DEVELOPMENT OF PROPOLIS POWDER FOR ENCAPSULATION VIA FREEZE DRYING METHOD : PURIFICATION AND FREEZE DRYING PROCESS OF EXTRACTED PROPOLIS

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ABSTRAK

Madu kelulut yang juga dikenali sebagai getah lebah merupakan sejenis resin yang diperolehi daripada sarang lebah pelbagai spesis berdasarkan kedudukan geografi lebah tersebut. Madu kelulut mempunyai pelbagai kebaikan termasuklah, antioksidan, antimikrobial, anti-diabetik dan anti-radang. Di Malaysia, terdapat sebanyak 32 jenis lebah tidak bersengat dan daripada itu spesis *Trigona Thoracica* merupakan spesis yang paling banyak terdapat di kebanyakan kawasan di Malaysia. Namun begitu, madu kelulut yang diperoleh daripada spesis *Trigona Thoracica* tersebut mempunyai satu kelemahan yang besar, di mana madu kelulut itu menjadi sangat melekit apabila digunakan pada suhu bilik. Bagi mengatasi masalah tersebut, proses pengeringan pembekuan dijalankan. Dalam kajian ini, madu kelulut telah diekstrak melalui proses purifikasi bagi memperoleh cecair madu kelulut yang asli. Cecair madu kelulut yang telah dipurifikasi telah dibekukan pada lima suhu pembekuan yang berbeza iaitu -40 °C, -50 °C, -60 °C, -70 °C dan -80 °C selama 48 jam. Kesemua sampel yang telah dibekukan kemudiannya melalui proses pengeringan pembekuan bagi menyingkirkan air dan juga bahan cecair yang lain untuk jangka masa 48 jam bagi memperoleh serbuk madu kelulut yang asli. Proses pengeringan pembekuan dikawal oleh dua parameter iaitu suhu dan juga tekanan. Serbuk madu kelulut yang diperoleh itu diuji mengunakan teknik sudut istirehat bagi memperoleh tahap keboleh aliran dan juga jumlah kehilangan kelembapan. Hasil kajian menunjukkan bahawa serbuk madu kelulut yang dibekukan pada suhu -80 °C mempunyai jumlah kehilangan kelembapan yang tertinggi dan juga tahap keboleh aliran yang cemerlang untuk digunakan dalam proses pengkapsulan.

ABSTRACT

Propolis or also known as the bee glue is a type of resin which is obtained from bee hives of various bees based on their geographical topography. The propolis is known for its various benefits such as antioxidant, antimicrobial, anti-diabetic and anti-inflammatory. In Malaysia, there are 32 types of stingless be with *Trigona Thoracica* being mostly found bee species at various parts of the country. However, propolis obtained from the *Trigona Thoracica* species has a major drawback where it is found to be sticky in normal temperature condition. To address the problem, freeze drying process is carried out. In this study, the water extracted propolis was purified to obtain pure propolis liquid. The purified propolis liquid was then freezed at five different freezing temperatures which were -40 °C, -50 °C, -60 °C, -70 °C and -80 °C for 48 hours period. The freezed propolis samples were then freezed dried to remove to freezed water and liquid substance for 48 hours period to obtain pure propolis powder. The process was controlled by two parameters, temperature and pressure. The obtained powder was then accessed by angle of repose method to determine the flowability and moisture content of the powder samples. Results indicated that the propolis powder sample of -80 °C freezing temperature to have the maximum loss in moisture content and the excellent powder flow flowability to be used for encapsulation.

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LIST OF SYMBOLS

g	Gram
%	Percentage
®	Registered trademark
°C	Degree centigrade or Celsius
µg mL^{-1}	microgram per milliliter
kPa	kilo Pascal
°	Degree
Θ	Theta
mL	Milliliter
mm	Millimeter
EC_{50}	Concentration of drug that gives half-maximal response
µm	Micrometer
mT	Millitorr
mg	Milligram

LIST OF ABBREVIATION

A.D.	Anno Domino
B.C.	Before Christ
DPPH	α,α-diphenyl-β-picrylhydrazyl
EEP	Ethanol extract of propolis
No	Number
USP	United States Pharmacopeia
WEP	Water extract of propolis
w/v	Weight per volume

CHAPTER 1

INTRODUCTION

1.1 Background Study

1.1.1 Propolis

Propolis or usually referred as bee glue is a type of resin. The propolis is collected from various species of bees based on the topography of the location where the bees originated. The bees use the propolis in construction and repair of their hives due to the stickiness nature of propolis. The United States Department of Agriculture described propolis as a gum that is gathered by the bees from various plants that may vary in color from light yellow to dark brown (United States Department of Agriculture, 1985). It also causes staining of the comb or frame and may be found in the extracted honey. The difference in the color is found to be due to the composition difference in the raw propolis. Although, the raw propolis content might vary as it changes with different location however, they are generally consist of five main materials which are 50% of resin and vegetable balsam, 30% of the wax, 10% of the essential and aromatic oils, 5% of the pollent and 5% of the other various substances such as the organic debris (Honary et al., 2011). The main nutritional compounds that can be found in the propolis are proteins (a maximum of 1 g/ 100 g), carbohydrates (a maximum of 1 g/ 100 g) and fat (a maximum of 1 g/ 100 g) (Bogdanov, 2016). Propolis is found to have lots of medicinal benefits for humans. Propolis is said to function as an anticancer, antibacterial, antiviral, antifungal, antioxidant and anti-diabetes properties (Król et al., 2013). All these health benefits are

subjected to certain types of propolis; for example, the anti-diabetes effect is only valid for *Poplar* and *Baccharis*-type of propolis (Bogdanov, 2016).

The usage of propolis started since 300 B.C. where it was usually used as home remedies and personal products (Ghisalberti, 1979). Since then, there is an increase in the usage of propolis in many fields such as pharmaceutical and food industry (Nori et al., 2011). The usage form is also differentiated as to aid the easiness among the users. There are various forms of propolis found in the market, from the fresh propolis to propolis in liquid and powder form. These various forms are to mask the weakness of the raw propolis as well as to aid the usage of the customers. The powder form of propolis is the famous type of propolis to be used in the manufacturing, considering the powder having a good stability. Various ways are being employed such as the spray and freeze drying to obtain the propolis powder. These drying processes produce a uniform size of the powder which will assist in the packaging of the powder such as tablet or capsule form.

1.1.2 Freeze Drying

Lyophilization or freeze drying process is one of the very popular method of producing powdered product or frozen items in food and pharmaceutical industry. It is a process where it involves removal of water or another solvent from a frozen pharmaceutical product (Labconco, 2010). This technique had been used for many years in both the industries. This technique is usually used to produce powder through rapid freezing and heating. Specialized equipments are involved in this technique to provide optimum temperature and pressure. The process could be divided into three parts which consist of freezing, primary drying and secondary drying. The freeze drying process consists of a clear process flow in which the water will be frozen first, continued by the removal of the water which has undergone the freezing process from the sample, initially be sublimation or know as the primary drying and then the secondary drying which is a desorption process (Nireesha et al., 2013). Freeze drying is regarded as the best method of water removal in order to obtain a high quality final product. This is due to the fact that the low temperature inhibits the microbiological activities of the product which provides an

excellent quality of powder. Besides that, this technique also protects the primary structure of the product as well as increases the shelf life of the product (Ratti, 2012). This criterion makes the freeze drying technique as the most suitable technique for production of pharmaceutical powdered based product.

