effect was determined based on a comparison of MIC value between Kelulut honey, *P.niruri* extract and the combined mixture. The combination was considered to be synergistic when the MIC values were lower than the MIC values of the individual components.

## **Results and discussion**

### **Antibacterial activity of Kelulut honey: Effect of thermal treatment**

Disc diffusion was employed to test the susceptibility of bacterial strains toward KH and the results are presented in **Table 1**. In general, all samples of KH showed varying inhibitory capacity towards all tested strains with higher concentrations being mostly potent than diluted samples. In order to rate the inhibitory reaction, inhibition zones were classified as not sensitive (< 8 mm), sensitive (8 to 14 mm), very sensitive (15 to 19 mm) and extremely sensitive (20 mm and above) [7]. Untreated KH (100 %) confers a sensitive reaction to all pathogenic strains tested with exception of *S.epidermidis* ( $7.00 \pm 0.00$  mm). On the other hand, at 70 % concentration, all strains were not sensitive except *P.aeruginosa* with an inhibition zone of  $9.50 \pm 0.87$  mm. Based on this observation, the inhibitory effect of untreated KH was more apparent in *P.aeruginosa* while *S.epidermidis* was the least susceptible.

**Table 1** Inhibition zone of untreated and thermally-treated Kelulut honey against bacterial strains.

Kelulut honey (KH)			Inhibition zone (mm)			
Sample	Treatment time (minutes)	Concentration (100 %)	Gram-positive bacteria		Gram-negative bacteria	
			<i>S.aureus</i>	<i>S.epidermidis</i>	<i>E.coli</i>	<i>P.aeruginosa</i>
Untreated	-	100	12.33 ± 0.58 <sup>a</sup>	7.00 ± 0.00 <sup>a</sup>	10.00 ± 1.73 <sup>a</sup>	12.00 ± 1.00 <sup>a</sup>
	-	70	6.50 ± 0.50 <sup>a,b</sup>	6.50 ± 0.50 <sup>a</sup>	7.83 ± 1.26 <sup>a</sup>	9.50 ± 0.87 <sup>a</sup>
Thermally treated 50 °C	5	100	6.75 ± 0.35 <sup>a,b</sup>	6.75 ± 0.35 <sup>a</sup>	3.75 ± 5.30 <sup>a</sup>	8.00 ± 1.41 <sup>a</sup>
		70	3.25 ± 4.60 <sup>b</sup>	3.50 ± 4.95 <sup>a</sup>	3.25 ± 4.60 <sup>a</sup>	7.25 ± 1.06 <sup>a</sup>
	10	100	7.25 ± 1.06 <sup>a,b</sup>	6.25 ± 0.35 <sup>a</sup>	6.00 ± 0.00 <sup>a</sup>	7.50 ± 0.71 <sup>a</sup>
		70	6.25 ± 0.35 <sup>a,b</sup>	3.00 ± 4.24 <sup>a</sup>	3.00 ± 4.24 <sup>a</sup>	7.00 ± 1.41 <sup>a</sup>
	20	100	7.00 ± 0.35 <sup>a,b</sup>	6.25 ± 0.35 <sup>a</sup>	3.25 ± 4.60 <sup>a</sup>	7.50 ± 2.12 <sup>a</sup>
		70	6.50 ± 0.71 <sup>a,b</sup>	3.00 ± 4.24 <sup>a</sup>	3.00 ± 4.24 <sup>a</sup>	7.00 ± 1.41 <sup>a</sup>
	30	100	7.25 ± 0.35 <sup>a,b</sup>	7.00 ± 0.00 <sup>a</sup>	6.25 ± 0.35 <sup>a</sup>	7.50 ± 0.71 <sup>a</sup>
		70	6.75 ± 1.06 <sup>a,b</sup>	3.25 ± 4.60 <sup>a</sup>	3.00 ± 4.24 <sup>a</sup>	6.75 ± 1.06 <sup>a</sup>
Thermally treated 60 °C	5	100	6.75 ± 0.35 <sup>a,b</sup>	3.25 ± 4.60 <sup>a</sup>	3.25 ± 4.60 <sup>a</sup>	7.00 ± 1.41 <sup>a</sup>
		70	6.25 ± 0.35 <sup>a,b</sup>	3.00 ± 4.24 <sup>a</sup>	3.25 ± 4.60 <sup>a</sup>	6.50 ± 0.71 <sup>a</sup>
	10	100	6.75 ± 0.35 <sup>a,b</sup>	7.00 ± 1.41 <sup>a</sup>	3.00 ± 4.24 <sup>a</sup>	7.00 ± 0.71 <sup>a</sup>
		70	6.25 ± 0.35 <sup>a,b</sup>	3.00 ± 4.24 <sup>a</sup>	3.25 ± 4.60 <sup>a</sup>	6.25 ± 0.35 <sup>a</sup>
	20	100	7.25 ± 1.06 <sup>a,b</sup>	8.00 ± 0.00 <sup>a</sup>	6.25 ± 0.35 <sup>a</sup>	6.75 ± 1.06 <sup>a</sup>
		70	6.50 ± 0.71 <sup>a,b</sup>	3.50 ± 4.95 <sup>a</sup>	3.25 ± 4.60 <sup>a</sup>	7.50 ± 2.12 <sup>a</sup>
	30	100	6.75 ± 0.35 <sup>a,b</sup>	6.25 ± 0.35 <sup>a</sup>	6.00 ± 0.00 <sup>a</sup>	4.50 ± 6.36 <sup>a</sup>
		70	3.25 ± 4.60 <sup>b</sup>	3.00 ± 4.24 <sup>a</sup>	0.00 ± 0.00 <sup>a</sup>	4.25 ± 6.36 <sup>a</sup>
Thermally treated 70 °C	5	100	6.50 ± 0.71 <sup>a,b</sup>	6.25 ± 0.35 <sup>a</sup>	6.25 ± 0.35 <sup>a</sup>	7.50 ± 0.71 <sup>a</sup>
		70	3.00 ± 4.24 <sup>b</sup>	3.75 ± 5.30 <sup>a</sup>	3.00 ± 4.24 <sup>a</sup>	7.25 ± 1.06 <sup>a</sup>
	10	100	9.75 ± 1.77 <sup>a,b</sup>	6.50 ± 0.71 <sup>a</sup>	6.50 ± 0.00 <sup>a</sup>	8.50 ± 1.41 <sup>a</sup>
		70	6.50 ± 0.00 <sup>a,b</sup>	6.00 ± 0.00 <sup>a</sup>	3.25 ± 4.60 <sup>a</sup>	7.50 ± 2.12 <sup>a</sup>
	20	100	4.00 ± 5.66 <sup>a,b</sup>	3.00 ± 4.24 <sup>a</sup>	7.00 ± 0.07 <sup>a</sup>	4.25 ± 6.01 <sup>a</sup>
		70	3.50 ± 4.95 <sup>b</sup>	6.50 ± 0.71 <sup>a</sup>	3.00 ± 4.24 <sup>a</sup>	4.25 ± 6.01 <sup>a</sup>
	30	100	3.00 ± 4.24 <sup>b</sup>	6.00 ± 0.00 <sup>a</sup>	7.00 ± 0.00 <sup>a</sup>	9.00 ± 0.00 <sup>a</sup>
		70	6.50 ± 0.70 <sup>a,b</sup>	0.00 ± 0.00 <sup>a</sup>	6.50 ± 0.00 <sup>a</sup>	8.00 ± 2.83 <sup>a</sup>

