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In vitro antioxidant activity of electrospun polyvinyl alcohol nanofiber mats containing stingless bees' propolis extracts

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Objectives: The goal of this study was to develop electrospun nanofiber mats containing stingless bee's propolis extracts and evaluated in vitro antioxidant activity via (2,2-diphenyl-1-picryhydrazyl (DPPH)) method.
Methods: Polyvinyl alcohol (PVA) was selected as polymer. The stingless bee's propolis extracts with the concentration of 0.5%, 1% and 2% wt alpha-mangostin were incorporated to PVA solution. Mixture solution was prepared to nanofibers via electrospinning process. The morphology and diameter of the electrospun nanofiber were observed using scanning electron microscope (SEM). The amount of alpha-mangostin was determined by high performance liquid chromatography (HPLC).The anti-oxidative activity of (2,2-diphenyl-1-picryhydrazyl (DPPH)) method were evaluated.
Results: The results showed that the diameters of the fibers were 184.0 ± 28 - 226.3 ± 64 nm and no crystal of the extract was detected in all concentrations. The α-mangostin content in electrospun nanofibers prepared by incorporating 0.5%, 1% and 2% wt were 64.2 ± 5.8 % 56.2 ± 5.3 % and 48.8 ± 1.9 % respectively. The antioxidant activities (IC_{50}) which evaluated by DPPH method of all electrospun nanofibers were 1.12-1.87 mg/ml.
Conclusion: In summary, Electrospun PVA nanofibers containing stingless bee's propolis may have potential for pharmaceutical application.

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Introduction

Stingless bees are a group of eusocial insects belonging to five different genera, viz. *Melipona*, *Trigona*, *Meliponula*, *Dectylurina* and *Lestrimelitta*[1]. All these genera also can produce propolis; a mixture of bees wax, plant exudates and pollens which are used for sealing their bee hives[2,3]. *Trigona* is the largest genus of stingless bees found in Mexico, Argentina, India, Sri Lanka, Taiwan, the Solomon Islands, South Indonesia, New Guinea and Australia. In Thailand, *Lepidotrigona ventralis* Smith, *Lepidotrigona terminata* Smith, and *Tetragonula pagdeni* Schwarz (Apidae) are commercially cultivated in artificial hives in fruit gardens. In a previous study, the composition and activities of the stingless bees' propolis collected in the same region of mangosteen garden from Thailand was reported. g- and α-mangostin was found as the major compounds in the stingless bees' propolis. It also had the antioxidant activities and alpha glucosidase inhibitory effect[4].

Electrospinning is a process that can be used to create fibers with very small diameters. This process can produce fibers with a diameter in the nanometer range. The polymer solution can be prepared to fibers under high voltage electrical power. The small fibers obtained from this process exhibit excellent properties such as a high surface area to volume ratio, high density of nanometer sized pores of the mat and surface functionalization. This process is versatility and has potential for various biomedical applications such as scaffold for tissue engineering, wound healing and carriers for delivery of drugs and substances. A large number of polymers can prepare nanofiber from this process such as polyvinyl alcohol (PVA), polyethylene oxide (PEO), poly caprolactone (PCL), polyvinyl pyrrolidone (PVP)[5,6]. Electrospinning process has been used to fabricate the fibers contained herbal substances such as curcumin[7], asiaticoside[8], capsaicin[9] and α-mangostin[10]. However, the study of electrospun which incorporated stingless bees' propolis has been few reported[11]. The objective of this study was to prepare to develop electrospun nanofiber mats containing stingless bee's propolis extracts and evaluated in vitro antioxidant activity. PVA was selected as a polymer for fabricated nanofibers. PVA is a water soluble synthetic polymer. PVA have a good fibers forming via electrospinning process and has the excellent properties as biocompatibility and biodegradability. The morphology of obtained nanofiber mats was observed under scanning electron microscope (SEM). The % loading efficacy of nanofiber mats containing stingless bees' propolis was determined using high performance liquid chromatography (HPLC) method. α-mangostin was used as a maker. The in vitro antioxidant activity was evaluated using a 2,2-diphenyl-1-picryhydrazyl (DPPH) method.