1.1.3 Capsule

Capsule which the name is derived from Latin was invented early in the 19th century when there was a need to remove obnoxious taste present in most medicinal substances. This is due to the reason that it usually cause nausea among patients consuming it (Swarbrick, 1996). The invention of this dosage form is due to certain disadvantages found in the tablet dosage forms such as tablet manufacturing requires many detailed steps which are a burden to the pharmaceutical companies. Besides, those tablets dosage forms are hard to swallow by the children and elderly people (Kathpalia, Sharma, and Doshi, 2014). Based on the research conducted by the CAPSUGEL®, it is found that out of 750 consumers, almost 57% prefer capsules over tablets or any other oral dosage (CAPSUGEL, 2010). Capsules can be divided into two types which are soft-shelled capsule and hard-shelled capsules. These two types of capsules are used for different types of drug ingredients, dry powders for hard capsules and oil for soft capsules. Both capsules have the same general benefits such as easier product identification, fewer developmental problems and consuming easiness (Qureshi, 2007).

1.2 Problem Statement

Raw propolis is sticky and gluey in normal environment condition. This condition may due to the presence of other substances such as resin, vegetable balsam and other refined propolis extract (Honary et al., 2011). This physical instability is found to cause a problem in the manufacturing of the propolis capsule. The propolis powder is packed in capsules rather than tableting or performing other method due to that capsules are more stable and have an accurate dosing as well (Qureshi, 2007). The sticky gel condition is found to affect the capsule that is being filled. The capsules tend to become oxidized and soften easily due to the reaction between the propolis with the capsule wall. Besides that,

the process of encapsulation of the propolis is also found to be hard as the propolis does not have good flowability properties. Poor flowability causes most capsules to not be filled completely. This issue tends to affect the quality and credibility of the propolis capsules that is being manufactured.

1.3 Research Objective

The group research objective of the project would be to develop the propolis powder for encapsulation by freeze drying method.

Meanwhile the individual research objective precising to the topic would be :

1. To optimize the freeze drying process condition by varying freezing temperature to obtain propolis powder.
2. To conduct flowability test on the propolis powder to determine the best powder condition for encapsulation.

1.4 Scope of Study

In this study, propolis extract is converted into powder through freeze drying process. The propolis bee hive was taken from the bee species of *Trigona Thoracica*. Prior to the freeze drying process, the extracted propolis is purified using filtration process to obtain pure propolis extract. The freeze drying process consists of three main steps which are the freezing, primary drying and secondary drying. The freeze drying process is selected because it is the only process that protects the primary structure of the product as well as increases the shelf life of the product (Ratti, 2012). The freezing temperatures are varied in order to obtain the suitable temperature that will produce a good yield of powder which could give good flowability properties.

CHAPTER 2

LITERATURE REVIEW

2.1 *Trigona Thoracica sp.*

Trigona (Geniotrigona) Thoracica is the subgenus of the Meliponini Tribe which originates from the Indo-Malayan group of bees (Michener, 2000). *Trigona Thoracica* is among the 32 species of stingless bee which have been documented in Malaysia (Mohd et al., 2010). This species is known as the pollination agent for fruit-based flowers. *Trigona Thoracica* is known to build its hive on the brunch or stump. The nest of this species occupies the cavity and the hive is constructed in layer based form as shown in Figure 2.1. It consist of stringed bags with propolis have been formed on the floor and wall parts of the hive. This multi-layered condition is favored by most breeders as the amount of the honey and propolis yield is higher than the normal layered condition (Ibrahim et al., 2016).

(a) (b)

Figure 2.1 *Trigona Thoracica sp.* (a) outer hive and (b) inner hive condition (Kelly et al., 2014)

Trigona Thoracica species is considered to be one of the unique species of bees compared to the other trigona bees. This is due to its body structure which is bigger than the other trigona type bees. *Trigona Thoracica* is easily identified and observed due to its brownish-black body with a brown thorax. The distinctive characteristic makes it to be easily identified as can be observed in Figure 2.2. Looking back on the history of these species, it was first discovered by Smith on 1857 and was named as *Heterotrigona* which was later changed to *Trigona Lacteifasciata Cameron* on 1902 by Cameron. There are also other names for these species such as *Trigona Ambusta* which was named by Cockerell in 1918 and *Trigona Borneensis* by Friese in 1933 due to discovery of *Trigona Thoracica* in Kalimantan seas (Roubik and Aluja, 2012).

(a) (b)

Figure 2.2 *Trigona Thoracica* sp. (a) aerial view and (b) side view (Michener, 2000)

2.2 Propolis

Propolis is a sub-product produced by the honeybee which is known for its biological as well as pharmacological properties. Research and studies had been conducted for centuries to identify its main properties and function but the researchers could still not find any kind of specific function (Bogdanov, 2016). Although many people are still unaware of the existence of propolis, however, there have been researchers that date far back to the 23 A.D. Propolis was first discovered in the ancient Egypt, where it was mostly used as an adhesive (Gesneri, 1558). Besides that, the Greeks used propolis as the main ingredient of an exquisite perfume called “polyanthus”, where it consists of a mixture of

propolis, olibanum styrax and aromatic herbs. As a matter of fact, the propolis name is said to be originated from the Greek, where “pro” indicates in front and “polis” indicates city. When combined it gives a meaning of ‘in front of the city which implies its function for the bees (Ghisalberti, 1979). Greek, as well as the Roman physicians, was the first one to use the propolis for medicinal values. They used it as a mouth disinfectant and also used it for antiseptic as well as healing products in wound treatment (Bankova, De Castro, and Marcucci, 2000). Propolis started to become very popular in Europe in between 17th and 20th century especially during the World War II (1939-1945), where the doctors used the propolis to treat wounds (Burdock, 1998). However, the first scientific work with propolis was only published in 1908 in which it included the chemical properties as well as the composition (Wagh, 2013).

2.2.1 Composition and Types of Propolis

Propolis is majorly found and used around the world for different aspect and function. However, there are no specific types of propolis that are present. The propolis constituents and types are based on the different geographical origin from where the propolis are being extracted (Wagh, 2013). The difference is due to the type of plants available at the countries as well as the temperature difference between countries. These differences affect the composition of the propolis making it to be functional in various aspects such as antimicrobial, anti-fungal, anti-inflammatory and anti-diabetic. The most common type of propolis which can be found in countries like China, Korea, Croatia and New Zealand is the poplar type propolis which originates from the Poplar tree species such as *Populus nigra L.* and *Populus alba L.*. The various types of propolis and its origin are listed in Table 2.1.

Although there are various types of propolis found throughout the globe, however, all the raw propolis has a fixed composition. These compositions are the standard in all the raw propolis which has been extracted. The composition, as well as the origin and source, are responsible for the characteristics of the propolis.