Mean values (means ± standard deviation), where n = 3 and the different letters in each column indicate a significant difference ( $p < 0.05$ ) based on Tukey's HSD test (Minitab v21.1.0, Statistical Software).

KH was also treated with thermal treatment at 50, 60 and 70 °C for 5, 10, 20 and 30 min where the resulting antibacterial activities are tabulated in **Table 1**. Likewise, all thermal-treated were mostly higher in activity with 100 % concentration. By looking roughly at thermal-treated KH with regards to untreated, the antibacterial activities showed decrement patterns in all strains tested. However, a significant difference ( $p < 0.05$ ) was only found with the inhibition zone shown in *S.aureus*. In particular, thermal-treated KH at 70 °C had a more significant reduction ( $p < 0.05$ ) in the inhibition zone from the untreated and it was mostly registered by diluted samples at 70 %. The lowest reduction was recorded in the thermal-treated sample at 70 °C with 5 min (70 % concentration) and 30 min (100 % concentration) treatment time.

KH in this study; either untreated or thermally treated; demonstrated various ranges of inhibitory activities that were dependent on the concentration. This is expected since several previous studies

demonstrated such results in which honey in its original amorphous form has higher antibacterial activity than diluted samples. Omar *et al.* [36], showed the undiluted *Geniotrigona thoracica* multi-floral honey (GTM) had a bigger inhibition zone than diluted samples. The same pattern was also reported by Roslic [37]. Their study showed that stingless bee honey with 50 % concentration had a bigger inhibition zone than 25 and 12.5 %. This, therefore, shows that concentration does play a role in the antibacterial activity of KH and it is the most potent in its original form.

Untreated KH as aforementioned in the results conferred sensitive reaction towards 3 out of 4 bacteria tested. Evidently, our KH possesses *in-vitro* antibacterial activity against pathogenic bacteria. However, a fair comparison with other studies on the potency of stingless bee honey can be hardly done as the inherent nature of honey is known to be vastly variable. Many studies reported that stingless bee honey has a vast spectrum of inhibition. For instance, *E.coli* was resistance to *H.itama* honey [36,37], but in other studies, *H.itama* honey did confer inhibition on *E.coli* with clear zone of  $0.50 \pm 0.87$  [38],  $1.0 \pm 0.1$ ,  $0.8 \pm 0.1$ ,  $1.3 \pm 0.3$  and  $1.3 \pm 0.2$  cm [6], as well as  $10.00 \pm 1.73$  mm from our samples. Even so, similar results have also been demonstrated. A study by Hasali *et al.* [39], generated similar results to our findings with their *Heterotrigona itama* honey conferred the biggest inhibition zone on *P.aeruginosa*.

In theory, gram-positive bacteria are more susceptible to antibacterial agents than gram-negative due to their permeable thick peptidoglycan that allows substances to pass thru easily. In contrast, gram-negative bacteria have 3 layers consisting of an outer membrane, peptidoglycan cell walls, and an inner membrane that determine the resistance with the outer membrane being mostly responsible for it [40]. Results for 100 % KH were mostly in line with the theory; with exception of *S.epidermidis*; in which *S. aureus* (gram-positive) had a bigger inhibition zone than both gram-negative bacteria (*E.coli* and *P.aeruginosa*). However, this was not the case with untreated KH 70 % as gram-negative bacteria (*P.aeruginosa*) was most susceptible. Among all strains, the resistance of *S.epidermidis* is not uncommon since it has been shown to be multi-drug resistant where many cases of isolates had up to 94 % resistance towards penicillin [41]. The resistance of *S. epidermidis* towards commercial antibiotics as well as its less sensitivity towards our KH might be contributed by its ability to manipulate its exopolysaccharide matrix or produce biofilms, reducing penetration and permeability of antibiotic agents [42]. Nevertheless, 3 out of 4 bacterial strains tested (*S.aureus*, *E.coli*, and *P.aeruginosa*) had sensitive reactions to our untreated samples (100 %) thus providing more evidence of the antibacterial capacity of KH and the potential to be diversified in a health-related product.

