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Dichloromethane-Extract of Propolis (DEP) and DEP/PLA Electrospun Fiber Membranes

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Abstract
Propolis is a waxy substance produced by the honeybee that has been used as a form of traditional medicine and natural medicine since ancient times. Propolis has a wide spectrum of alleged applications, including potential anti-infection and anti-cancer effects. The following paper used a propolis extract containing 90% ethanol solution, 70% ethanol solution, ligarine, and dichloromethane as solvents that extracted the bioactive components. The highest yield of the propolis was obtained via the 70% ethanol leaching method and dichloromethane immersion stirring method. Fourier Transform Infrared (FTIR) analysis proved that the extracted propolis with dichloromethane had the highest methylene content and the maximum types of effective propolis components. A Propolis/PLA electrospinning solution was prepared by adding PLA powder into the supernatant of the dichloromethane -extract of propolis (DEP) directly, with there being no need for purification of the propolis extract and thus reducing the loss of active ingredients. DEP/PLA nanofiber was prepared via the electrospinning process, where it was found that with additional 4% PLA, the final electrospun fiber membrane was stabilised. tStudy of the antibacterial performance of the DEP/PLA electrospun membrane showed that the membrane affected some of the antibacterial properties. It was particularly effective when inhibiting Staphylococcus aureus, but not as effective when inhibiting Escherichia coli. This electrostatic spinning membrane could be used for food preservation, wound healing, and tissue engineering.

Key words: *propolis, dichloromethane-extract of propolis (DEP), electrospun, antibacterial.*

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Introduction

Propolis is a bee product, composed primarily of plant resins and beeswax. It is thought that this compound is used in beehives to seal holes, stop drafts, and protect against external invaders. In regions of temperate climate, such as China, *Apis cerana* bees primarily obtain resins from the buds of populus, birch, elm, and pine, where the main bioactive components are flavonoids [1-3]. There are differences in the chemical composition of resins from different regions, however the principal components are consistently flavonoids, mushroom alkenes, organic acids, aromatic aldehydes, alcohols, lipids, amino acids, enzymes, vitamins, and minerals [4]. The main function of propolis is to prevent the decomposition of organic matter (i.e. creatures that have been killed by bees in response to an invasion) within the hive by inhibiting microbial growth. In countries like China, where equatorial, tropical and sub-tropical climates are found, the plant origin and chemical composition of propolis vary. In addition to *A. cerana*, there are hundreds of species of native Chinese stingless bees that mix plant resins with wax (cerumen) and occasionally clay (geopropolis) for use as a construction material, as well as defense against predators and diseases [5]. Propolis from various origins has been used as a popular remedy for several centuries as the antimicrobial properties are quite effective [6]. Propolis extract has strong inhibitory effects against fungi, ringworm fungus, and microspore fungi. Propolis is effective at killing the cucumber mosaic virus, the tobacco mottle virus, the tobacco necrosis virus, and the influenza A virus [7-10]. Propolis extract could inhibit the reproduction of the herpes virus and could significantly reduce vaccinia virus infection, while having a neutralising effect on diphtheria, tetanus exotoxin, and edema [11]. The inhibitory effect that propolis has on *Staphylococcus aureus* and *Bacillus subtilis* is the strongest [12]. Propolis has a good antibacterial effect and could be used in combination with certain antibiotics to improve their antibacterial activity and prolong their life. Propolis has been shown to have a moderate synergistic effect on the antibacterial activity of penicillin, streptomycin, tetracycline, chloramphenicol, erythromycin, neomycin, kanamycin, barone neriantin, and polymycin E within a culture medium and ointment formulation [13-16].

