# ELUCIDATING THE PHYSICOCHEMICAL PROPERTIES OF STINGLESS BEE HONEY AS CRYOPROTECTANT AND THERMOREGULATED CONSTITUENTS

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***ABSTRACT:*** Stingless bee honey had been studied for their unique properties and their potential in medical aspects. In this study, the physicochemical properties of the stingless bee honey of *Heterotrigona itama* and *Geniotrigona thoracica* were investigated for their potential as cryoprotectant. The water content of the stingless bee honey was determined through water removal process where *G. thoracica* and *H. itama* contained 26.8% and 29.68% of water, respectively. The freezing point and properties of the stingless bee honey under different temperatures between 0ºC, -20ºC and -80ºC were studied and the results showed that *G. thoracica* stingless bee honey had a slower freezing rate compared to *H .itama.* Thawing experiment of the stingless bee honey was conducted at room temperature in order to find the rate of thawing for the stingless bee honey. It was found that the melting rate of *H. itama* was slower compared to *G. thoracica*. Image analysis was done in order to analyze the freezing and melting rate of the stingless bee honey from *G. thoracica* and *H. itama*. DSC analysis thermograms of pure *G. thoracica* and pure *H. itama* stingless bee honey showed that the $T_g$ value for *G. thoracica* was 36.64ºC and *H. itama* was 58.06ºC, while the $T_m$ value of *G. thoracica* was 122.33ºC and *H. itama* was 153.27ºC. Finally, the $T_c$ value for *G. thoracica* was 89ºC, while for *H. itama*, the value was 129.47ºC. Our study has shown that these stingless bee honey contained high water content and low $T_g$ value. Stingless bee honey's amorphous solid physicochemistry merit for study and should be further elucidated for its potential in cells and macromolecules such as mRNA and DNA protection and preservation under low temperature and extreme conditions.

KEY WORDS: Stingless bee honey, Stingless bee, *Heterotrigona itama*, *Geniotrigona thoracica*, cryoprotectant, DSC Analysis.

## 1. INTRODUCTION

Researchers have explored the best method of preserving cells, organs, and biological components in order to find the best method of preserving the cells, and thanks to chemical cryoprotectants, a method of preserving biological components at low temperature for a long period of time has been discovered [1], [2], [3], [4]. Cryopreservation, or the method of maintaining biological components by storing them at a very low temperature, was often

employed at temperatures ranging from -20°C to -80°C to store cells for a long time and ensure their stability [5], [6], [7].

The mRNA vaccine for Covid-19 contains messenger RNA (mRNA) that instruct the cytoplasm in the cells to produce specific proteins required in the immunomodulation to train the body immune system to fight off the virus infection. However, mRNA has short life of a few hours and vulnerable to insult from stresses and oxidation from the chemicals in cryoprotectants such as glycerol, dimethyl siloxane and others. While thermostable mRNA vaccine development was still at infancy, cryofreezing at a temperature less than -20 to -80ºC was the most commonly used methods for long term storage and stability of the vaccines. The changes in mRNA can have detrimental impact on cellular function and may impair the effectiveness of the vaccine. Safer and reliable cryoprotectant technology for mRNA vaccine was outmost important especially for our tropical weather and high humidity. Trehalose and sucrose are used as crystalisation inhibitor agent in protein based vaccine formulations to protect the molecules and cellular membrane from osmolytic pressure and heat shock during storage and transportation at temperature of 0-10ºC. There was a need for a protectant that is able to protect mRNA structural integrity and biological functions at regular room temperature and longer storage time.

Trehalulose was a unique newly found sugar compound from stingless bee with amorphous solid physicochemistry[1] and shorter molecular packing that was useful to mRNA protection and structural mobility, which stabilise partially folded protein against stresses. The protective properties of trehalulose are based on its intermolecular interactions in macrobiomolecules as a result of preferential exclusion with water and its hydroxyl group. In this study, we elucidated the potential of stingless bee honey from two species, *G. thoracica* and *H. itama* in cryopreservation and protection of cell membrane by examining the honeys‘ chemical structure and its relations when stored at different set of temperatures and timing. Trehalose and sucrose are two sugars that can be found in stingless honey that exhibit cryoprotectant qualities and are utilized as crystallization inhibitors in cell preservation to protect molecules and the cellular membrane from osmolytic pressure during low-temperature storage [1], [8].