In the case of thermal-treated KH, the varying decrement in antibacterial activity of different concentrations indicated the impact of prolonged time and the influence of concentration on the bactericidal capability. This decrement in antibacterial activities as portrayed by all strains tested was actually in-line with what has been reported previously in heated Kelulut honey [32], heated Malaysia and Australian stingless bee honey [43], and heat-treated Polish honey [44]. Sulaiman and Sarbon [32], reported that their heated Kelulut honey (50, 70 and 90 °C) showed a reduction in antibacterial activities when tested against *Staphylococcus aureus*, *Escherichia coli*, *Salmonella*, and *Pseudomonas aeruginosa*. Similar results were also demonstrated by Ramlan *et al.* [43], as their thermally-treated Malaysian and Australian stingless bee honey had higher minimum inhibitory value (MIC) than untreated samples when tested against *Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella pneumoniae*, *Salmonella typhimurium*, *Bacillus cereus* and *Staphylococcus aureus*. The higher MIC value consequently signifies the reduced capacity of heated stingless bee honey to inhibit bacterial growth. A study done by Majkut *et al.* [44], also revealed the same reduction in inhibition capacity as ours with their Rapeseed honey rapidly losing its antibacterial properties and at the same time having the lowest contents of vitamin C, polyphenols content and FRAP value.

The reduction in inhibition capacity of our thermal-treated KH is postulated to be due to the detrimental effect brought about by heat. The consensus behind this is that it is mainly contributed by the deterioration of thermolabile components that can be found abundantly in honey. Honey's antibacterial activity is influenced by high osmolarity, acidity (low pH), peroxide and non-peroxide components [14], in which the latter 2 tend to deteriorate upon heat. Non-peroxide components such as polyphenols (phenolic acid and flavonoids) have been reported to influence the capacity of honey to inhibit bacterial growth. Mono-floral *Meliponini* honey from Brazil had high total phenolic contents (TPC) which in turn exhibited high antibacterial activities [45]. On the other hand, peroxide activity contributed by hydrogen peroxide ( $H_2O_2$ ) is generated in honey when it is diluted as enzyme glucose oxidase oxidizes glucose to gluconic acid and  $H_2O_2$  is activated [14]. As honey is actually a complex ecosystem on its own, the inherent components are bound to interact with one another and consequently influenced the antibacterial capacity. This conjecture has been corroborated through multivariate analysis of data generated by total fluorescence spectroscopy, in which phenolic compounds and Maillard products were shown to be related to the

sustained chemical production of hydrogen peroxide over time and subsequently enhance the antibacterial activity of honey, primarily against *S. aureus* [46]. It alludes that non-peroxide is interlinked with hydrogen peroxide, particularly phenolic compounds and Maillard reaction products. Hence, detrimental degradation of any of these 2 components by heat can potentially reduce the inhibition capacity greatly. This assumption is evidently possible since the previous study on the impact of industrial heat treatment on Spanish honey revealed the significant reduction ( $p < 0.05$ ) of 4 phenolic compounds namely the galangin, kaempferol, myricetin and p-coumaric acid [47]. In addition, a significant reduction in flavonoid content ( $p < 0.01$ ) has been documented in thermally treated (121 °C for 30 min) Moroccan Zantaz' honey albeit with an insignificant variation of polyphenol content post-thermal treatment [48]. Peroxide activity has also been reported to be negatively impacted by heat as well as the presence of catalase [14]. This is markedly true since honey heated at 50 °C for 20 min had reduced enzyme activity where glucose oxidase was affected [49], consequently disrupting the generation of hydrogen peroxide in honey. Therefore, the probability of peroxide and non-peroxide components in our thermally-treated KH to be degraded was high, thus explaining the reduction in bactericidal, especially against *S.aureus*. This particular hypothesis for our significant reduction ( $p < 0.05$ ) demonstrated by *S.aureus* can be supported by results obtained through Cebrero *et al.* [46], study as aforementioned. The negative impact of thermal treatment on the antibacterial activity of KH further proved the need to find an alternative to counteract it. Incorporation with other natural products is therefore potentially beneficial with diversification prospects in mind.

#### **Incorporation of *Phyllanthus niruri* extract to Kelulut honey (KH) and its resulting effect on antibacterial activity**

Aqueous *Phyllanthus niruri* extract was incorporated into untreated KH with ratios of 70:30, 50:50 and 30:70 of KH to *P.niruri* (v/v) resulting in KH mixture of 70, 50 and 30 %. The KH mixtures were then tested against 4 strains of bacteria (*S.aureus*, *S.epidermidis*, *E.coli* and *P.aeruginosa*) and the results are tabulated in **Table 2**. With regards to untreated KH, significant differences ( $p < 0.05$ ) were recorded for mixture samples and *P.niruri* extract tested against all bacteria except *E.coli*. For *S.aureus* and *P.aeruginosa*, the combination mixture did not out-performed the inhibition prowess of untreated KH but it was definitely better than *P.niruri* extract. The same can be said with *E.coli*, however, the differences between the inhibition zone of all samples were not statistically significant ( $p > 0.05$ ). Interestingly, the combination mixture of 70 % had a slightly bigger inhibition zone than untreated KH (100 %) when tested against *S.epidermidis*. By comparing the capacity to inhibit bacterial strains tested, KH seems to be more bactericidal than *P.niruri* extract with an average difference of 6.25 mm. The bactericidal of mixture samples on the other hand seems to be on average for all bacteria tested except *S. epidermidis*, thus giving an initial indication that the interaction might be an addition. Intriguingly, the results show that a higher concentration of KH in the mixture exhibit more potent antibacterial prowess than the opposite mixture (*P.niruri* > KH).