Previous research on the antibacterial aspects of propolis has primarily focused on the antibacterial experimental study of propolis extracts, while studies on propolis monomer compounds have focused on the study of individual monomer compounds, where a majority of the mechanisms of action remain unclear [17]. More than 100 phenolic compounds have been identified from propolis, which are recognised as one of the main active ingredients in propolis. The propolis antioxidant as well as the antibacterial and anti-tumour activities are shown to be related to the phenolic acids, particularly the biological activity of caffeic acid phenethyl ester (CAPE) [18-19]. Due to different vegetation, climate, and bee species, phenethyl caffeine varies from region to region. Propolis from South Korea contains phenethyl caffeate, scavenges oxygen free radicals and protects against DNA damage [20]. The antibacterial activity and spectrum of propolis extracts from three different regions were studied, and results showed that the antibacterial effect of propolis extracts in the Urez region was stronger against G+bacteria than G-bacteria,

where CAPE was the main antibacterial component [21]. The main skeleton types of phenolic compounds found in propolis are two: the C6-C1 type, where the basic skeleton is a benzoic acid, such as gallic acid, and the C6-C3 type, where the basic skeleton is a phenylpropionic acid, such as Caffeic acid [22-23].

Propolis nanofiber fiber membrane with antibacterial activity were prepared by an electrospinning technique for different application areas. Propolis extract was used as an active ingredient, and polyvinylpyrrolidone (PVP) K90, polycaprolactone (PCL), polyurethane (PU) , polyvinyl alcohol (PVA) and Polycaprolactone (PCL) were used as the polymer matrix [24-29]. The antibacterial activity could be used against Streptococcus mutans, *Staphylococcus aureus (S. aureus*), *Staphylococcus epidermidis*, *Proteus mirabilis*, *Bacillus cereus*, and *Escherichia coli*, as well as against gram positive bacteria (*S. aureus*), gram negative bacteria (*A. baumannii* and *P. aeruginosa*) [31]. Previous mechanical characterisation showed that propolis reduces the tensile strength. Fiber mats exhibit worse mechanical performance when the propolis concentration increases [27]. Propolis has an effect on the mechanical properties of the fiber mat, hence fiber mat mechanical properties are improved by adding nanocellulose and polyurethane PU [25-26]. The incorporation of propolis into PU and PLA microfibers could increase its cell compatibility, such as HaCaT cells [26, 30].

Electrospun fibers with propolis extract dissolve and release the propolis in water [14, 2, 28-29, 32]. Biopharmaceutical characterisation of electrospun PVP mats with propolis demonstrates the quick release of propolis phenolic compounds [32]. Polyvinyl pyrrolidone (PVP) and polyvinyl alcohol (PVA) were chosen as fiber-forming polymers that dissolve rapidly in water, within 10 seconds [29]. The phenolic compound release kinetics show that up to 86-96% of vanilic acid, caffeic acid, vanillin acid, p-coumaric acid, and ferulic acid is released from electrospun PVA/aqueous propolis solution mats within 15 min [13].

In this study, we compared the contents of propolis extracts that were extracted by four different methods: the 90% ethanol leaching method, 70% ethanol leaching method, ethanol-ligarine extraction method, and dichloromethane immersion stirring method. FTIR graphs were used to find a suitable method for extracting propolis components. The components and amount of propolis extracted via the dichloromethane immersion stirring method were the best. After the propolis was extracted with dichloromethane, PLA was directly added to the propolis dichloromethane solution to prepare an electrospinning solution. A DEP/PLA nanofiber membrane was prepared via the electrospinning process, which has antibacterial properties. We then tested the antibacterial properties of the electrospun fiber membrane. The DEP/PLA nanofiber membrane could be used in tissue engineering or food preservation.

Materials and methods

Materials

The propolis was purchased from Qiupu Healthy Product Co., Ltd. (Anhui, China). The polylactic acid (PLA, MW = 170000) was purchased from Hisun Chemical Co., Ltd (Zhejiang, China). The analytical grade anhydrous ethanol was purchased from Prospect Chemical Reagent Co., Ltd. (Jiangsu, China). The ligarine was purchased from Sanhe Chemical Co., Ltd (Jiansu,China). The dichloromethane was purchased from Qiaosun Fine Chemical Co., Ltd. All other chemicals and solvents were analytical grade. Double-distilled water was used in this study. The *Penicillium, mucor*, A*spergillus niger, S. aureus, Escherichia coli*, beef extract peptone medium, and malt-agar medium for the fungi were purchased from Fuxiang Biological Technology Co., Ltd (Shanghai, China). No chemicals or solvents were altered.