Stingless bee honey comprises a few medicinal components, the most important of which are sugars, primarily fructose, glucose, and trehalulose, with enzymes, proteins, organic acid, minerals, pollen grains, waxes, and phytochemicals making up the minor components [5], [9]. Honey's composition and functional qualities vary based on the components brought in by the bees during the maturation process, and the honey's physicochemical properties should be investigated in order to evaluate the floral and quality of honey. The floral source, storage conditions, bee species, and geographical region all have an impact on the taste and quality of stingless bee honey [8], [10], [11]. Stingless bee honey contains amorphous solid physicochemistry that was useful for cell protection under low temperature and extreme conditions [1], [11]. Cryopreservation techniques are commonly utilized to store biological components like organs, cells, and vaccines. Cryopreservation necessitates the correct use of cryoprotectants to ensure cell stability, as some cryoprotectants can cause toxicity in warm temperatures and cell instability[12]. Because of our country's tropical climate, a more secure and reliable cryoprotectant method for cell storage was necessary. Since there was a need for a cryoprotectant to protect the structure of the cells and its biological function at normal room temperature and for a longer storage time, we have to study the potential substance that can act as cryoprotectant. In this study, we examined the potential of stingless bee honey and elucidate the chemical structure and its relations to cold temperature and thawing at ambient temperature to explore its potential as cryoprotectant.

# 2. MATERIALS AND METHOD

## 2.1. Collection of stingless bee honey

A stingless bee farm, Madu Kelulut D'Serendah Bullah Garden, Serendah, Selangor provided two samples of stingless bee honey from *G. thoracica* and *H. itama*. The stingless bee honey samples were collected in November 2022 and were kept at room temperature until the studies were carried out.

## 2.2. Water removal

Water was removed from stingless bee honey from *G. thoracica* and *H. itama* in order to determine the honey's total moisture content. First, an empty petri dish was weighed and the weight was recorded. After that, 9mL of stingless bee honey from both species were placed onto two petri plates, weighed, and stored in an incubator. To avoid honey degradation, the stingless bee honey was dried in an incubator at 40°C, and the weight of the samples was monitored every 24 hours to assess the percentage of water loss in both samples. The proportion of water loss represents the amount of water available in both *G. thoracica* and *H. itama,* as determined by the formula below.

$$Percentage\ water\ loss = \frac{(W_{h0}-W_p)-(W_{h1}-W_p)}{(W_{h0}-W_p)} \times 100 \quad (1)$$

## 2.3 Freezing and thawing

The stingless bee honey was initially diluted with distilled water into five different concentrations: 5, 10, 15, and 20%. Distilled water was also utilized as a reference because it has a freezing point of 0°C and a boiling point of 100°C. Pure stingless bee honey was frozen to study the honey's behavior at three different temperatures of 0°C, -20°C, and -80°C. Image analysis was used to observe ice formation in the samples, with the size of the ice formation being measured every hour.

After frozen, the stingless bee honey was thawed at normal temperature. The rate of thawing was measured. The thawing rate was observed every 3 minutes and analyzed through image analysis by using the “ImageJ” application for the measurement of aspect ratio.

# 3. RESULTS

## 3.1 Water removal

In this study, the stingless bee honey extracted from *G. thoracica* and *H. itama* were examined on their physicochemical properties to be used as cryoprotectant. The water content of the stingless bee honey was very high at 30%. High water content supports the growth of bacteria and other microorganisms which may not be suitable for cryoprotection while forming ice crystalisation that may implicate with protein denaturation and osmotic lysis.

Fig. 1 shows that stingless bee honey from *H.itama* lost water more quickly than *G. thoracica.* As the experiment progresses, the rate of water loss for both species decreased until it becomes constant. This was due to the differences in sugar component in the stingless

bee honey. Sugars interact with polar groups to stabilize them at lower water concentration as water content decreases, sugar was thought to take the place of the hydrogen bond that was normally formed by water to avoid dessication.

The proportion of water loss for both stingless bee honeys was detected when the water content of *G. thoracica* and *H. itama* was compared. *G. thoracica* had a final percentage of water loss of 29.68%, while *H. itama* had a final percentage of water loss of 26.38%. This suggests that *H. itama* contained more water than *G. thoracica*. The floral source and how the stingless bees digest the honey had an impact on the high water content between the two stingless bees honey.

It was critical to understand the water content of stingless bee honey because of crystallinity influences in osmolysis and protein denaturation. The differences in water content for *G. thoracica* and *H. itama* was determined by heating experiment in an oven at temperature of 90ºC. Because of the high water content (>25%) for both stingless bee honey, it is important that these two honey need to be dried prior utilisation for experimentation as cryoprotectant.