Table 2.1 Propolis distribution around the world and its main bioactive compound

No.	Geographic Origin	Plant Source	Main Bioactive Compound
1	Europe, North America and nontropic regions of Asia	*Populus spp.*	Polyphenols
2	Russia	*Betula verrucosa*.	Polyphenols
3	Brazil	*Baccharis spp.*	Prenylated p-coumaric acids, diterpenic acids
4	Cuba, Venezuela	*Chusia spp.*	Polyprenylated benzophenones
5	Pacific region (Okinawa, Taiwan)	Unknown	C-prenylflavanones Furofuran lignans
6	Canary Islands	Unknown	Furofuran lignans
7	Kenya	Unknown	Polyphenols
8	Greece and Cyprus	Unknown	Flavonoids, terpenes

Source: (Wagh, 2013)

2.2.2 Characteristics of Propolis

Propolis is a sticky material which is mixed by the honeybees to be utilized to protect their hive. Besides being sticky, there are various characteristics of propolis. Propolis has a melting point between 25°C to 45°C where the propolis tends to become soft, pliable and sticky. On the other hand, propolis becomes liquid at a temperature of 60°C to 70°C (Wagh, 2013). However, if the propolis is in the frozen condition, it will become brittle and hard and any high temperature treatment will not have any effect on the condition of the propolis. The temperatures are important as it ensures that the component and composition of the propolis are in optimum desirable state. Besides the melting point, the solubility of the propolis is one of the major factors which have a direct relation with the function of the propolis. The raw propolis consists of many complex structures making it difficult to be used straight away. The propolis usually undergoes extraction process in order to obtain the pure propolis. The type of solvent selected will affect the solubility of

the components present in the raw propolis as the bactericidal components are soluble in water and alcohol (Wagh, 2013). This solubility factor will affect the quality of the extracted propolis.

2.2.3 Extraction of Propolis

Raw propolis will undergo the extraction process. There are various types of solvent used to extract the propolis which includes vegetable oil (Tosi, et al., 1996), ethanol and also water (Park and Ikegaki, 1998). Among the extraction method available, the common solvent that is usually used would be ethanol as the constituents that is found in the propolis such as the benzoic acid and cinnamic acid derivatives are lipophilic compounds (Bankova, De Castro, and Marcucci, 2000). These active ingredients are easily soluble in ethanol (Krell, 1996). However the ethanol extract of propolis (EEP) has a major drawback as it posses immunological properties which could have serious effect to the animals and patients (Scheller et al., 1990). Another major issue is the Halal issue. Ethanol is regarded as the Khamr which signifies alcoholic beverage from fermented fruits or other items. Existance of this content of more than 15% (w/v) is strongly prohibited and are marked as non-halal (Alzeer and Abou Hadeed, 2016). These issues are the main reason why many pharmaceutical and food based industry prefers the water extract of propolis (WEP). WEP method is known to have a greater antioxidative effects, greater inhibitory activity against some enzyme and possesses greater absorbency when compared to the EEP (Matsui et al., 2004). The extraction process is conducted based on 1:2 proportion of propolis and water. This ratio is found to be the best for the extraction of propolis (Sosnowski, 1981).

2.2.4 Antioxidant Studies on Propolis

Antioxidant activity is one of the functions and constituents of the propolis. Antioxidant is defined as any substance which present at low concentration when compared with that of an oxidizable substrate, will significantly delay or inhibit oxidation of that

substrate (Halliwell and Gutteridge, 1995). The oxidation reaction that occurs under natural condition produces free radicals that can cause the start of multiple chain reactions that will cause damage or death to the propolis contents. The antioxidant property will ensure that the chain reaction is removed. The test is conducted in 1,1-diphenyl-2-picrylhydrazyl (DPPH) using the mechanism of free radical scavenging activity. The test is conducted in the dark at room temperature. The results were expressed by EC_{50} value which is known as the half maximal effective concentration that determines the extract concentration (μg mL^{-1}) by providing 50% inhibition (Cottica et al., 2011). The efficiency of the antioxidant is determined by the lowest value of the extract concentration.

2.3 Freeze Drying

2.3.1 History and Development

Air-drying is an ancient process which is usually used to preserve foods, where the material to be dried will be exposed to a continuously flowing hot stream of air, which causes all the moisture to evaporate. This process ensures the shelf life of the product to be extended by a year (Ratti, 2001). However, the quality of the dried product is found to be drastically reduced when compared to the original stuff. The decreased quality is due to the physical alterations, chemical reactions as well as biochemical effects. The changes are the one responsible for the damage to the microscopic structure of the materials. Constant modification in this process gave rise to the revolution of freeze drying or lyophilization process. Freeze drying is defined as a process involving a solvent and/or suspension medium to be crystallized at a low temperature than the triple point and thereafter sublimated from the solid state directly into the vapor phase (Liu, Zhao and Feng, 2008). Since its existence, freeze-drying has become one of the most important processes in preserving heat-sensitive biological matters (George and Datta, 2002). The development of this freeze drying process gives an advantage in the various field nowadays such as in food, pharmaceutical, cosmetic as well as biotechnological (Ratti, 2012; Ciurzyńska and Lenart, 2011; Labconco, 2010). These advances made this freeze-drying a systematic and well planned process.

2.3.2 Characteristic of Freeze Drying

The freeze drying process is a complex process which involves various parameters such as temperature and pressure. The general principle of freeze drying is the conversion of solid state product into powdered form without crossing the liquid state. Generally, the process is conducted at a temperature and pressure below the triple point. The triple point of a substance is known as the temperature and pressure at which the three phases which are gas, liquid and solid coexist in a thermodynamic equilibrium. The triple point plays a major role in determining the temperature and pressure of the freeze drying process that should be less than 0.01 ℃ and 0.603 kPa (Ratti, 2012) to ensure that the conversion between phases happens from ice to vapor without passing through the liquid phase. Figure 2.3 shows the location of the triple point in the phase diagram.

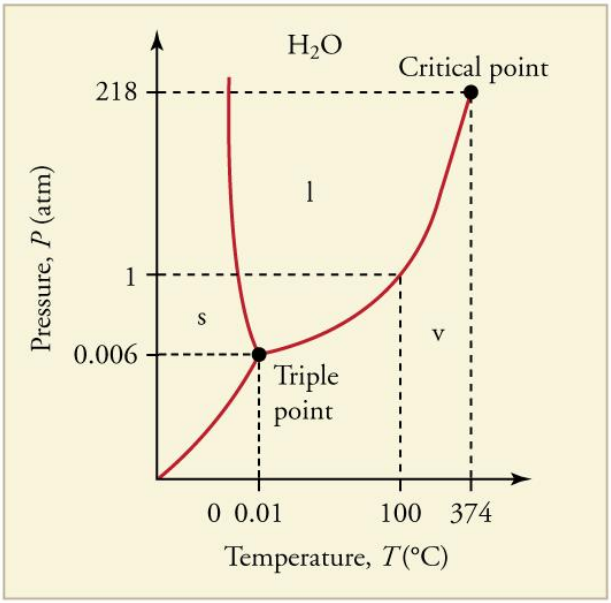


Figure 2.3 Phase diagram showing the triple point of water (Nireesha et al., 2013)

Freeze drying process has a detailed flow which makes it very hard to be done precisely. There are many companies which prefer to take the easy route by opting for drum-drying and spray-drying (Desobry, Netto and Labuza, 1997) which is not as stringent as freeze drying. However, when compared to the other methods, freeze drying produces a stable product that is easy to be used and aesthetic in appearance (Labconco, 2010). Besides that, it preserves the odor, flavor and colour as the original material. Most

importantly, freeze drying process retains the materials' nutritional value and structure (Fellows and Fellows, 2009). This is very true as the freeze drying process retains the substance pattern throughout the process as the process is a surface-tension-free process (Tanaka et al., 1993). It is also mentioned that the freeze drying products have high rehydration capacity as it maintains the products freshness (Hammami and René, 1997). Therefore, freeze drying is the best method to retain the freshness and the functionality of a product.