Methods

Propolis extraction

The crude propolis contained plant resins, beeswax, and insoluble material. The four procedures used to extract the bioactive components were the 90% ethanol leaching agitation method, 70% ethanol leaching method, ethanol-ligarine extraction method, and dichloromethane immersion stirring method. The crude propolis was solid and had a high viscosity at room temperature, making mechanical disintegration difficult. This meant that the crude propolis required cooling (preferably 5 °C) in a refrigerator, where it could be crushed into a powder and then filtered with a mesh (the 14 mesh sieve (1.4 mm)).

The 90% ethanol leaching agitation method was performed with 10 g of propolis powder that was placed into a sealed glass. The absolute ethanol was diluted to 90% ethanol, 40 ml of which was added to 10 g of propolis and then stirred with a magnetic stirrer for 6 hours. The supernatant was filtered with a glass filter, then 40 ml of 90% ethanol was added, and stirring again for 6 hours. The second supernatant was extracted with the glass filter and mixed with the previous mixture. The residual impurities were weighed after being dried naturally outdoors for 2 days. The 70% ethanol leaching method was performed with 10 g of propolis powder placed in a sealable glass and mixed with 40 ml of 70% ethanol. The mixture was extracted after being leached outdoors for 3 days with a glass filter. The remaining impurities were dried and then weighed, as seen in the first method. The ethanol-ligarine extraction method was performed by putting 20 g of propolis powder in a sealable glass together with 80 ml of 90% ethanol and 40 ml of 60~90 ligarine. The mixture was stirred for 4 hours with the magnetic stirrer, then filtered, dried, and weighed (as seen with the first method). The dichloromethane immersion stirring method was performed by adding 10 g of propolis into 100 ml of dichloromethane in a sealed glass bottle. The mixture was stirred by magnetic stirrer agitation for 4 hours, after which the supernatant was filtered using a glass filter, and the remaining impurities were weighed after naturally drying for 2 days [33].

Preparation of DEP/PLA electrospun fiber membrane

The control PLA polymer base electrospun fibers had either 1%, 2%, 3%, or 4% (w/v) PLA dissolved in supernatants of the propolis extraction and dichloromethane mixture, which was subjected to 15 kV voltage, with an injection time of 20 h, flow rate of 0.7 ml/h, and receiving distance of 10 cm. In this paper, fibres were electrospun from a single syringe needle.

The antibacterial test

The antibacterial test required the preparation of a bacterium suspension with 10^6~10^7 living bacteria per milliliter. This was done by joining sterilised normal saline in a beef extract peptone medium and a malt-agar medium. The test-bacteria required a cooling down period, where the ready-prepared sterilised medium was cooled to 50~60 °C, and then