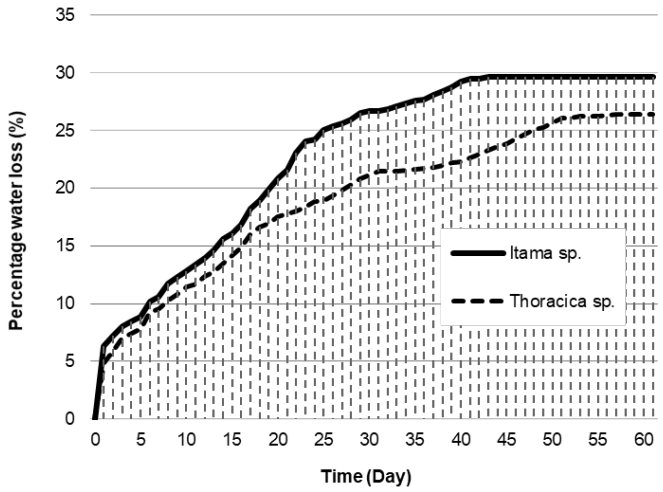


Fig.1. The percentage of water loss in stingless bee honey of *G. thoracica* and *H. itama* over 60 days (n=3)

### 3.2 Freezing

The freezing rate of 100% stingless bee honey from *H. itama* and *G. thoracica* in 0ºC are shown in Fig. 2, where it was obvious that the honey from *G. thoracica* was unable to solidify completely even after 432 hours. We then studied the freezing rate of these two honey species at different concentrations; 5, 10, 15, 20 and 100% at different temperatures of 0ºC, -20ºC and -80ºC to find the optimum freezing conditions for these honey. Fig. 3 a and b show the rate of ice formation from these two stingless bee honey species in different concentrations at 0ºC, where the measurement of the size of ice formed were measured using ImageJ application to determine the percentage of ice formation.

At -20ºC, the freezing rate of 100%. stingless bee honey from *H. itama* and *G. thoracica* differed strikingly at 220 and 450 minutes to be completely frozen (Fig. 4). Fig. 5 a and b show the rate of ice formation from these two stingless bee honey species in different concentrations at -20ºC.

Distilled water stored in -80ºC, completely frozen after 4 minutes which was the fastest compared to the honey which had been stored at -20ºC and 0ºC (Fig. 6). For *H. itama* the concentration of 5, 10, 15 and 20% were completely frozen after 8 minutes, while the honey from *G. thoracica* was completely frozen after 50 minutes (Fig. 7 a and b).

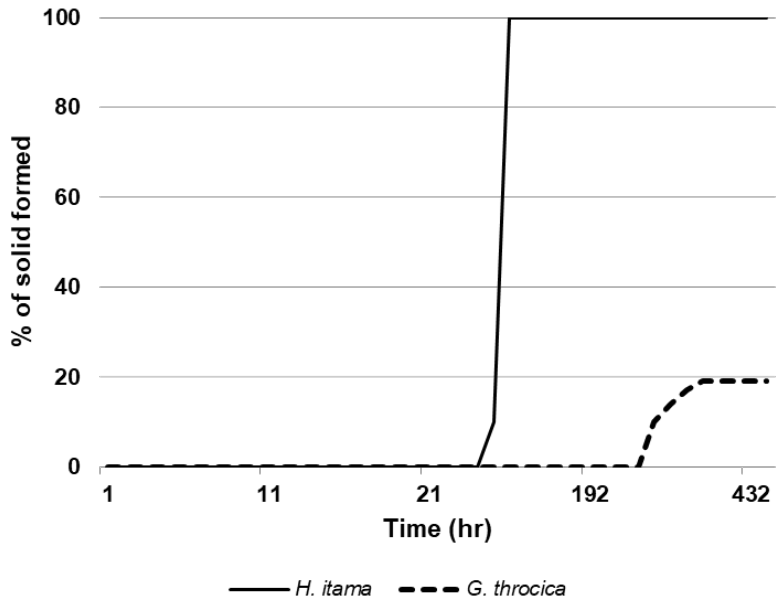


Fig. 2. The percentage of ice formed in pure (100*%) H. itama* and *G. thoracica* which kept at 0ºC up to 432 hours. (n=3)