Methods

Materials: Stingless bees' propolis was collected form Makham district, Chantaburi province, Thailand. Polyvinyl alcohol (PVA) (degree of polymerization » 1600, degree of hydrolysis » 97.5-99.5 mol%) was purchased from Fluka, Switzerland. All other reagents and solvents were of analytical grade.

Stingless bees' propolis extraction: Stingless bees' propolis was washed and grinded to into small pieces and dry in a hot air oven at 50 °C for 24 h. Dried pieces of stingless bees' propolis was separately macerated with methanol at room temperature until the extraction was exhausted. Methanol extract was combined and filtered through a Whatman no. 1 filter paper under suction. The filtrate was concentrated on water bath and evaporates solvent in rotary evaporator to obtain the dry crude extracts. The stingless bees' propolis extracts was analyzed by the amount of α-mangostin determined by HPLC. A VertiSep® AQS C18 column (250 mm × 4.6 mm, 5 µm particle size) with a C18 guard column was used. The HPLC analysis was performed according to the method of Pothitirat et al., 2009 with a slightly modification[12]. The elution was performed using gradient solvent systems that consisted of acetonitrile (mobile A) and 0.1% v/v ortho phosphoric acid (mobile B) with a flow rate of 1 mL/min at ambient temperature. The gradient program was as follows: 70% A for 0–15 min, 70% A to 75% A in 3 min, 75% A to 80% A in 1 min, constant at 80% A for 6 min, and 80% A to 70% A in 1 min. The wavelength of the UV–visible detector was set at 320 nm.

Preparation of electrospun nanofiber mats: The PVA solution (10% w/v) was prepared by dissolving PVA in distilled water at 80°C and then allowing the solution to stir for 4 h. Stingless bees' propolis extracts solutions in methanol were loaded to PVA solution at various concentration (0.5%, 1%, 2%wt a mangostin) and stirred for 24 h. The mixed solution was prepared nanofiber mats via an electrospinning process. All of the mixed solution were taken up in a 5 mL glass syringe equipped with a 20-gauge, stainless steel needle (diameter = 0.9 mm) at the nozzle. The needle was connected to the emitting electrode of positive polarity of a Gamma High Voltage Research device. The electric potential was fixed at 15 kV. The nanofibers were collected as-spun on an aluminum thin foil that was wrapped on a rotating collector. The speed of rotating collector was fixed at 60 rpm. The solution was electrospun at room temperature, and the collection distance was fixed at 15 cm. The solution feed was driven by a syringe pump, and the feed rate was fixed at 0.25 mL/h. The process duration was fixed at 24 h in each different weight ratios that provide a thickness approximately 20-30 µm.

Morphology of nanofiber mats: The morphology and diameter of the nanofiber mats was examined using scanning electron microscopy (SEM; LEO 1450VP, EDAX®, USA). For this process, a small section of the electrospun nanofiber mats was sputtered with a thin layer of gold prior to SEM observation. The accelerating voltage was fixed at 15 kV. The diameter of nanofibers was measured using software JmicroVision, collected 100 measurements for each sample.

Loading efficacy: The loading efficacy of the stingless bees' propolis extracts into the PVA nanofiber mats was determined by submerging the mats (5 mg) into 5 mL of a phosphate buffer (pH 7.4) and methanol (50:50) for 24 h. Then, 1 mL of the solution was analyzed using HPLC to determine the amount of α-mangostin. The amount of α-mangostin was used to calculate the amount of extracts in the nanofiber mats. The % loading efficacy was calculated using equation 1:

$$\text{Loading efficacy (\%)} = (La/Lt) \times 100 \qquad (1)$$

where La is the amount of the stingless bees' propolis extracts that are embedded in the nanofibers and Lt is the theoretical amount of stingless bees' propolis extracts (obtained from the feeding condition) incorporated into the nanofibers.