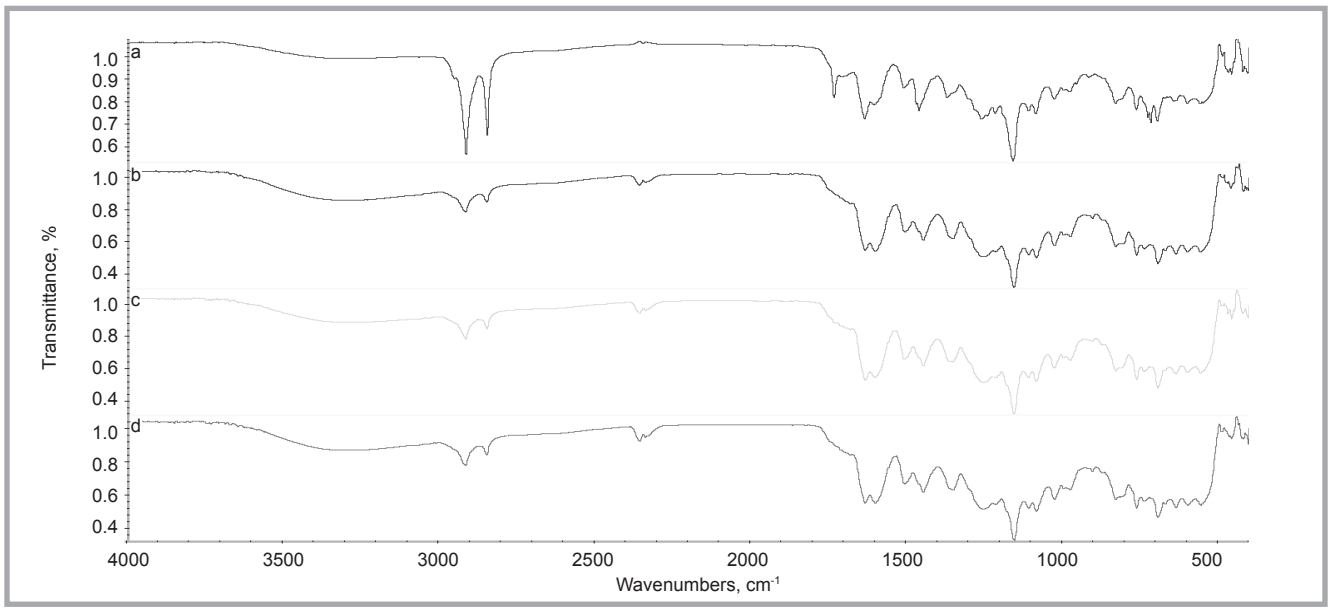

Figure 1. *FTIR of propolis extract.*

infused on petri dishes to obtain a platy form. Each type of test-bacteria was placed on a sterilised plate with sterilised droppers at a volume of 0.2 ml, where the glass was spread evenly using a sterilised glass spatula. Three pieces of the DEP/PLA electrospun fiber membrane were placed on the test plates equidistant to each other, which was repeated thrice. Each processed culture dish was put inversely under the optimum temperature for the test-bacteria inside, the bacteria was then incubated at 37 °C for 24 hours and the mold at 28 °C for 48 hours. The diameter of the bacteriostatic ring was measured after incubation [34].

Fourier transform infrared spectroscopy(FTIR) analysis

The supernatants of the four procedures were placed outdoors to obtain crude propolis extraction via the solvent evaporation method. FTIR spectra were obtained on a BRUKER TENSOR 27 FTIR meter, with an annex ATR acquisition of the spectrum that scanned 64 times, at a resolution of 4 wave numbers.

Morphological characterisation of the DEP/PLA electrospun fiber membrane

A scanning electron microscope (SEM) (Hitachi S-4800) was used to quantify the morphology and surface of the electrospun fibers. Samples were sputtered with gold and pictures taken. The diameters of more than 50 electrospun fibers were measured with the SEM images using Image J software. The average diameters and standard deviations were calculated.

Results and discussion

Comparison of propolis extracting yield

The propolis extraction yield was calculated by comparing the original weight of the propolis with the impurities extracted. Each testing method was performed 5 times and averaged. Results are shown in ***Table 1***.

Table 1 depicts the yields, which were similar across the different extraction methods. The highest yields of propolis were obtained by the 70% ethanol leaching method and dichloromethane immersion stirring method. The lowest yield was obtained by the 90% ethanol leaching agitation method. The propolis had a complex composition, with hundreds of ingredients, which varied by area. The types of propolis included flavonoids, terpene compounds, organic acids, lipids, alcohols, aldehydes, ketones, steroids, vitamin B, vitamin A, amino acids, enzymes, polysaccharides, and minerals [24]. The data from ***Table 1*** shows that the extraction yield via 70% ethanol-water solution with a smaller mass fraction was higher than that via the 90% ethanol-water solution method. This could be because of the dissolution rate of water-soluble substances in propolis, including amino acids, vitamins, and microelements, when in water-soluble salt.