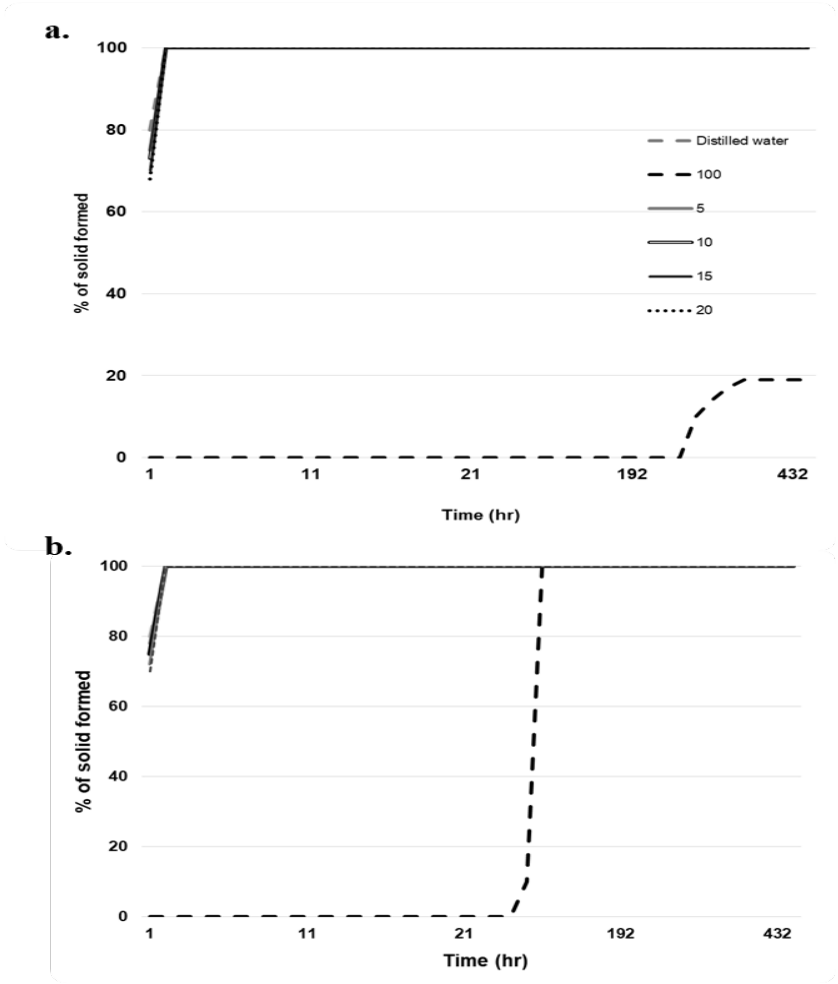


Fig. 3. The percentage of ice formed in a. *H.itama* and b.*G. thoracica* honey with different concentration (from 5-100) % and distilled water as control over time when kept in at 0ºC up to 430 hours. (n=3)

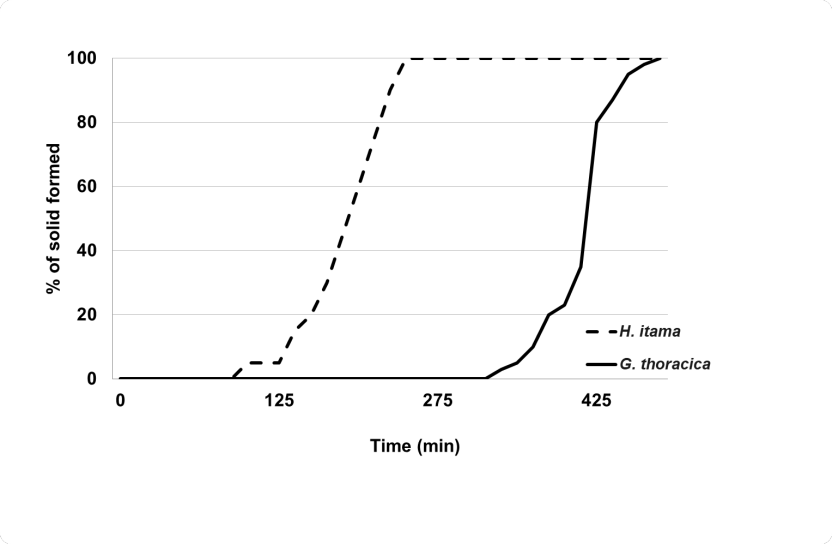


Fig. 4. The percentage of ice formed in pure (100%) *Heterotrigona itama* and *Geniotrigona thoracica* which kept at -20ºC up to 400 mins. (n=3)