2.3.3 Eutectic Temperature

Eutectic temperature or also known as collapse temperature is defined as the temperature above which a collapse happens (Labconco, 2010). The eutectic temperature is the most vital temperature in determining the condition of the material during freezing as well as primary drying (Shalaev, Franks, and Franks, 2002). Therefore, before running the freeze dryer, the collapse temperature of the material must be determined. This is due to the fact that the collapse can result in loss of structure and porosity, a significant decrease in water sublimation rate, increase in product density and residual water content, change in colour and also loss in the aroma of the material (Ratti, 2012). The collapse temperature will increase as the sublimation rate increases. During constant sublimation rate, the collapse temperature will increase in the surface area of the solid which is known as the glass transition (Pikal and Shah, 1990). There are three methods used to estimate the collapse temperature of an unknown substance which are the direct microscopic observation (Pikal et al., 1983), thermal analysis method (Franks, 1986) and the electrical resistance method (Nail and Gatlin, 1985).

2.3.4 Primary Drying

Ice sublimation or also known as the primary drying is the second part of the freeze drying process. Before a material is fit for a primary drying, it will be freezed to a temperature below the triple point to ensure all the materials are in rigid solid form.

Primary drying is one of the most time consuming portion of the entire process. The process must always operate at almost the maximum allowable product temperature (Pikal and Shah, 1990). The maximum allowable product temperature indicates that the substances crystallizes and not remain amorphous. If the substance remains amorphous, then the substances had collapsed (Mackenzie, 1996). There are several factors which could affect the ability to freeze dry a substance of suspension. The factors are temperature and pressure. The general pressure used is 20 to eight mT while the temperature is in the range of -120 °C to 80°C (Nireesha et al., 2013). The balances between the parameter are the key for an optimum sublimation process.

2.3.5 Secondary Drying

Secondary drying is also referred as removal of unfrozen water. Unfrozen water is water that had been adsorbed on the surface of the crystalline product or still remain in the solute phase, either as hydrate or dissolved in the amorphous solid (Pikal and Shah, 1990). Secondary drying begins at the end of primary drying. Although the primary drying might give a dry powder, however, there will be a residual moisture content which could go up to 7-8% (Labconco, 2010). In this process, the remaining water content will be desorbed from the glass as the temperature of the sample gradually increases while maintaining a low pressure (Sugimoto et al., 1981). The secondary drying cycle will progress for about 1/3 to ½ of the time taken for primary drying. The process is usually carried out at shelf temperature which is 25 °C or higher with a very low chamber pressure even though this practice can cause a problem in the transfer of volatile stopper components to the product (Pikal et al., 1983). At the end of the process, the moisture content of the substance will typically be between 0.5% and 3% (Ratti, 2012).

2.4 Flowability Test

Powder flowability is the ability of a powder to flow. However, the characteristic of the powder is not as simple as its definition. Most people assumes that the flowability

defines one-dimensional characteristics but the powder behavior is much more multidimensional and follows its individual characteristic (James and Roger, 2000). This is the key requirement in the pharmaceutical manufacturing process, which are related to powder tableting and capsulation. There are various methods that could be used in determining the powder flow that is; (1) angle of repose (United States Pharmacopeia, 2016), (2) bulk density (United States Pharmacopeia, 2012), (3) tapped density, (4) Carr's compressibility index (Carr, 1965), or (5) Hausner ratio (Hausner, 1967). The easiest and cost saving process would be the angle of repose method. Although it is a reasonable process, however, it gives the same quality of powder flow as other methods. In the angle of repose, the angle formed by the horizontal base of the bench surface and the edge of a cone-like pile of granules. It forms cone shape granules collection which can be calculated using the angle of repose formula (Shah, Tawakkul and Khan, 2008). The formula will give about the angle of repose of the powder tested which can be compared with the properties as listed in Table 2.2. Although there is some variation as in the qualitative description of powder flow when using the angle of repose, but most pharmaceutical literatures appear to be consistent with this classification.

Table 2.2 Flow properties and corresponding angle of repose

Flow Property	**Angle of Repose (degrees)**
Excellent	25 – 30
Good	31 – 35
Fair – aid not needed	36 – 40
Passable – may hang up	41 – 45
Poor – must agitate, vibrate	46 – 55
Very poor	56 – 65
Very, very poor	>66

Source: (United States Pharmacopeia, 2016)

2.5 Encapsulation

Capsule is a dosage form where the drug is enclosed in a hard or soft soluble container. Hard container or capsule is usually made up of gelatin or a small soluble container, that are used to enclose a dose of an oral medicine or product (Afzal et al., 2014).

2.5.1 Principles of Capsules

Capsules, in general, exist in two main types which are the soft gelatin capsules and hard gelatin capsules. Soft gelatin capsules are the more flexible type of capsules that consist of various types of shapes like spherical, ovoid, or cylindrical in shape. Soft gelatin capsules are more prone to be created, filled and sealed in one production flow. This is different from the hard capsule, where it is made up of hard gelatin which is formed in two halves. The medication or drug will usually be inserted in the long portion of the capsules the other half is fitted after the filling process (Marriott, Wilson, and Langley, 2010). Figure 2.4 shows the example of soft and hard capsules.

(a) (b)

Figure 2.4 The example of (a) soft gelatin capsule and (b) hard capsule (Gelatin Manufacturers Institute of America, 2012)

Aside from the different shapes and sizes of the soft gelatin capsules, the hard capsules consist of a set of capsule sizes which are usually selected based on the amount of

material or powder to be filled. Table 2.3 shows the size of capsules and its approximate content capacity.

Table 2.3 Size of hard gelatin capsules and its approximate content capacity.

Size of capsule	**Content capacity (mg)**
000	950
00	650
0	450
1	300
2	250
3	200
4	150
5	100

Source: (Marriott, Wilson and Langley, 2010)

2.5.2 Evaluation of Capsules

Quality control is one of the most vital parts in the pharmaceutical field. Therefore, for all the capsules produced, there are certain evaluation tests that must be conducted before the capsules are certified to be safe for release from the plant. The test for the capsules consists of two parts, which are the physical and chemical test. The physical test includes disintegration test and weight variation while the chemical test includes dissolution test, assay, content uniformity, stability testing and moisture permeation test (Leonorus et al., 2006). Weight variation test is conducted where a random of 20 capsules are chosen based on the amount of capsules produced. The weights of the capsules are taken in three conditions: (1) capsules with powder, (2) empty capsule, and (3) powder only. Then the mean values of these capsules are calculated. Dissolution test is conducted to ensure that the capsules are able to breakdown in 60 minutes in a buffer liquid that represents the human body temperature and pH. The disintegration test, on the other hand, is done the same way as the dissolution test except, in this test, the time for the capsules to completely disintegrate are recorded and the mean disintegration time of the capsules are calculated (Kumadoh, 2011).