Fourier transform infrared spectroscopy (FTIR) analysis of propolis extract (PE)

Figure 1 graph ***a)*** depicts the infrared spectrum of the propolis extract from the dichloromethane method, graph ***b)*** the infrared spectrum of the propolis extract from the 70% ethanol method, graph ***c)*** the infrared spectrum of the propolis extract from the 90% ethanol method, and graph ***d)*** shows the infrared spectrum of the propolis extract from ligarine. ***Figure 1*** demonstrates that there was a significant difference between graph a) and the other curves. Graphs ***b)***, ***c)*** and graph ***d)*** were similar.

The ***Figure 1.a*** shows infrared spectrum analysis of the propolis extract from dichloromethane. The absorption peak of the alcohol-ether associated -OH stretching vibration absorption occurred at 3301.87 cm^{-1}. The peak shift of the alcohol and phenol's C-O stretching vibration absorption occurred at 1088.10 cm^{-1}. The asymmetric stretching vibration of

Table 1. *Propolis extraction yield.*

Experimental Method	Original weight	Impurity content	Standard deviation	Yield of propolis
90% ethanol leaching method	10g	2.13g	0.011	78.7%
70% ethanol leaching method	10g	2.02g	0.013	79.8%
Ethanol-ligarine extraction method	20g	4.17g	0.027	79.2%
Dichloromethane immersion stirring method	10g	2.03g	0.013	79.7%