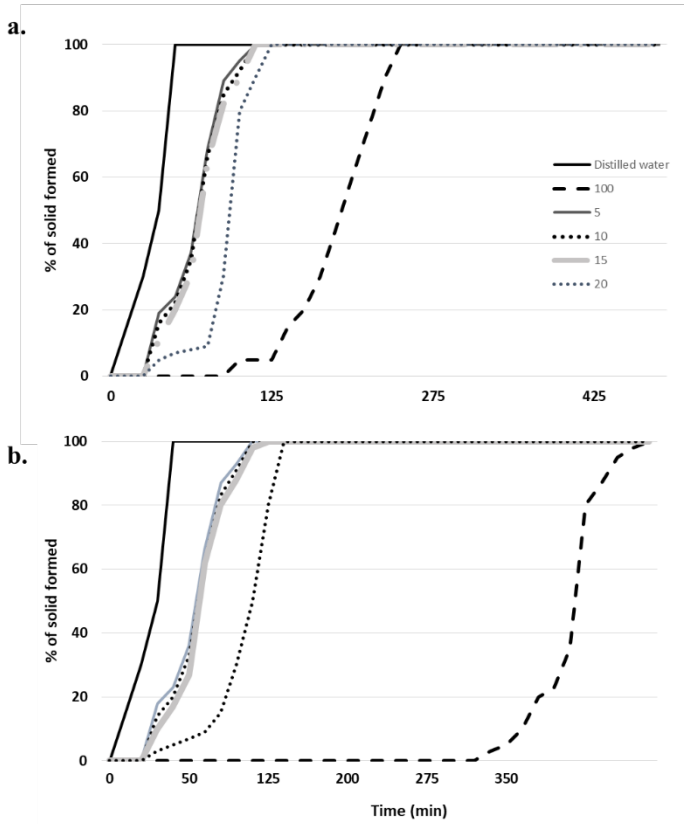


Fig. 5. The percentage of ice formed i*n* a *H.itama* and b. *G. Thoracica* honey with different concentration (from 5-100) % and distilled water as control over time when kept in -20ºC up to 430 mins. (n=3)

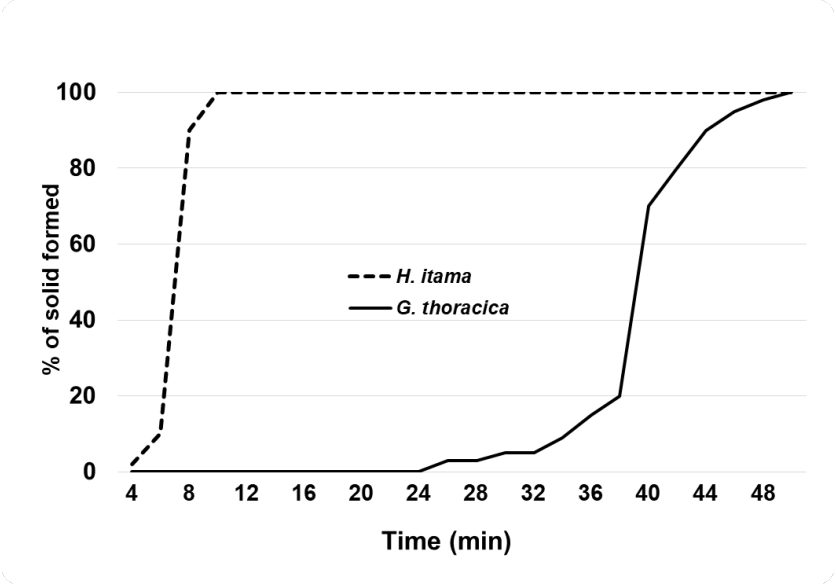


Fig. 6. The percentage of ice formed in pure (100%) *H. itama* and *G. thoracica* which kept at -80ºC up to 40 mins. (n=3)

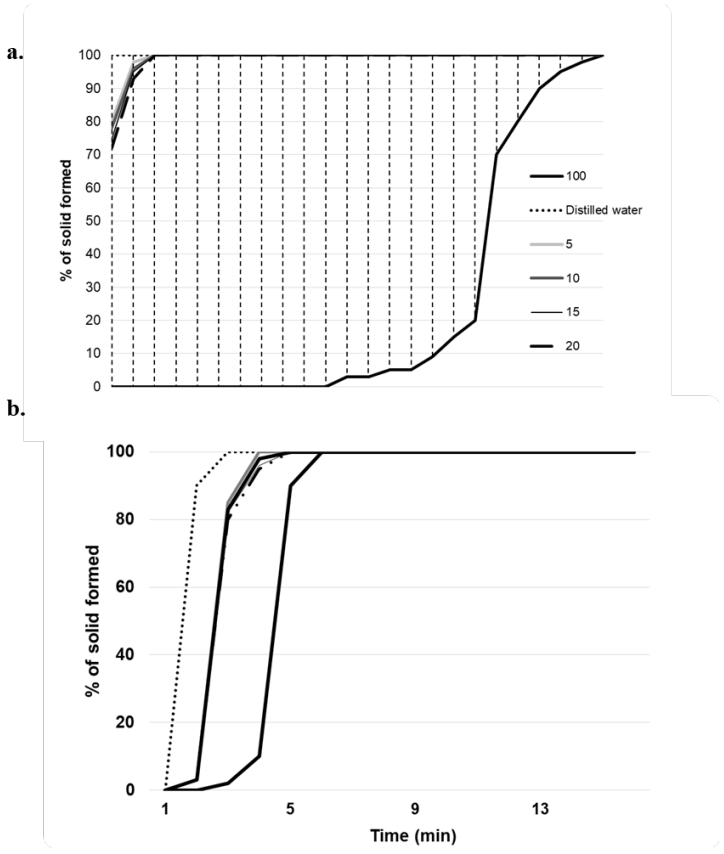


Fig. 7. The percentage of ice formed in a. *H. itama* and *G. thoracica* honey with different concentration (from 5-100) % and distilled water as control over time when kept in -80ºC up to 13 mins. (n=3)

### 3.3 Thawing

In a thawing experiment, the melting rate of stingless bee honey from *H. itama* and *G. thoracica* which was kept in 0, -20, and -80°C was examined when thawed at room temperature. The goal of the experiment was to determine the melting rate of stingless bee honey in various concentrations after being stored at low temperatures.