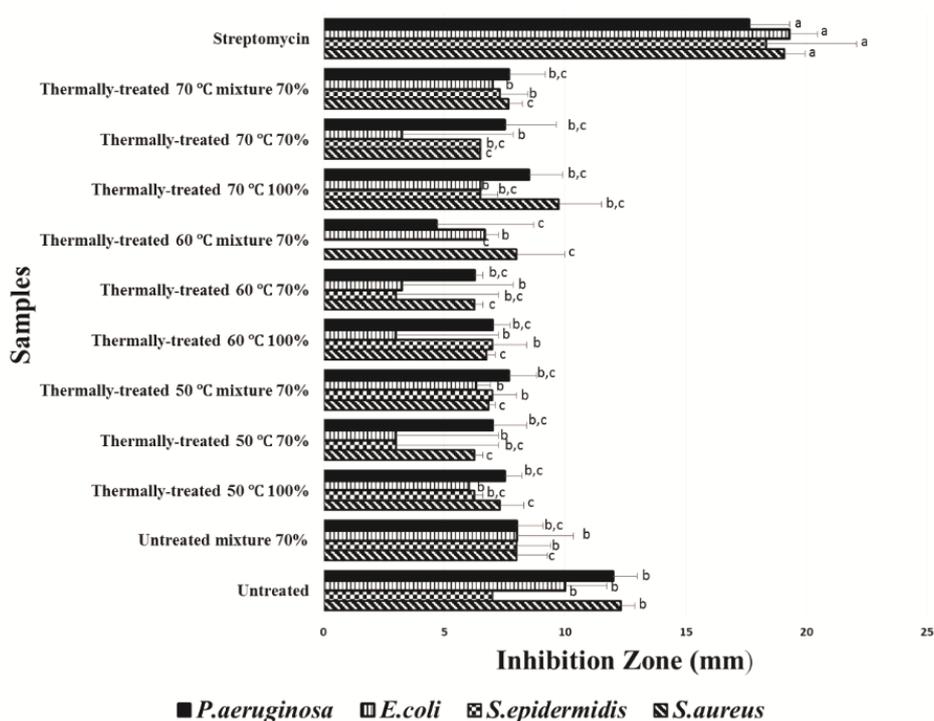
**Table 2** Inhibition zone of untreated Kelulut honey, incorporated mixture with *Phyllanthus niruri* extract and crude aqueous *Phyllanthus niruri* extract against bacterial strains.

Samples	Ratio (v/v %)		Inhibition zone (mm)			
	Kelulut honey	<i>P.niruri</i> extract	Gram-positive		Gram-negative	
			<i>S.aureus</i>	<i>S.epidermidis</i>	<i>E.coli</i>	<i>P.aeruginosa</i>
Untreated	100	0	12.33 ± 0.58 <sub>a</sub>	7.00 ± 0.00 <sub>a,b</sub>	10.00 ± 1.73 <sub>a</sub>	12.00 ± 1.00 <sub>a</sub>
	70	30	8.00 ± 1.26 <sub>b</sub>	8.00 ± 1.41 <sub>a</sub>	8.00 ± 2.36 <sub>a</sub>	8.00 ± 1.09 <sub>a,b</sub>
Mixture	50	50	6.83 ± 0.98 <sub>b</sub>	5.50 ± 2.74 <sub>a,b</sub>	6.50 ± 3.89 <sub>a</sub>	5.17 ± 4.17 <sub>b</sub>
	30	70	6.5 ± 0.84 <sub>b</sub>	3.50 ± 3.89 <sub>b,c</sub>	4.50 ± 4.97 <sub>a</sub>	7.33 ± 1.51 <sub>a,b</sub>
<i>P.niruri</i> extract	0	100	5.00 ± 4.58 <sub>b</sub>	0.00 ± 0.00 <sub>c</sub>	6.00 ± 5.29 <sub>a</sub>	5.33 ± 4.73 <sub>a,b</sub>

Mean values (means standard deviation), where n = 3 and the different letters in each column indicate a significant difference ( $p < 0.05$ ) based on Tukey's HSD test (Minitab v21.1.0, Statistical Software).

Since the incorporation of *P.niruri* extract with untreated KH revealed that the ratio of 70:30 (KH:*P.niruri*) exhibited the highest inhibition zone among the mixtures, the same ratio was applied to thermal-treated KH. This was carried out to evaluate if the addition of *P.niruri* extract will somehow offset

the impact of thermal treatment. The extract was incorporated with KH samples thermally treated at 50, 60 and 70 °C for 10 min, as on average it was deemed to be the best treatment time based on the disc-diffusion results. **Figure 2** compares the inhibition zone of the thermal-treated mixture to thermal-treated KH, untreated KH, untreated mixture (70 %) and Streptomycin (positive control). Based on the graph, the addition of *P.niruri* extract to thermal-treated KH did not produce statistically significant ( $p > 0.05$ ) differences in the inhibition zone as compared to thermal-treated alone when tested against all bacterial strains. However, in the case of *P.aeruginosa*, the thermal-treated mixture of 50 and 70 °C had slightly bigger inhibition zone than thermal-treated KH (70 % concentration) with differences of 0.67 and 0.17 mm, respectively. *E.coli* on the contrast were shown to be slightly more susceptible to the thermal mixture than the individual thermal-treated KH across all temperatures as depicted in **Figure 2**. This shows that *E.coli* in general was more susceptible to the mixture than *P.aeruginosa*., therefore insinuating the beneficial improvement made by *P.niruri* incorporation towards thermal-treated KH.



**Figure 2** Comparison of the thermal-treated mixture to other samples against bacterial strains tested. Mean values (means standard deviation), where  $n = 3$  and the different letters in each similar pattern bar indicate a significant difference ( $p < 0.05$ ) based on Tukey's HSD test (Minitab v21.1.0, Statistical Software).