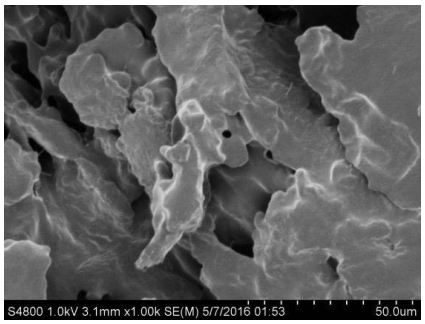

Figure 2. *SEM image of DEP/1% PLA membrane.*

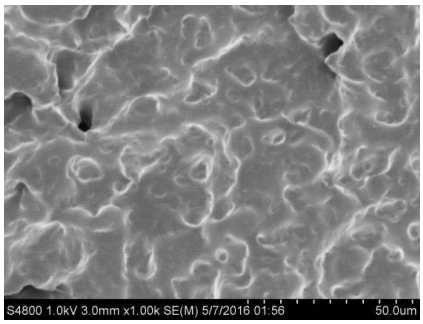

Figure 3. *SEM image of DEP/2% PLA membrane.*

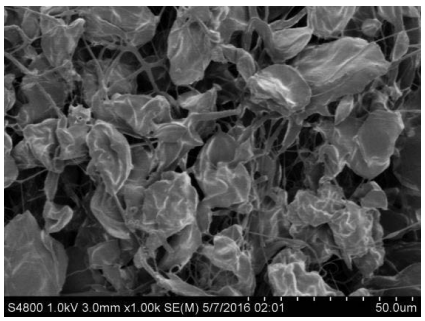

Figure 4. *SEM image of DEP/3% PLA membrane.*

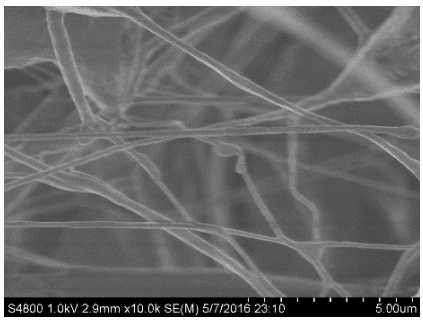

Figure 5. *SEM amplification image of DEP/3% PLA fiber.*

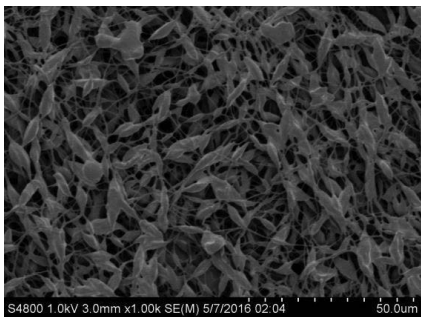

Figure 6. *SEM image of DEP/4% PLA membrane.*

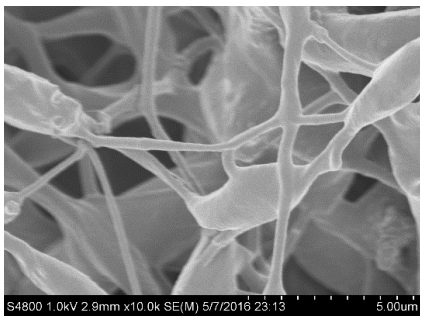

Figure 7. *SEM amplification image of DEP/4% PLA fiber.*

the lipidic and ether methine -CH_2 occurred at 2916.87 cm^{-1}. The symmetric stretching vibration of the methine -CH_2 occurred at 2849.01 cm^{-1}. The stretching band of the C = O from the aldehydes, ketones, carboxylic acids, and the carboxylic acid derivatives occurred between 1736.10 cm^{-1} to 1637.59 cm^{-1}. The vibration observed at 1607.08 cm^{-1} was attributed to the asymmetric stretching absorption of the C-C of the terpenoids. The asymmetric stretching vibration absorption peak of the aromatic nitro compounds' nitro occurred at 1511.96 cm^{-1}. The peak that occurred at 1462.94 cm^{-1} appeared to be caused by the out-of-plane bending absorption peak of the -CH_3 of lipids and saccharides (glycosides). The peak that occurred at 1373.55 cm^{-1} was a result of the bending absorption peak of the lipidic -CH_3. The C-O and C-OH of terpenoids, phenols, and glycosides had a stretching vibration absorption peak at 1219.09 cm^{-1}. The stretching vibration of the aromatic ethers (Ar-O) had an absorption peak at 1263.24 cm^{-1}. The R-O had an absorption peak at 1088.12 cm^{-1}. The polyether construction -C-O-C-O-C- of the acetal compositions had absorption peaks at 1161.91 cm^{-1} and 1029.26 cm^{-1}. There was a characteristic absorption peak at 1111.58 cm^{-1}, which was caused by the bending vibration absorption of the C-H becoming linked with oxygen.

Figure 1.b has characteristic absorption peaks at 2916.87 cm^{-1} and 2849.01 cm^{-1}, where the transmittance of the -CH_2 of methine was less than that seen at the characteristic absorption peaks that occurred at 2916.87 cm^{-1} and 2849.01 cm^{-1}. This showed that the propolis that was extracted with dichloromethane had a higher content of methylene compounds. ***Figure 1.b*** shows the lack of a stretching vibration absorption peak of carbon based C = O at 1736.10 cm^{-1}, which further demonstrated that the propolis extracted with dichloromethane had richer types of compounds. ***Figure 1.a*** shows graphs comparing the other extraction methods, where the method with dichloromethane received more types of compounds. In the other methods, the components extracted had no significant difference.

SEM analysis of DEP/PLA

Figure 2 depicts SEM images of the DEP/PLA membrane, which is an electrostatic spun thin-membrane made from the propolis solution that was extracted by dichloromethane with 1% PLA. The SEM images demonstrated that the membrane surface was composed of irregular particles, which was a significant feature of the electrostatic spraying. The membrane surface was uneven and rough because the irregular particles had thin gaps between them. The lumps were joined together through contact points, with empty spaces persisting between them, which caused the entire structure to seem loose, and the membrane was brittle with poor elasticity.

Figure 3 depicts SEM images of the electrospun membrane composed of DEP/2% PLA. The figures show that the membranes still did not have obvious fibers, and the structure was composed of particles that were overlying and leaning on each other. The membrane surface was relatively smooth, with some small concave and convex points. There were some raised and lowered holes on the contact points, but the membrane seemed coagulated instead of loose.