Fig. 8 a and b show the melting rate of stingless bee honey from *H. itama* and *G. thoracica* stored at 0ºC and the reduction of the size of the frozen honey was measured using ImageJ to determine the aspect ratio. The melting rate for 100% honey could not be recorded because of its inability to freeze, compared to other samples which were mixed with water. The 20% concentration was completely thawed after 16 minutes while the distilled water completely thawed after 26 minutes. For *G. thoracica*, the stingless bee honey with the concentration of 20%, was completely thawed after 12 minutes which was faster compared to 20% of *H. itama*, while the pure honey thawed completely after 4 minutes.

Referring to Fig. 9 a and b, the stingless bee honey samples were stored under -20ºC in different concentrations before being thawed. The distilled water was completely thawed after 18 minutes at room temperature while for pure *H. itama*, the ice started to melt after 2 minutes and was completely thawed after 6 minutes. For the samples of 5%, 10% and 15% of *H. itama*, they showed a decreasing pattern of time taken to be completely thawed as the honey concentration was lowered.

*G. thoracica* honey that was kept in -20ºC, began to melt after 2 minutes. The 100% concentration of this sample was completely thawed by 6 minutes. The same observation was seen in *H. itama* honey. The stingless bee honey was kept in -80ºC before being thawed and the result was shown in Fig. 10 a and b. For distilled water stored in -80ºC, it was completely thawed after 16 minutes, while for *G. thoracica* the 20% concentration was completely thawed after 14 minutes. The 20% of *H. itama* took 10 minutes to be thawed. Comparing the data from the thawing experiment of 100% *G. thoracica* and *H. itama*, the melting rate oft he latter was slower compared to *G. thoracica.*

On the other hand, comparing the freezing and thawing data, it was observed that the honey concentration affects the freezing and melting point of the solutions and this was due to the impurities concentration in the samples. Since the stingless bee honey is composed of more than one material, the time taken for the solutions to freeze was longer while the time taken for the solutions to melt will be faster as the amount of impurities in the solution increases. Therefore, it can be suggested from this experiment that the stingless bee honey of *G.thoracica* contains more impurities compared to *H. itama*. Moreover, when the samples were frozen, the attraction that holds the particles together was strong and even though these particles vibrate, the energy produced was not enough to disrupt the structure. However, when the frozen samples were left to thaw at room temperature, the particles gained energy, and the vibration of the particles would increase and the sample turned to liquid form.

The stingless bee honey kept at three different temperatures, which were 0ºC, -20ºC and -80ºC before the thawing experiment show different melting rate from each other sample and concentration. At lower storage temperature, the time taken for ice to be formed in the stingless bee honey was faster as the freezing rate was affected by the temperature of the surroundings. However, as observed from Fig. 8, 9 and 10, the 100% concentration stingless bee honey from *G. thoracica* that were stored at different temperatures were fully thawed at the same range of time regardless of the difference in the storage temperature during freezing. The same occurrence was also observed for pure stingless bee honey from *H.*

*itama.* The thawing of the stingless bee honey was not affected by the storage temperature during freezing, but they were affected by the temperature of the surrounding which the samples were exposed to during thawing.