CHAPTER 3

METHODOLOGY

The purification and freeze drying technique are the main processes that will be done to obtain the propolis powder of good flowability. Parameter such as the temperature was varied in order to obtain different texture and flow of the powder. The summary of the entire process is shown in Figure 3.1.

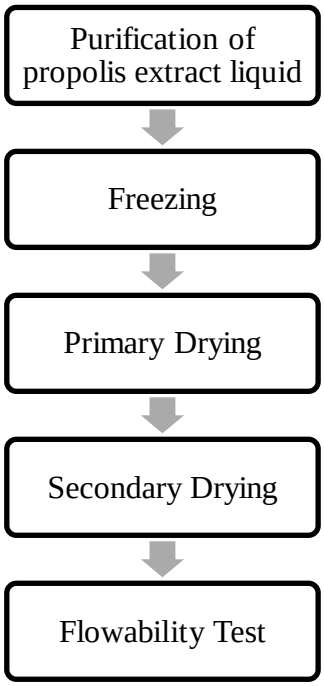


Figure 3.1 Flowchart for the whole process

3.1 Materials/Chemicals

The materials used in this study are listed in Table 3.1.

Table 3.1 Material list and quantity

Material	Quantity
Purified Propolis	4000 ml
Whatman No.1 filter paper (11µm pore size)	2 boxes x 100

3.2 Equipments

The equipments used in this study are summarized in Table 3.2.

Table 3.2 Equipment List

Equipment	Brand	Purpose/ Usage
Freeze Dryer	VirTis BenchTop Pro	Freeze drying the purified propolis sample
Refrigerator	Thermo Scientific-906	Freezing of propolis sample

3.3 Purification of Propolis Extract

The extracted propolis liquid was filtered using Whatman No.1 filter paper. The purification process basic setup can be observed in Figure 3.2. A clear brown-yellowish filtrate was obtained, that ensures the purity of the propolis extract (Sosnowski, 1981).

Figure 3.2 The general setup of the propolis extract

3.4 Basic Operation of the Freeze Dryer

Before starting the freeze dryer, the condenser was checked to ensure it was clean, dry and empty. The drain line was checked for the residual moisture which can cause the slowdown of the vacuum pump. Besides that, the plastic quick-connect drain fitting was ensured to not be inserted into the drain fitting receptacle on the front unit. The pump oil was ensured to be in the safe level to avoid pump malfunction. The freeze dryer was then switched on. The freeze dryer was left to set up where the condenser and vacuum pump condition were ensured to be in green signal together with the pressure and temperature values. When all the conditions were meet and the connection and ports were checked, the sample was placed in the condenser. The Quickseal valves were then closed. After ensuring all the parts and connections were checked, the 'AUTO' button was pressed and the system started with the freeze drying process by using automatic setting. The entire critical system parameters such as the refrigeration and vacuum were ensured to be within the acceptable ranges by checking the System Status screen, whether the signal appears to be green or red. Moreover, the condenser was checked periodically for the ice build-up and defrosts. After the process completion, the 'AUTO' was pressed again to switch-off the vacuum and the refrigeration. The remaining vacuum was released by inserting the drain plug into the drain fitting or by opening the Quickseal valve. The 'DEFR' button was pressed to start the defrosting process. The defrost system turned-off automatically after one hour (Dryer and Dryer, 2016). The equipment that used the study was the VirTis Benchtop Pro Freeze Dryer as shown in Figure 3.3.

Figure 3.3 VirTis Benchtop Pro Freeze Dryer

3.5 Freezing of Propolis Extract

The purified propolis extract was freezed in the laboratory refrigerator (Thermo Scientific-906, The United States). The freezing temperatures were varied from -40 °C, -50 °C, -60 °C, -70 °C and -80 °C. The 25 mL of propolis liquid extract was transferred in a 50 mL centrifuge tube and was placed in the laboratory refrigerator at a temperature of -40 °C. The centrifuge tube was covered with an aluminum foil to ease the freezing process, to reduce the pressure in the centrifuge tube that could affect the freezing quality and also to avoid contamination. The freezing process was conducted for a 48 hour period. The same process was repeated for the freezing temperatures of -50 °C, -60 °C, -70 °C and -80 °C.

3.6 Primary Drying of the Propolis Extract

The 25 mL of freezed propolis was transferred into to the condenser type freeze dryer to undergo the primary freezing which is the sublimation process. The setup of freeze dryer was mentioned in Section 3.4. The temperature and pressure for the primary drying process were set automatically by the equipment and the process commenced until the status bar turned from red to green. The process proceeded for 24 hours (Gomes do Nascimento et al., 2016). The temperature and pressure were maintained throughout the process in order to ensure a smooth process. The same process was repeated for the freezing temperatures of -50 °C, -60 °C, -70 °C and -80 °C.

3.7 Secondary Drying of the Propolis Extract.

At the end of primary drying, the secondary drying process began. In the secondary drying step, the extracted propolis temperature was increased at a slow rate to avoid the propolis collapse by the equipment automatically. The temperature can be increased to a maximum of 34 °C, as 35 °C is the maximum temperature limit for the propolis (Buchwald, Breed, and Greenberg, 2007). Any increase in temperature more than that will cause the propolis content to collapse. The vacuum pressure was reduced and maintained at the same level by the equipment automatically. The secondary process continued for 24

hour time frame to complete the entire process (Gomes do Nascimento et al., 2016). The process was completed when the status bar showed changes from red to green and the product appearance indicated dryness, where there were no powder residue sticking at the inner surface of the centrifuge tube (Labconco, 2010). The same process was repeated for the freezing temperatures of -50 °C, -60 °C, -70 °C and -80 °C.

3.8 Flowability Test

The flowability test conducted was the angle of repose method with fixed height to obtain the angle of repose, θ. Prior to that, moisture content loss of the propolis powder after freeze drying was calculated by using Equation 3.1 (Roongruangsri and Bronlund, 2016). About 5 g of the obtained propolis powder from different freezing conditions was poured from a fixed height of 40 mm above the bench surface. A cone was formed and the height, h of the granules forming the cone and the horizontal length of the base, r were measured. The setup of the entire process is as shown in Figure 3.4. The angle of repose, θ was calculated in triplicate using the Equation 3.2 and the value was compared with the values in Table 2.2 to determine the smoothness of the powder.