Figure 5 depicts an SEM image of the electrospun membrane composed of DEP/3% PLA. The Figure demonstrates that fibers and particles co-existed in the membrane. The fibers were distributed with large differences in diameter, ranging from 52~462 nm, where the average diameter of fibers was 172 nm. The obvious differences in diameter were attributed to the diverse oligomers in the propolis. Sometimes the oligomers with high viscosity attached to the fibers or adhered to them to create a bolder fiber, thus increasing the diameter. ***Figure 4*** depicts fibers within the DEP/3% PLA membrane, where the membrane formed

approached combined electrospun and electrostatic sprayed membranes, with electrostatically sprayed being the primary similarity. This shows that as the major components in the propolis, the oligomers and small molecules, were unable to form an electrostatically spun membrane without a polymer like PLA. PLA was the key in membrane formation. The 3% content of PLA was a low value, and there were only few fibers that were formed from the spinning solution via electrostatic spinning.

Figure 6 illustrates an SEM image of the membrane comprised of DEP/4% PLA. These two Figures show that the 4% PLA membrane had a higher fiber content. Within the entire electrospun membrane, there was an even distribution of spindle fibers, which showed a comparative uniformity. The other shaped fibers were also distributed more evenly. The fiber diameter distribution ranging between 200~540 nm had a mean value of diameter of 315 nm, as seen in ***Figure 7***. The width of the spindle fibers were between 0.5~2 μm. The formation of a DEP/PLA membrane with 4% PLA was characteristic of electrostatic spinning. There could be two main reasons for such an increase in spindle fibers in the 4% PLA membrane: Many oligomers and small molecules in the propolis could be stretched evenly by the electrostatic field and thus were often clumped together. The formation of some special gloss spindle nanofibers was caused by the propolis extract, which had some adhesive properties, adhering the fibers together.

Antibacterial test of DEP/PLA electrospun membrane

As shown in ***Table 2***, the electrospun membrane of pure 4% PLA had no anti-bacterial properties. In contrast, the DEP/PLA electrospun membrane had satisfactory antibacterial performance. The DEP/PLA membrane was able to restrain *Staphylococcus aureus* most successfully, although the suppression between *Escherichia coli* was the worst. The strong antibacterial activity of propolis was the result of a synergistic effect between the components [35].

Table 2. *Antibacterial performance of DEP/4% PLA Electrospun membrane.*

Inhibition zone diameter	Penicillium, mm	Mucor, mm	Aspergillus niger, mm	Staphylococcus aureus, mm	Escherichia coli, mm	Bacillus subtilis, mm
DEP/PLA	9.5	15	11	19	8	11
PLA	0	0	0	0	0	0

Conclusions

This article used four different methods to extract the active ingredients of propolis (the 90% ethanol leaching method, 70% ethanol leaching method, Ethanol-ligarine extraction method, and Dichloromethane immersion stirring method. Among these methods, dichloromethane extracted more components from the propolis and obtained more methylene compounds. Then we took DEP supernatants and added PLA directly as the electrospinning solution for electrostatic spinning. This method did not require the distillation of chloroform to purify the propolis, which ensured that the active ingredients of the propolis were not lost. By adding 4% PLA to the DEP supernatants, we produced stable nonwoven membranes by the electrospinning process. This type of DEP/PLA membrane had a good antibacterial effect, particularly against *Staphylococcus aureus*. The PLA and propolis extract could be absorbed and degraded by the organism. The DEP/PLA membrane had a good anti-bacterial effect, which could be used as raw material for food preservation and wound dressings, such as trauma, burns, and mouth ulcers. The membrane could also be used in tissue engineering. However, this article lacks a detailed antibacterial process and the antibacterial properties of other important strains, although a suitable biological laboratory is being set up to perform related antibacterial demonstration experiments. In the future, our group will study the effects of various factors on the morphology of propolis electrospun membranes, in order to study the antibacterial properties of propolis on more strains, to research the antibacterial mechanism of propolis electrospun membranes, to systematically study the components of propolis and propolis electrospun membranes, as well as to investigate the effect that propolis and its conductive composites have on stimulating the growth of tissue cells.

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