The same pattern was also seen in the reaction of gram-positive bacteria to the thermal-treated mixture. For *S.aureus*, it was more susceptible to combination mixture for all temperature than thermal-treated (70 %), whereas, for *S.epidermidis*, the slightly bigger inhibition zone than thermal-treated (100 and 70 %) were registered by the mixture of 50 and 70 °C. This hence showed the advantages of *P.niruri* incorporation into thermal-treated KH, with it being particularly true for *S.epidermidis*. Nevertheless, when being compared to the untreated mixture, the thermal-treated mixture produced comparable results for all bacteria tested albeit the fact that it was still lower than the bactericidal of pure untreated KH. The incorporation of *P.niruri* extract into KH reveals that the ratio of 70:30 (KH:*P.niruri*) exhibited the biggest inhibition zone among all other ratios. This implies that a higher concentration of KH in the mixture exhibit more potent antibacterial prowess than the opposite mixture (*P.niruri* > KH). To put more perspective, a previous study on acetone and ethanol extract of *P.niruri* with the concentration of 1.0 µg/discs registered inhibition zone of 0.1 and 0.01 mm, respectively against wild strains of *Staphylococcus sp.* and *E.coli* as well as 0.01 mm against mutant strains of *E.coli* [50]. In addition, the crude methanolic extract of *P.niruri* studied by Ibrahim and team [34], showed results within range with our aqueous extract as it registered inhibition zone of  $7.0 \pm 0.3$  and  $7.0 \pm 0.2$  mm for *E. coli* and *P.aeruginosa*, respectively. This hence supported the inhibition capacity of our aqueous extracts despite it being lower than KH. The differences were somehow expected since honey is a complex ecosystem with many compounds working in tandem,

whereas plant extract mainly has phytochemicals such as lignans (like phyllanthin and hypophyllanthin), flavonoids (like quercetin), astragalins, triterpenoids, glycosides and tannins (ellagitannins) [34], that contributed to the bactericidal capacity.

#### Minimum inhibitory concentration (MIC) and determination of synergism

Minimum Inhibitory Concentration (MIC) was identified and the results are tabulated in **Table 3**. In this experiment, the MIC was determined visually by observing the presence of cloudiness within each of the solutions, and the first solution that has the cloudiness characteristic, the solution before that shall be determined as the MIC.

**Table 3** Minimum inhibitory concentration (MIC) of untreated Kelulut honey, *P.niruri* extract and combination mixture.

Sample	Initial concentration of sample (%)		Minimum inhibitory concentration (MIC) (%)			
	Kelulut Honey	<i>P.niruri</i> extract	<i>S.aureus</i>	<i>S.epidermidis</i>	<i>E.coli</i>	<i>P.aeruginosa</i>
Untreated KH	100	0	6.25	6.25	3.13	6.25
<i>P.niruri</i> extract	0	100	50.00	25.00	25.00	25.00
Combination mixture	70	30	3.13	6.25	3.13	3.13
	30	70	12.50	12.50	12.50	12.50

MIC for untreated KH was mostly at 6.25 % with exception of *E. coli* which recorded the lowest MIC with a value of 3.13 %. The low MIC, therefore, revealed that our untreated KH was a strong bactericidal. Contrarily, *P.niruri* registered higher MIC, mostly at 25.00 % with exception of *S.aureus* with a MIC value of 50.00 %. When in combination, the value of MIC was dependent on the final ratio of the mixture. The mixture with combinations of 70 % KH and 30 % *P.niruri* had much lower MIC as compared to the mixture of 30 % KH and 70 % *P.niruri* as tabulated in **Table 3**. The mixture of 70 % KH had as low as 3.13 % MIC for 3 bacteria, namely *S.aureus*, *E.coli* and *P.aeruginosa*. Whereas the combination mixture of 30 % KH had a MIC value of 12.50 % for all bacteria tested.

In order to evaluate if synergism exists between those 2 components, the MIC value of the combined mixture must be lower than the MIC value of the individual components. Based on our results (**Table 3**), it can be deduced that a synergistic relationship exists when the concentration of KH was much higher than the *P.niruri* extract. This is particularly true for *S.aureus* and *P.aeruginosa* as the MIC combination of 70 KH:30 *P.niruri* (3.13 %) was much lower than the MIC of individual components (6.25 % KH, 50.00 and 25.00 % for *P.niruri*). Intriguingly, the MIC of the thermally treated and its mixture were somehow different from than untreated as tabulated in **Table 4**. For the sake of coherent comparison, the MIC was determined for thermal-treated at 70 °C/10 min and its mixture, against *S. aureus* (gram-positive) and *P. aeruginosa* (gram-negative). The treatment setting was chosen based on disc diffusion results which produced the best inhibition results for those 2 bacterial strains. Thermal-treated KH had the same MIC value as untreated KH which is 6.25 % and it was much lower than the MIC of *P.niruri*. Fascinatingly, the thermal-treated mixture (70 %) registered the same MIC value as the thermal-treated and untreated KH. Since the same value of MIC was registered, the incorporation of *P.niruri* extract could not be deemed as synergistic but merely additive. This observation is very intriguing since the combination of untreated KH and *P.niruri* produces a synergistic antibacterial effect, principally against *S.aureus* and *P.aeruginosa*. Yet, when the KH was thermally treated, the combination was merely additive.

**Table 4** Minimum Inhibitory Concentration (MIC) of thermally-treated Kelulut honey, *P.niruri* extract and combination mixture.

Sample	Initial concentration of sample (%)		Minimum inhibitory concentration (MIC %)	
	Thermally-treated Kelulut Honey	<i>P.niruri</i> extract	<i>S.aureus</i>	<i>P.aeruginosa</i>
Thermal-treated KH	100	0	6.25	6.25
<i>P.niruri</i> extract	0	100	50.00	25.00
Combination mixture	70	30	6.25	6.25

The bactericidal of both KH and *P.niruri* extract is therefore further proved by the MIC values. KH registered values much lower than *P.niruri* extract as expected. As above-mentioned, this is most probably because KH has much more complex inherent compounds of peroxide and non-peroxide (phenolic compound) that enables the ability [14]. *P.niruri* in contrast might be a stronger antioxidant due to the presence of methanolic content such as beta-sitosterol, gallic acid, ellagic acid and alkaloids-4-methoxy-nor-securinine [51]. Thus, the combination of Kelulut honey and *Phyllanthus niruri* could prove to have a synergistic effect as each compound could complement the other in regards to being both a potent antibacterial and antioxidant agent.