$$\% \text{ Moisture content loss} = \frac{\text{mass}_{\text{initial}} - \text{mass}_{\text{dried}}}{\text{mass}_{\text{initial}}} \times 100 \quad (3.1)$$

$$\theta = \tan^{-1}\left(\frac{h}{r}\right) \quad (3.2)$$

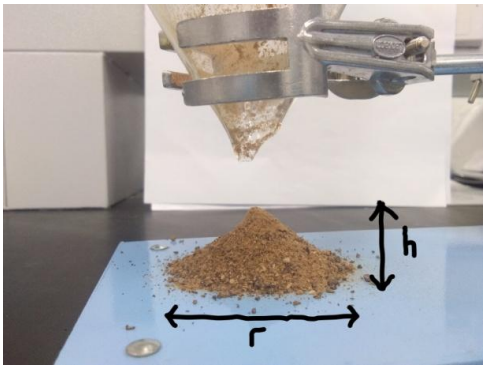


Figure 3.4 Angle of repose basic measurement setup

3.9 Propolis Powder Bulk Production

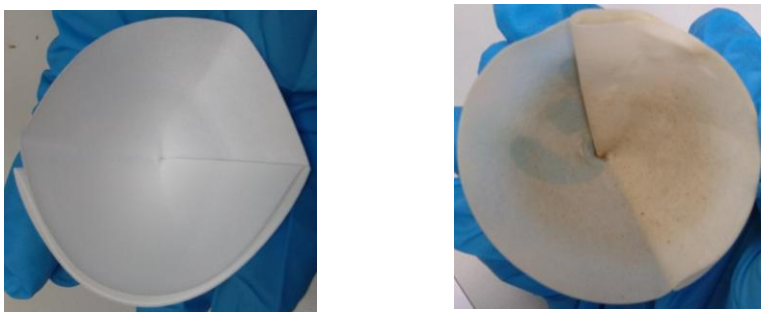
Based on the flowability test results, bulk production of propolis powder was conducted. The entire process flow was repeated using the most suitable temperature to obtain the best powder smoothness. About 30 g of propolis powder were produced by using 3000 mL of purified propolis extract.

CHAPTER 4

RESULT & DISCUSSION

4.1 Purification of Propolis

Based on the antioxidant activity study it was found that the ethanol extract of propolis (EEP) have a higher activity compared to the water extract of the propolis (WEP). However, due to the Halal issues, WEP method was chosen as the extraction method. After 24 hours of extraction by water, the propolis content that is extracted out underwent the purification step by filtration method. From the observation, the filtration process for propolis purification took a longer period of time due to particle size complexion, where the larger sized particle more the 11 µm that blocks the filter pore caused delay. The condition of filter paper before and after filtration can be observed in Figure 4.1. The brownish condition of the filter paper after undergoing the filtration process verifies that other components of the extracted propolis such as the waxy substance are totally removed giving a purified propolis intended for the study.



(a) (b)

Figure 4.1 Condition of filter paper (a) before and (b) after the purification process

4.2 Freeze Drying Condition and Control

The freeze drying condition is represented in this part. The entire samples were freeze dried in three batches in order to be used in the flowability testing. The parameters involved during the freeze drying process were temperature and pressure. Table 4.1 shows the changes in the pressure and temperature difference for every 12 hours period.

Table 4.1 Pressure and temperature change in 12 hours interval of the 48 hours freeze drying process

Sample	**Temperature (°C)**					**Pressure (millitorr, mT)**				
	Time Interval (hours)									
	0	**12**	**24**	**36**	**48**	**0**	**12**	**24**	**36**	**48**
Batch 1	-100.6	-101.2	-102.0	-98.0	-80.1	197	18	9	8	8
Batch 2	-98.4	-101.1	-102.8	-100.7	-99.7	187	20	8	7	7
Batch 3	-101.1	-102.7	-103.1	-101.7	-100.1	200	25	10	8	8

The freeze dryer pressure and temperature initial value were automated by the freeze dryer. The difference in the initial value is due to the buffer period where the equipment is paused to allow the sample to be placed before the process begins at the zero hours. During this buffer period, there would be a rapid increase in the temperature and the pressure value from the initial pre-setup, as the equipment is exposed to ambient temperature which was 22 °C. Therefore, precaution should be taken when placing the sample, where the temperature range should not increase to more than -80 °C and the pressure should not increase more than 250 mT. Any increase from the mentioned temperature and pressure value will affect the process of freeze drying as well as the condition of the powder formed for all the temperature samples.

Based on Table 4.1, a line graph was formed to differentiate the increase and decrease of the temperature and pressure value as shown in Figure 4.2 and Figure 4.3 respectively. The fluctuation in the temperature and pressure values throughout the freeze drying process was due to the different condition needs during the primary drying and

secondary drying. During primary drying, the temperature should be in a decreasing rate but not more than -120 °C for propolis (Nireesha et al., 2013). This ensures that the ice sublimation process occurs in an optimum condition (-80 °C < -120 °C) and the water content in the propolis sample were sublimated without affecting the structure of the propolis. Based on the graph on Figure 4.2, from the zero hour to the 24th hour, there is a decrease in the temperature which verifies that the samples are undergoing the primary drying process.

Observing the graph in Figure 4.3, there is a major decrease in pressure from the zero hour to the 24th hour. This decrease verifies that the primary drying process was being conducted by the freeze dryer as the pressure during ice sublimation should be below 200 mT. Meanwhile, the low temperature ensured that all the water content in the propolis sample was sublimated without affecting the structure of the propolis. The decrease in value observed from the 12th to the 24th hour signifies that the primary drying process was reaching completion. Based on both the graph, it could be concluded that the primary drying process completed at the 24th hour. The condition of the propolis sample during the completion of primary drying is as shown in Figure 4.4 (a).

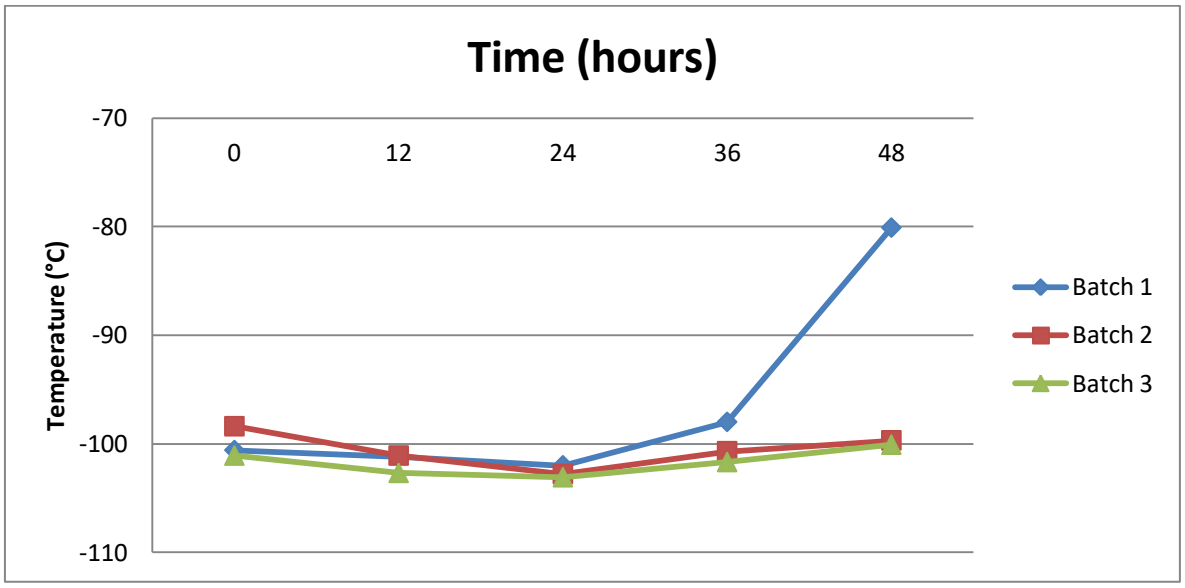


Figure 4.2 Graph of temperature difference at different time interval of the freeze drying process