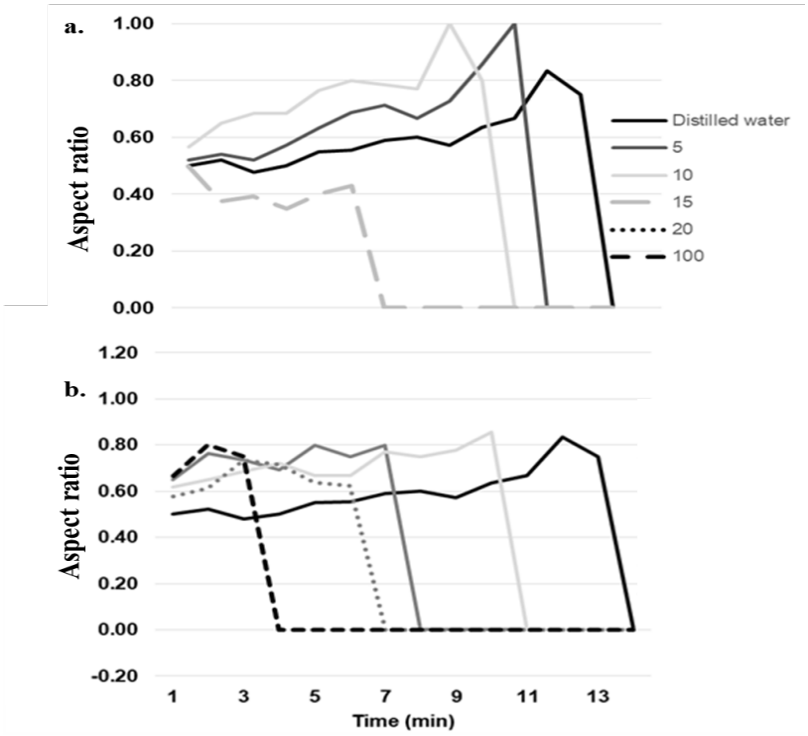


Fig. 8. The aspect ratio of a. *H.itama* and b. *G. thoracica* at different concentrations and distilled water stored at $0^0$C over time.

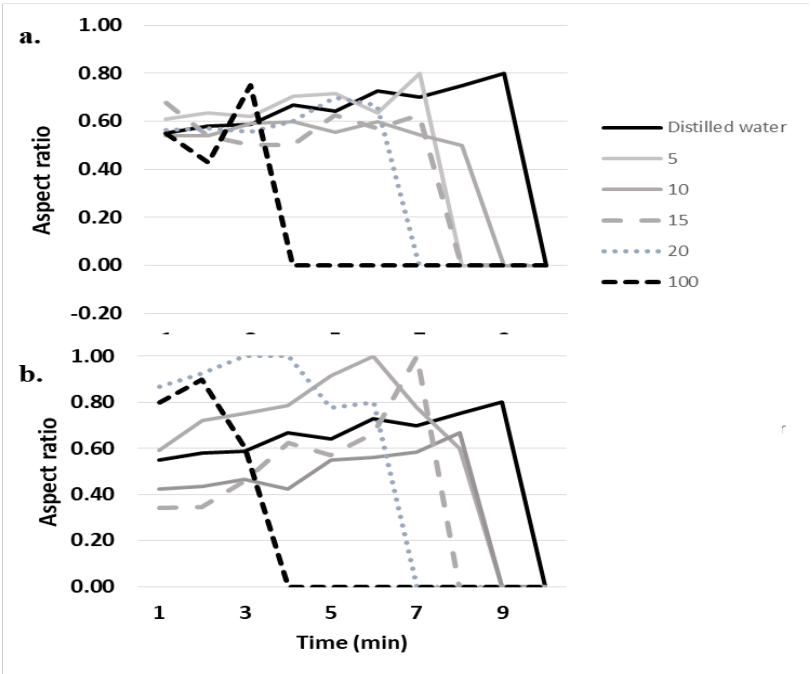


Fig. 9. The aspect ratio of a. *H.itama* and b *G. thoracica* at different concenttions and distilled water stored at -$20^0$C over time.

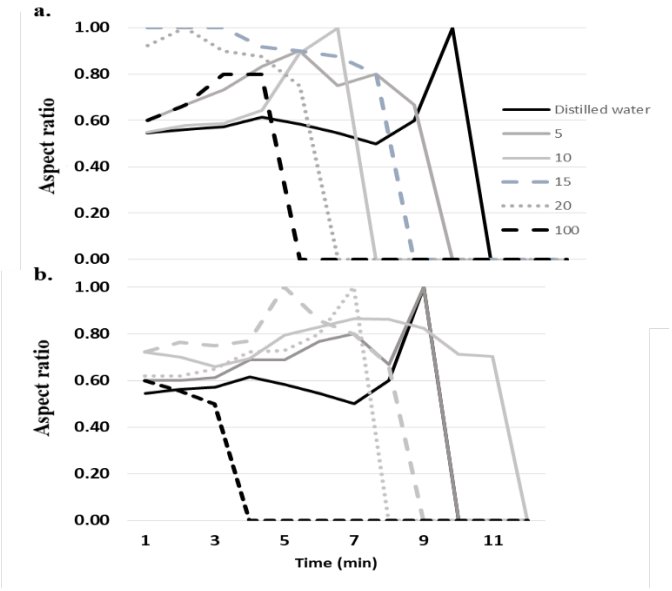


Fig. 10. The aspect ratio of a. *H.itama* and b *G. thoracica* at different concenttions and distilled water stored at -80$^0$C over time.

### 3.4 DSC Analysis

The DSC analysis was was performed to gain a better knowledge of the structural behavior of stingless bee honey as well as their thermal behavior. when subjected to temperatures ranging from -200 to 300ºC. The data gained through DSC analysis was more precise, and complements the data from water removal, freezing, and thawing of stingless bee honey. By analyzing the DSC thermogram of the stingless bee honey, the value of glass transition temperature ($T_g$), melting temperature ($T_m$) and crystallization temperature ($T_c$) of the stingless bee honey will be obtained from the peaks of the DSC thermograms.