In vitro antioxidant of nanofiber mats: the antioxidant activity of stingless bees' propolis extracts and nanofiber mats containing Stingless bees' propolis extract were evaluated using DPPH method. A 200 µM aliquot of DPPH in methanol (100 µL) was added to 100 µL of the GM extract. The extracts were dissolved by their solvent and then diluted to the desired concentration with methanol. The mixture was held at 37 °C for 30 min. The absorbance was measured at 520 nm by a microplate analyzer. The results of the assay were expressed as IC_{50}, which represents the concentration of the extract (µg/mL) required to inhibit 50% of the free radical scavenging activity. The free radical scavenging activity was assessed using equation 2:

$$\text{\% Inhibition} = [(A_{\text{control 520 nm}} - A_{\text{sample 520 nm}})/A_{\text{control 520 nm}}] \times 100 \qquad (2)$$

where $A_{\text{sample 520 nm}}$ is the absorbance in the presence of the extracts and $A_{\text{control 520 nm}}$ is the absorbance of the control. The IC_{50} values were calculated by linear regression of the plots where the x-axis represented the various concentrations (µg/mL) of the GM extracts and the y-axis represented the % inhibition.

Results

After extraction, the crude extract was collected and calculated to % yield. The yield was 23.07 ± 1.2 %. The content of α-mangostin in the stingless bees' propolis extracts was 4.93 ± 0.5 % w/w.

Morphology of nanofiber mats: The SEM image of electrospun PVA nanofiber mats containing stingless bees' propolis extracts at a various concentration of α-mangostin shown in Figure 1. The average diameter of 0%, 0.5%, 1% and 2%wt α-mangostin was 184.0 ± 28, 203.4 ± 54, 226.3 ± 50 and 238.7 ± 64 nm, respectively. When the concentration of stingless bees' propolis increased the average diameter of fibers slightly increased. In a preliminary study, the maximum concentrations that can prepare nanofiber using electrospinning process were 6%wt.

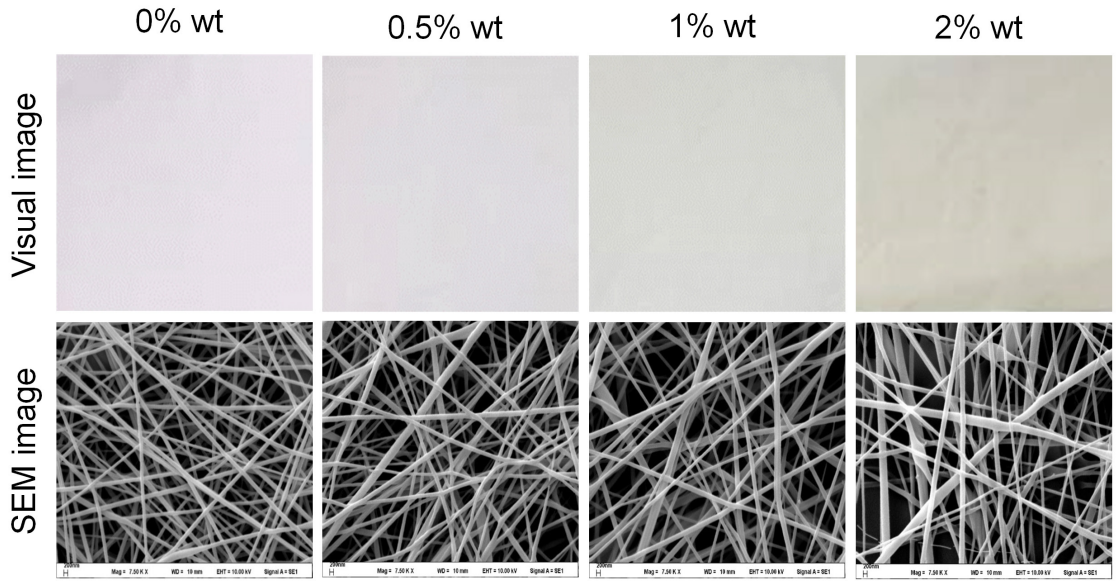

Figure 1. Visual image and SEM image of electrospun PVA nanofiber mats containing stingless bees' propolis extracts (0, 0.5, 1 and 2%wt α-mangostin)