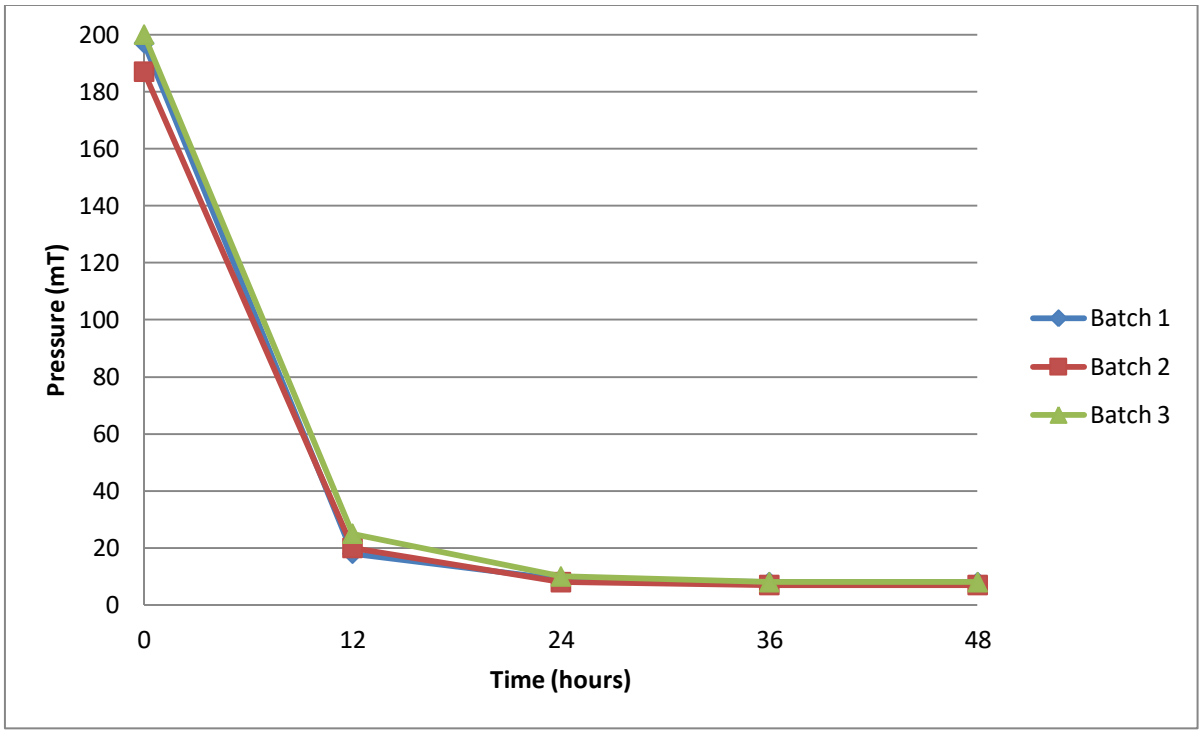
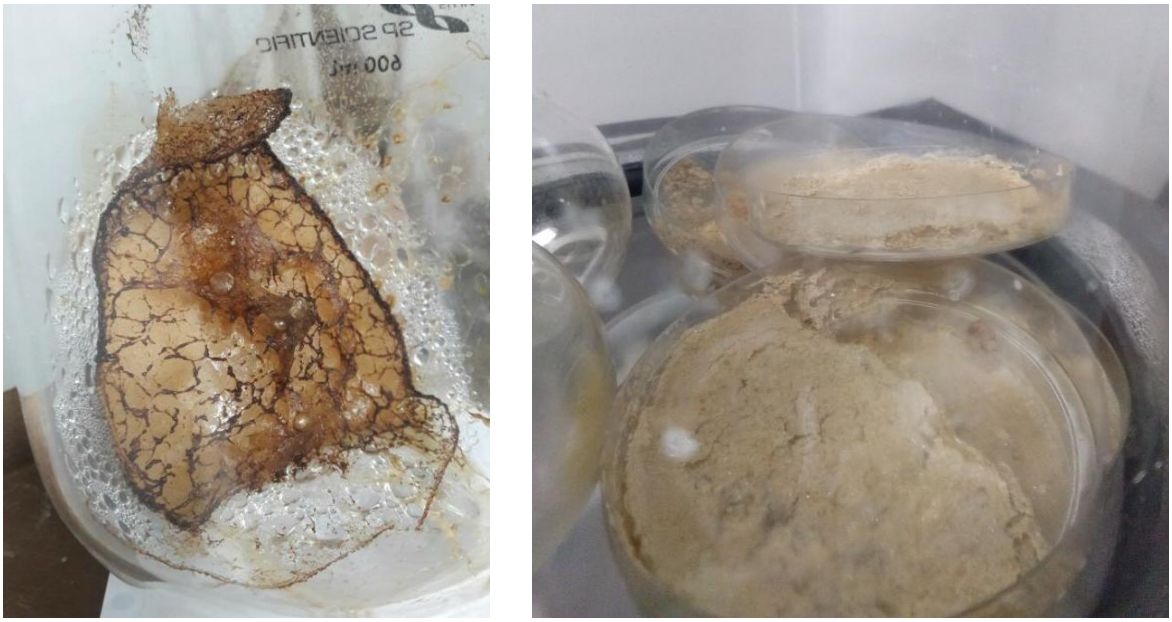


Figure 4.3 Graph of pressure difference at different time interval during the freeze drying process



(a) (b)

Figure 4.4 Condition of propolis sample during (a) primary drying and (b) secondary drying

The secondary drying began at the 24th hour right after the completion of the primary drying. This was proved where there is an increase in temperature and the constant low pressure from the 24th hour to the 48th hour which signifies and verifies that removal of unfrozen water was going on as per theoretical standards. Based on the theoretical standards, during the secondary process there is an increase in the temperature until the maximum allowable temperature while maintaining a constant low pressure (Sugimoto, Ishihara and Habata, 1981). The maximum allowable increase in temperature for propolis sample would be -80 °C. The condition of sample was observed to reach the powder state during the secondary drying due to the desorption of the remaining water crystals in from the surface of the propolis sample. The major difference in the increase of temperature of the 1st batch sample during this process is due to the increase in the ambient temperature that caused the heating up of the freeze dryer. Therefore as precautionary step, the ambient temperature was ensured to be maintained at 22 °C throughout the freeze drying process. At the 48th hour, the freeze drying process was completed as it had maintained a constant pressure reading and there is an increase in the temperature to a maximum allowable temperature of -80 °C. The condition of the propolis powder during the secondary drying process is as shown in Figure 4.4 (b) and the powder appearance after the completion of the entire freeze drying process is as in Figure 4.5.

Figure 4.5 Final propolis powder appearances after the completion of freeze drying process

4.3 Effect of Temperature towards Weight of Propolis Powder

The powder obtained from the different freezing conditions was weighed to identify the ratio of the purified propolis volume to propolis powder formation. The results were tabulated in Table 4.2. The volume of each sample of purified propolis was 100 mL and the weight was measure in triplicate to obtain an average weight.