The thermogram obtained from the DSC analysis of the stingless bee honey from *G. thoracica* and *H. itama* are shown in Fig. 11. The value of the Tg, Tm, and Tc of the stingless bee honey was determined from the peaks in the thermogram. The heating temperature range from -30ºC to 200ºC, where we could observe the molecular structure of the stingless bee honey changes as the temperature gave energy to the molecules to vibrate and break their molecular bond.

Fig. 11 shows that both stingless bee honeys had a "V-form" with a pronounced start at low temperatures, declining to a large minimum, and then climbing back up to higher values. Exothermic phenomena were seen in both honey samples. *G. thoracica* had a minimum onset temperature of 107.130°C, while *H. itama* had a minimum onset temperature of 132.870°C, with an end set temperature of 131.020°C for *H. itama* and 168.840°C for *G. thoracica*. The enthalpy values for *G. thoracica* and *H. itama* were 542.77J/g and -650.22J/g, respectively. The melting points of *G. thoracica* and *H. itama* were 122.33°C and 153.27°C, respectively. This may explain the thawing experiment where *H. itama* took longer to melt than *G. thoracica*. The crystallization temperature for *H. itama* and *G. thoracica were* 129.47°C, and 89°C, repectively whereas glass transition temperature, $T_g$ for *G. thoracica* was 36.64ºC and *H. itama* was 58.06ºC. The glass transition temperature is the time where the mechanical properties of the honey shift from elastic to brittle due to

changes in the mobility chain in the molecular structure. Once the $T_g$ chains obtained enough energy from the increasing of the temperature, the samples would undergo crystallization which was an exothermic process and required more energy.

By integrating the area under the peak and the transition temperature yields, the integral was determined. For *G. thoracica*, the integral was -12.08e$^3$mJ while *H. itama* have the integral value of -17.39e$^3$mJ. The onset value for *G. thoracica* was 107.13ºC, while for *H. itama* the onset value was 132.87ºC. The peak value of *G. thoracica* and *H. itama* were 115.04ºC and 146.02ºC, respectively. The endset value *of H. itama* and *G. thoracica* were 168.84ºC and 131.02ºC, respectively.

The DSC analysis values are complementary to the results of the preceding studies, which included water removal, freezing and thawing, as well as melting experiment. The melting rate of the stingless bee honey for *G. thoracica* was faster than that of *H. itama*, as evidenced by the DSC study.

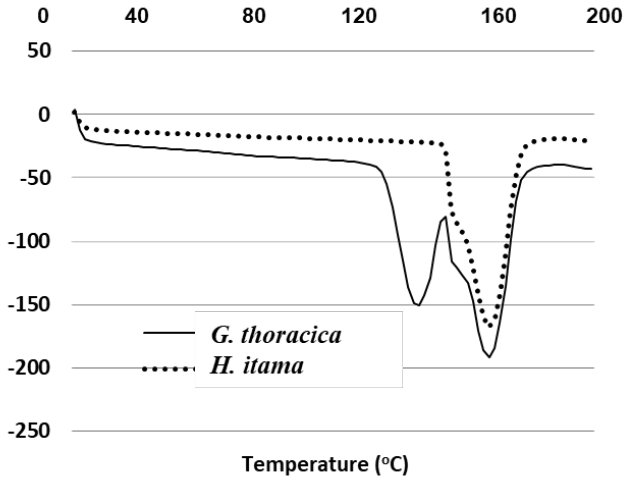


Fig. 11. The DSC Thermograms of pure *H. itama* and pure *G. thoracica* stingless bee honey

## 4. CONCLUSION

Cryopreservation was a process of preserving biological components to a very low temperature for long-term preservation with the help of cryoprotectants. However, to most living organism, freezing was lethal since intra- and extracellular ice crystals would be formed and cause cryoinjury in the cells and high concentration of cryoprotectants might cause toxicity to the cells. The study of stingless bee honey as cryoprotectant had never been done before and in order for us to study its properties as cryoprotectant, it is important that we study the physicochemical properties of the stingless bee honey.

The potential of the stingless bee honey lies in the cryoprotective behavior, the value of glass transition temperature ($T_g$), crystallization temperature ($T_c$) and melting temperature ($T_m$). The value of the glass transition temperature ($T_g$) affect the degree of crystallization and can be influenced by the presence of sugar in the honey. As the amount of water in the

sample increases, the value of the $T_g$ was lower. By determining the value of $T_c$, we can avoid intracellular crystallization and reduce the size of crystals formed and the rate of extracellular ice propagation.

## ACKNOWLEDGEMENT

We would like to thank Madu Kelulut D'Serendah Bullah Garden, Serendah, Selangor for the stingless bee honey samples from *G. thoracica* and *H. itama.*

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