The combination mixture was observed to have a much lower MIC in the mixture of 70 % KH instead of 30 %. This further signifies the importance of KH being higher in concentration than *P.niruri* extract in order for this combination to work well. The ratios were then deemed to be synergistic, principally against *S.aures* and *P.aeruginosa*. In contrast, the incorporation of *P.niruri* extract into thermal-treated KH was deemed to be a mere addition as the MIC of the mixture was the same as the MIC of thermal-treated KH alone. Since no plausible previous study has reported on the combination of honey and *P.niruri* extract, a comparison can be hardly done. However, other combinations of honey and plant material revealed that different ratios had different inhibition activities. For instance, stingless Bee Honey (*Trigona itama*) in combination with Ajwa Date (*Phoenix dactylifera L.*) seeds registered the highest inhibition zone when the combinations were done in a 1:2 ratio (*Trigona itama*:Ajwa date seeds) [52]. On the other hand, a study on the combined mixture of stingless bee (*Apis mellipodae*) honey and garlic (*Allium sativum*) extracts showed that an equal amount of both individual components had a synergistic effect on the antibacterial activities against the standard and clinical pathogenic bacteria tested [53]. A study on ginger extract and honey on bacteria isolates from extracted carious teeth also revealed that the MIC of the combined mixture was in the range of 15.63 - 31.25 mg/mL, in which lower than the MIC of the individual components alone [54]. These ratios are not in-line with ours, therefore suggesting that the bactericidal of mixtures does not heavily dependent on the amount of honey alone. However, all these studies are in agreement with our findings in that honey in combination usually has a synergistic effect on antibacterial activity. Nonetheless, our study demonstrated the combination of KH and aqueous *P.niruri* extract produced a higher inhibition zone when KH concentration is much higher than *P.niruri* extract.

## Conclusions

Kelulut honey either untreated or thermally treated has the capacity to inhibit bacteria in which this ability is much dependent on the concentration and the species of bacteria tested. However, thermal treatment is detrimental to the bactericidal capacity as treated KH had lower inhibition zones in all bacteria tested. In the case of incorporation with *Phyllanthus niruri* extract, the combined mixture was deemed to be synergistic with the ratio of 70 % untreated KH to 30 % *P.niruri* extract, principally against *S.aureus* and *P.aeruginosa*. In conclusion, the combination of Kelulut honey and *P.niruri* extract is feasible and the potential to diversify this combination should be further explored.

## Acknowledgments

The first author would like to thank The Ministry of Higher Education of Malaysia for the Fundamental Research Grant Scheme (FRGS) K302. The supply of honey samples from Nyaleh Trigona Farm, Melaka, Malaysia is much appreciated. Special thanks to staff from the Department of Chemical Engineering Technology, Faculty of Engineering Technology for their technical support.

## References

- [1] FAZ Amin, S Sabri, SM Mohammad, M Ismail, KW Chan, N Ismail, ME Norhaizan and N Zawawi. Therapeutic properties of stingless bee honey in comparison with European bee honey. *Adv. Pharmacol. Pharmaceut. Sci.* 2018; **2018**, 6179596.
- [2] A Nordin, N Omar, NQ Sainik, SR Chowdhury, E Omar, AB Saim and RB Idrus. Low-dose stingless bee honey increases viability of human dermal fibroblasts that could potentially promote wound healing. *Wound Med.* 2018; **23**, 22-7.
- [3] NZ Ramli, KY Chin, KA Zarkasi and F Ahmad. The beneficial effects of stingless bee honey from *Heterotrigona itama* against metabolic changes in rats fed with high-carbohydrate and high-fat diet. *Int. J. Environ. Res. Publ. Health* 2019; **16**, 4987.
- [4] NE Hau-Yama, D Magaña-Ortiz, AI Oliva and E Ortiz-Vázquez. Antifungal activity of honey from stingless bee *Melipona beecheii* against *Candida albicans*. *J. Apicult. Res.* 2020; **59**, 12-8.