Table 4.2 Weight of propolis powder obtained at different freezing temperature

Freezing Temperature (°C)	Weight (g)				Ratio (volume:weight)
	1	2	3	Average (4 s.f)	
-40	1.6034	1.6037	1.6035	1.6035	1:0160
-50	1.4720	1.4719	1.4721	1.4720	1:0147
-60	1.2998	1.2995	1.2997	1.2997	1:0130
-70	1.1923	1.1920	1.1922	1.1922	1:0119
-80	1.0110	1.0107	1.0105	1.0107	1:0101

As shown in Table 4.2, as the freezing temperatures decrease, the amount of propolis powder produced also decrease. This is due to the moisture content level that was removed during the freeze drying process. The temperature of the freezed propolis affected the freeze drying process efficiency which affects the amount of propolis powder produced. The percentage of moisture content loss in each sample can be seen in Table 4.3

Table 4.3 Percentage of moisture content loss at different freezing temperature

Freezing Temperature (°C)	Moisture content loss (%)
-40	98.39
-50	98.52
-60	98.70
-70	98.81
-80	98.99

Based on Table 4.3, all the samples were verified to be completely freezed dried as the moisture content of the powder after freeze drying is between 0.5% to 3% as per standard method (Ratti, 2012). When compared to all freezing samples, the propolis powder produced at -80 °C freezing condition, showed the maximum loss of moisture content. This was due the high amount of freezed water in the sample compared to the other samples. In order to avoid the propolis becoming sticky in room condition, freezing at -80 °C would be the best freezing temperature for the samples due to the maximum moisture content amount.

4.4 Flowability Test on Propolis Powder

Propolis powder from different freezing temperatures was tested for its respective flowability capacity based on angle of repose formula as in Equation 3.1. Each test was repeated trice to ensure reproducibility of the data and the results are shown in Table 4.4.

Based on Table 4.4, the flowability of the propolis powder obtained at freezing temperatures of -40 °C did not pass the Unites States Pharmacopeia (USP) standard as per shown in Table 2.2. However, the freezing temperatures of -50 °C, -60 °C and -70 °C produced the propolis powder with passable and fair flowability characteristic. The propolis powder with excellent flowability characteristics was obtained at -80 °C. The excellent form of powder is required for the encapsulation process. At the -80 °C of freezing temperature, all the water particle present in the purified propolis sample were completely freezed which was then removed through freeze drying process giving it the maximum water content lost ensuring the powder to flow smoothly in the test conducted. Figure 4.7 shows the angle of repose of propolis powder obtained at different freezing temperature. From Figure 4.7, the close gap between the standard errors verifies and validates that the angle of repose values are not spread widely and are of specification. Through this test, the best freezing temperature to obtain an excellent flow of propolis powder is verified to be -80 °C. Therefore 30 g of bulk propolis powder were produced using the -80 °C freezing temperature and was used for encapsulation purposes.

Table 4.4 Angle of repose and flowability characteristic of propolis powder

Freezing Temperature (°C)	Angle of Repose (degree,°)				Flowability Characteristic
	1	2	3	Average	
-40	52.43	51.71	53.13	52.42	Poor
-50	46.59	50.96	47.64	48.40	Passable
-60	43.09	44.06	41.18	42.78	Passable
-70	36.63	37.87	38.09	37.53	Fair
-80	25.64	26.56	28.37	26.86	Excellent

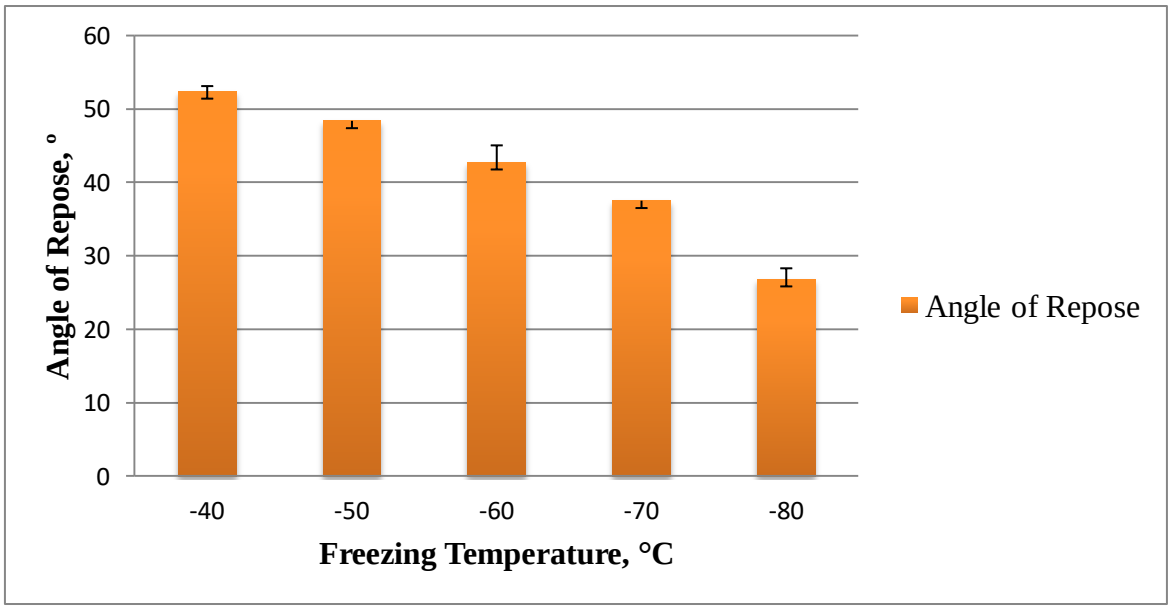


Figure 4.6 Graph of angle of repose for its respective freezing temperature

CHAPTER 5

CONCLUSION & RECOMMENDATION

5.1 Conclusion

Based on results obtained, the purified propolis was subjected to the freezing drying process based on different freezing temperature, which were -40 °C, -50 °C, -60 °C, -70 °C and -80 °C. The primary drying was conducted in the temperature range of -98.4 °C to -103.1°C and pressure ranging from 8-200 mT. The secondary drying was conducted in a higher temperature of between – 80 °C to -102 °C. Both the process of the freeze drying occurred in the maximum allowable range as per the standard. The angle of repose and moisture content testing conducted on the obtained propolis powder proved and verified that the propolis powder sample of -80°C have the maximum loss in moisture content about 98.99% and have an excellent flowability characteristic with an average of 26.86º. Therefore the propolis powder of -80°C freezing temperature is best used for the encapsulation process.

5.2 Recommendation

It is recommended that the freezing volume must be placed in a large area volume apparatus in order to gain more exposed area for freeze drying. Then, the condition of the room equipped with freeze dryer must be ensured to always be less than 22 °C. Moreover, due to the distance difference between the freezer and the freeze dryer, it caused some of the sample to have increase in temperature that affected the quality of powder. Therefore,

in future researches to be done ensure the timing and distance gap before using the freeze dryer. Due to time limitation, the freeze dryer temperature and pressure was totally automated. In the future researches, it is recommended to try different temperature or pressure to understand the powder formation and quality. Besides that, additional testing is suggested to be conducted such as 'flow through orifice method', tapped density and Hausner ratio. This is because this testing will further verify the powder quality and flowability that will be useful for validation purpose in the future.

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