%Loading efficacy: The % loading efficacy of the stingless bees' propolis extracts in the nanofiber mats slightly decreased from 64.19 to 48.78%, respectively, when the concentration of extracts was increased. Data are shown in Table 1.

Table 1. Loading efficacy (%) of electrospun PVA nanofiber mats containing stingless bees' propolis extracts (0.5, 1 and 2%wt α-mangostin) Data are express as mean ± SD (n = 3).

Samples	%Loading efficacy
0.5% wt α-mangostin nanofiber mats	64.19 ± 5.8
1% wt α-mangostin nanofiber mats	56.16 ± 5.3
2% wt α-mangostin nanofiber mats	48.78 ± 1.9

In vitro antioxidant of nanofiber mats: Table 2 shows the DPPH radical antioxidant activity of stingless bees' propolis extracts and nanofiber mats with difference concentration of extracts. The antioxidant of nanofiber mats depended on the concentration of extracts.

Table 2. The antioxidant capacity of Stingless bees' propolis and electrospun PVA nanofiber mats containing stingless bees' propolis extracts (0, 0.5, 1 and 2%wt α-mangostin) using DPPH method. Data are express as mean ± SD (n = 3).

Samples	IC_{50} (µg/ml)
Stingless bees' propolis extracts	112.31 ± 14.1
0% wt α-mangostin nanofiber mats	N/A
0.5% wt α-mangostin nanofiber mats	1844.6 ± 21.1
1% wt α-mangostin nanofiber mats	1120.2 ± 35.6
2% wt α-mangostin nanofiber mats	464.5 ± 6.6
Ascorbic acid	3.95 ± 0.4

N/A = the inhibition (%) was not reach to 50%

Discussion

The morphology of nanofiber mats containing stingless bees' propolis extracts was smooth and without bead structure in all concentration. The average diameter of fibers was in nanometer scale. However, the average diameter of fibers slightly increased and the amounts of extract increased. This result was in accordance with our previous study, which indicated that the incorporation of *Garcinia mangostana* (GM) extracts into the electrospun chitosan/PVA nanofibers did not affect their morphology and the average diameters of both the bare and the PVA fibers loaded with GM extracts ranged between 205.56 to 251.35 nm[10]. The % loading efficacy of stingless bees' propolis in nanofiber mats was approximately 50%. This result indicates the limited incorporation of stingless bees' propolis extracts into the nanofiber mats. Because of the stingless bees' propolis extracts was hydrophobic compound it may be poorly incorporated into hydrophilic PVA solution. Therefore, the stingless bees' propolis extract may be losing from fibers during the electrospinning process. For the antioxidant activity, the stingless bees' propolis extracts exhibited the DPPH radical scavenging activity with the IC_{50} value of 112.3 mg/ml. This result corresponding with a previous study, the DPPH radicals scavenging activity of extract

from the stingless bees' propolis (*Tetragonula pagdeni*) showed the IC_{50} value of 122.7 mg/ml[4]. When the stingless bees' propolis extracts were incorporated into nanofiber mats, it still remains the antioxidant activity depends on the concentration. This result illustrated that the antioxidant activity of the extract was not eliminated under high voltage via the electrospinning process.

Conclusion

The electrospun nanofiber mats containing stingless bee's propolis extracts were successfully fabricated using the electrospinning process. The fibers obtained from this process were in the nanometer range and without bead formation. The loading efficacy was around 50%. The nanofiber mats still remain the antioxidant activity. In summary, this nanofiber mats may have a potential for pharmaceutical application.

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