- [5] SCB Domingos, VH Clebis, G Nakazato, AGD Oliveira, RKT Kobayashi, RC Peruquetti, CD Pereira, MTS Rosa and LDS Medeiros. Antibacterial activity of honeys from Amazonian stingless bees of *Melipona* spp. and its effects on bacterial cell morphology. *J. Sci. Food Agr.* 2021; **101**, 2072-7.
- [6] WJ Ng, NW Sit, PA Ooi, KY Ee and TM Lim. The antibacterial potential of honeydew honey produced by stingless bee (*Heterotrigona itama*) against antibiotic-resistant bacteria. *Antibiotics* 2020; **9**, 871.
- [7] MM Aspar, R Edros and NA Hamzah. Antibacterial evaluation of Malaysian Kelulut, Tualang, and Acacia honey against wound infecting bacteria. *IOP Conf. Ser. Mater. Sci. Eng.* 2020; **991**, 012065.
- [8] M Sahlan, O Rahmawati, DK Pratami, R Raffiudin, RR Mukti and H Hermasyah. The effects of stingless bee (*Tetragonula biroi*) honey on streptozotocin-induced diabetes mellitus in rats. *Saudi J. Biol. Sci.* 2020; **27**, 2025-30.
- [9] O Rahmawati, DK Pratami, R Raffiudin and M Sahlan. Alpha-glucosidase inhibitory activity of stingless bee honey from *Tetragonula biroi* and *Tetragonula laeviceps*. *AIP Conf. Proc.* 2019; **2092**, 030001.
- [10] NA Malik, M Mohamed, MZ Mustafa and A Zainuddin. *In vitro* modulation of extracellular matrix genes by stingless bee honey in cellular aging of human dermal fibroblast cells. *J. Food Biochem.* 2020; **44**, 13098.
- [11] NS Nayan, MA Yazid, K Nallappan, AA Amran, NS Zaidi, F Zakaria, H Hassan and NA Karim. *In vitro* modulation of endogenous antioxidant enzyme activities and oxidative stress in autism lymphoblastoid cell line (ALCL) by stingless bee honey treatment. *Oxidative Med. Cell. Longevity* 2020; **2020**, 4539891.
- [12] SB Budin, FF Jubaidi, SN Azam, NL Yusof, IS Taib and J Mohamed. Kelulut honey supplementation prevents sperm and testicular oxidative damage in streptozotocin-induced diabetic rats (*in Indonesian*). *Jurnal Teknologi* 2017; **79**, 89-95.
- [13] MA Mohamad, MA Mazlan, M Ibrahim, AM Yusof, SA Shamsuddin, NF Hassan, H Muhammad and ML Isa. The effect of Malaysian stingless bee, *Trigona* spp. honey in promoting proliferation of the undifferentiated stem cell. *Asia Pac. J. Mol. Biol. Biotechnol.* 2018; **27**, 10-9.
- [14] MD Mandal and S Mandal. Honey: Its medicinal property and antibacterial activity. *Asian Pac. J. Trop. Biomed.* 2011; **1**, 154-60.
- [15] P McLoone, D Tabys and L Fyfe. Honey combination therapies for skin and wound infections: A systematic review of the literature. *Clin. Cosmet. Investig. Dermatol.* 2020; **13**, 875.
- [16] O Sherlock, A Dolan, R Athman, A Power, G Gethin, S Cowman and H Humphreys. Comparison of the antimicrobial activity of Ulmo honey from Chile and Manuka honey against methicillin-resistant *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa*. *BMC Compl. Alternative Med.* 2010; **10**, 47.
- [17] RN Pereira and AA Vicente. Environmental impact of novel thermal and non-thermal technologies in food processing. *Food Res. Int.* 2010; **43**, 1936-43.
- [18] X Zhang, N Tao, X Wang, F Chen and M Wang. The colorants, antioxidants, and toxicants from nonenzymatic browning reactions and the impacts of dietary polyphenols on their thermal formation. *Food Funct.* 2015; **6**, 345-55.
- [19] S Bliidi, P Gotsiou, S Loupassaki, S Grigorakis and AC Calokerinos. Effect of thermal treatment on the quality of honey samples from Crete. *Adv. Food Sci. Eng.* 2017; **1**, 1-8.
- [20] Y Ewnetu, W Lemma and N Birhane. Synergetic antimicrobial effects of mixtures of Ethiopian honeys and ginger powder extracts on standard and resistant clinical bacteria isolates. *Evid. Base. Compl. Alternative Med.* 2014; **2014**, 562804.
- [21] AT Khalil, I Khan, K Ahmad, YA Khan, M Khan and MJ Khan. Synergistic antibacterial effect of honey and *Herba Ocimi Basilici* against some bacterial pathogens. *J. Tradit. Chin. Med.* 2013; **33**, 810-4.
- [22] K Khosravi-Darani, R Khaksar, S Esmaeili, F Seyed-Reihani, A Zoghi and S Shahbazizadeh. Antifungal and anti-bacterial synergistic effects of mixture of honey and herbal extracts. *Zahedan J. Res. Med. Sci.* 2013; **15**, 30-3.
- [23] FI Akinnibosun and E Itedjere. Evaluation of the antibacterial properties and synergistic effect of *Garcinia kola* Heckel (Family: Guttiferae) seed extract and honey on some bacteria. *Afr. J. Microbiol. Res.* 2013; **7**, 174-80.
- [24] AL Noori, A Al-Ghamdi, MJ Ansari, Y Al-Attal and K Salom. Synergistic effects of honey and propolis toward drug multi-resistant *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans* isolates in single and polymicrobial cultures. *Int. J. Med. Sci.* 2012; **9**, 793.

- [25] J Dahanayake, P Perera, P Galappaththy and M Arawwawala. A mini review on therapeutic potentials of *Phyllanthus niruri* L. *Trends Phytochemical Res.* 2020; **4**, 101-8.
- [26] NY Lee, WK Khoo, MA Adnan, TP Mahalingam, AR Fernandez and K Jeevaratnam. The pharmacological potential of *Phyllanthus niruri*. *J. Pharm. Pharmacol.* 2016; **68**, 953-69.
- [27] D Rusmana, R Wahyudianingsih, M Elisabeth, B Balqis, M Maesaroh and W Widowati. Antioxidant activity of *Phyllanthus niruri* extract, rutin and quercetin. *Indonesian Biomed. J.* 2017; **9**, 84-90.
- [28] A Sood, R Kaur, J Kaur and S Kaur. *In vitro* and *in vivo* evaluation of antileishmanial activity of *Phyllanthus niruri* (Family: Euphorbiaceae). *Int. J. Trop. Dis. Health* 2016; **13**, 21450.
- [29] VA Ajibade, OA Ajenifuja, FT Akinruli, FA Ajayi and O Famurewa. Antifungal efficacy of saponin extracted from *Phyllanthus niruri*. *Int. J. Pathogen Res.* 2018; **1**, 44703.
- [30] K Ramandeep, A Nahid, C Neelabh and K Navneet. Phytochemical screening of *Phyllanthus niruri* collected from Kerala region and its antioxidant and antimicrobial potentials. *J. Pharmaceut. Sci. Res.* 2017; **9**, 1312.
- [31] OH Basir, NA Zaid, NAM Fauzi and AHA Razak. Characterisation of extracted *Phyllanthus niruri*: Total phenolic content, antioxidant and antibacterial activities. *J. Sustain. Nat. Res.* 2020; **1**, 32-6.
- [32] NH Sulaiman and NM Sarbon. Physicochemical, antioxidant and antimicrobial properties of selected Malaysian honey as treated at different temperature: A comparative study. *J. Apicult. Res.* 2022; **61**, 567-75.
- [33] NN Zakaria, NM Jaafar and AZ Mohamad. Antioxidant, antibacterial and anti-diabetic activities of stingless bee honey from selected areas in Peninsular Malaysia. *IOP Conf. Ser. Earth Env. Sci.* 2020; **596**, 012093.
- [34] D Ibrahim, LS Hong and N Kuppan. Antimicrobial activity of crude methanolic extract from *Phyllanthus niruri*. *Nat. Prod. Comm.* 2013; **8**, 493-6.
- [35] AL Noori, A Al-Ghamdi, MJ Ansari, Y Al-Attal and K Salom. Synergistic effects of honey and propolis toward drug multi-resistant *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans* isolates in single and polymicrobial cultures. *Int. J. Med. Sci.* 2012; **9**, 793.
- [36] SY Omar, NF Mat-Kamir and MA Sanny. Antibacterial activity of Malaysian produced stingless-bee honey on wound pathogens. *J. Sustain. Sci. Manag.* 2019; **14**, 67-79.
- [37] FN Rosli, MH Hazemi, MA Akbar, S Basir, H Kassim and H Bunawan. Stingless bee honey: Evaluating its antibacterial activity and bacterial diversity. *Insects* 2020; **11**, 500.
- [38] L Tuksitha, YL Chen, YL Chen, KY Wong and CC Peng. Antioxidant and antibacterial capacity of stingless bee honey from Borneo (Sarawak). *J. Asia Pac. Entomol.* 2018; **21**, 563-70.
- [39] NH Hasali, AI Zamri, MN Lani, A Mubarak, F Ahmad and TZ Chilek. Physico-chemical analysis and antibacterial activity of raw honey of stingless bee farmed in coastal areas in Kelantan and Terengganu. *Malays. Appl. Biol.* 2018; **47**, 145-51.
- [40] Z Breijyeh, B Jubeh and R Karaman. Resistance of gram-negative bacteria to current antibacterial agents and approaches to resolve it. *Molecules* 2020; **25**, 1340.
- [41] N Haque, MA Hossain, L Bilkis, AK Musa, C Mahamud, MS Bari, N Muhammad, US Parvin, MT Islam, SI Khan and MA Islam. Antibiotic susceptibility pattern of *Staphylococcus epidermidis*. *Mymensingh Med. J.* 2009; **18**, 142-7.
- [42] AE Namvar, S Bastarahang, N Abbasi, GS Ghehi, S Farhadbakhtarian, P Arezi, M Hosseini, SZ Baravati, Z Jokar and SG Chermahin. Clinical characteristics of *Staphylococcus epidermidis*: A systematic review. *GMS Hyg. Infect. Contr.* 2014; **9**, Doc23.
- [43] NAM Ramlan, AS Zin, NF Safari, KW Chan and N Zawawi. Application of heating on the antioxidant and antibacterial properties of Malaysian and Australian stingless bee honey. *Antibiotics* 2021; **10**, 1365.
- [44] M Majkut, J Kwiecińska-Piróg, E Wszelaczyńska, J Pobereźny, E Gospodarek-Komkowska, K Wojtacki and T Barczak. Antimicrobial activity of heat-treated Polish honeys. *Food Chem.* 2021; **343**, 128561.
- [45] JM Sousa, ELD Souza, G Marques, B Meireles, ÂTDM Cordeiro, B Gullón, MM Pintado and M Magnani. Polyphenolic profile and antioxidant and antibacterial activities of monofloral honeys produced by Meliponini in the Brazilian semiarid region. *Food Res. Int.* 2016; **8**, 61-8.
- [46] G Cebrero, O Sanhueza, M Pezoa, ME Báez, J Martínez, M Báez and E Fuentes. Relationship among the minor constituents, antibacterial activity and geographical origin of honey: A multifactor perspective. *Food Chem.* 2020; **315**, 126296.
- [47] I Eseriche, M Kadar, M Juan-Borrás and E Domenech. Suitability of antioxidant capacity, flavonoids and phenolic acids for floral authentication of honey. Impact of industrial thermal treatment. *Food Chem.* 2014; **142**, 135-43.

- [48] Y Elamine, O Anjos, LM Estevinho, B Lyoussi, S Aazza and MG Miguel. Effect of extreme heat processing on the Moroccan Zantaz' honey antioxidant activities. *J. Food Sci. Tech.* 2020; **57**, 3323-33.
- [49] AI Schepartz and MH Subers. The glucose oxidase of honey I. Purification and some general properties of the enzyme. *Biochim. Biophys. Acta Specialized Enzymological Subj.* 1964; **85**, 228-37.
- [50] KS Divya, AG Duth and JB Chauhan. A study on the phytochemical and antibacterial activity of *Phyllanthus niruri* against the isolates of poultry feeds. *J. Phcog. Phytochemistry* 2018; **7**, 910-6.
- [51] CO Okoli, AC Ezike, PA Akah, SO Udegbonam, TC Okoye, TP Mbanu and E Ugwu. Studies on wound healing and antiulcer activities of extract of aerial parts of *Phyllanthus niruri* L. (Euphorbiaceae). *Am. J. Pharmacol. Toxicol.* 2009; **4**, 118-26.
- [52] NS Roslan, NS Rahim, Z Razali and MA Zulkifli. *Anti-microbial properties and toxicity test of stingless bee honey (Trigona itama), Ajwa date (Phoenix dactylifera L.) seeds and their combinations.* In: N Alias and R Yusof (Eds.). *Charting the sustainable future of ASEAN in science and technology.* Springer, Singapore, 2020.
- [53] B Andualem. Combined antibacterial activity of stingless bee (*Apis mellipodae*) honey and garlic (*Allium sativum*) extracts against standard and clinical pathogenic bacteria. *Asian Pac. J. Trop. Biomed.* 2013; **3**, 725-31.
- [54] RV Patel, VT Thaker and VK Patel. Antimicrobial activity of ginger and honey on isolates of extracted carious teeth during orthodontic treatment. *Asian Pac. J. Trop. Biomed.* 2011; **1**, S58-S61.