THESIS

NANOSPRAY-ON DRESSING FROM BIODEGRADABLE POLYMER AND THAI HERBAL EXTRACT

WHIJITRA SUVANDEE

GRADUATE SCHOOL, KASETSART UNIVERSITY
Academic Year 2021

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THESIS

NANOSPRAY-ON DRESSING FROM BIODEGRADABLE POLYMER AND THAI HERBAL EXTRACT

WHIJITRA SUVANDEE

A Thesis Submitted in Partial Fulfillment of
the Requirements for the Degree of
Master of Science (Nanomaterials Science)
Graduate School, Kasetsart University
Academic Year 2021

Whijitra Suvandee : Nanospray-on Dressing from Biodegradable Polymer and Thai Herbal Extract. Master of Science (Nanomaterials Science), Major Field: Nanomaterials Science, Department of Materials Science.
Thesis Advisor: Assistant Professor Decha Dechtrirat, Dr.rer.nat.
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In this work, a facile one-pot preparation of spray-on wound dressing based on polyvinylpyrrolidone (PVP) hydrogel containing silver nanoparticles as a broad-spectrum antimicrobial agent and antioxidant *Phyllanthus emblica* extract with wound healing properties was demonstrated. Silver nanoparticles were synthesized through a green chemical method using *Phyllanthus emblica* extract as a biogenic reducing agent in the preformed polyvinylpyrrolidone solution. The biocompatible PVP was used as a film-forming agent to produce an adhesive hydrogel-based dressing matrix to provide a moisture balance and a protective barrier for the wound bed and to control the release of fruit extract to promote wound healing. The presence of silver nanoparticles in the dressing solution was confirmed using UV-VIS spectroscopy, X-ray diffraction (XRD), and transmission electron microscopy (TEM). The surface morphology of the sprayed film was assessed using scanning electron microscopy (SEM), respectively. After being sprayed, the adhesive hydrogel film was rapidly formed within seconds. Further *in vitro* studies demonstrated that the prepared dressing film showed a controlled release of the fruit extract, antioxidant activities, and a potent antibacterial effect against *S. aureus*, *P. aeruginosa*, *E. coli*, and MRSA. Moreover, the investigation on biocompatibility also revealed that the dressing film exhibited no toxicity to both human fibroblasts and keratinocytes. According to the aforementioned results, the present spray-on solution could be a promising candidate for antibacterial wound dressing.

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Student's signature Thesis Advisor's signature

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I want to express my sincere appreciation and credit to the following that helped make this research possible and successful throughout the course of my master's degree.

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INTRODUCTION

Conventional wound dressings such as cotton wool, bandages, lint, gauze, and plasters are often used as main or supplementary dressings for clean and dry wounds with low discharge levels to safeguard the site from contamination (Dhivya et al., 2015). Conventional dressings, on the other hand, do not offer a moist environment for the wound. They are normally prefabricated and need a layer of adhesive covering for attachment. They are also difficult to put on wounds with uneven forms and wide regions. Such dressings typically need replacement often to avoid fluid buildup, which is unpleasant, disrupts the healing skin, and may boost the risk of contamination (Daristotle et al., 2020). Modern dressings, on the other hand, are meant to keep the wound moisturized and encourage healing as well as protect it from infection. Semi-porous films, semi-porous foams, hydrogels, hydrocolloids, and alginates are some examples of modern wound dressings. Nonetheless, concerns about the aforementioned dressings exist, such as user-friendliness, flexibility, and transportability (Islam et al., 2021).

Such problems can be handled by inventing a quick gel-forming spray that can be put directly to the wound without coming into contact with hands or cotton swabs, lowering the risk of infection or contaminants. Typically, these sprays generate thin, adhesive coatings, eliminating the risk of further trauma while dressing or re-dressing the wound (Retnowati et al., 2021). Intimate engagement with the injured area, simple and fast treatment to vast and uneven wounds, minimal discomforts during treatment, many uses with a portable container, and ultimately improved patient compliance are all advantages of this approach (Boonmak et al., 2018). In addition to promoting tissue repair, the spray-on wound dressing can deliver drugs that limit the chance of infection and inflammation. Drug dosages in a film-forming spray can also be changed depending on the volume of solution in each spray (Umar et al., 2021). In comparison to patches, a thin film created from the spray is effortless to wash off with just water (Ranade et al., 2017) and can improve patient comfort while moving (Devaux et al., 2012; Tan et al., 2012). Due to their ease of use and benefits, spray-on dressings are becoming progressively popular (Boonmak et al., 2018; Catanzano et

al., 2015; Daristotle et al., 2020; Du et al., 2020; Islam et al., 2021; Li, Y. et al., 2021; Ranade et al., 2017; Retnowati et al., 2021; Sritharadol et al., 2017; Umar et al., 2021).

Antimicrobial drugs serve a critical function in bacterial burden reduction. Silver nanoparticles (AgNPs) have been shown to successfully limit the growth of both common and drug-resistant bacterial strains in many studies. It exhibits broad anti-bacterial efficacy against both gram-positive and gram-negative bacteria, with less bacterial resistance formation (Bruna et al., 2021). Lately, nanotechnology has made it possible to produce many kinds of AgNPs. Because of their small size, AgNPs have a higher sur-face area-to-mass ratio, which allows them to make better contact with bacteria and have a stronger antibacterial effect as a result (Wang et al., 2017). It is worth noting that the typical chemical reducing agents used in AgNP synthesis are toxic and could cause health problems. As a consequence, researchers have documented the utilization of plant extracts in the green synthesis approach to manufacture and stabilize metallic nanoparticles (Vanlalveni et al., 2021). Many publications have detailed the addition of AgNPs into dressings in varying forms, such as foams, hydrogels, and polymeric films, each claiming to have different advantages, but all claiming silver's bactericidal effectiveness (Kalantari et al., 2020; Nqakala et al., 2021). In addition to assisting in the creation of metallic nanoparticles, plant extracts are a good source of bioactive chemicals, with a wide diversity of secondary metabolites that have a wide range of pharmacological effects.

Phyllanthus emblica is a tropical and subtropical plant used in folk medicine for a long time to treat common colds and fevers, sore throats, coughs, dry mouth, diarrhea, inflammation, and wounds, either alone or in combination with other components (Variya et al., 2016; Zhang et al., 2017). The effect of *Phyllanthus emblica* extract on wound healing has been documented, including mechanisms involving collagen growth, extracellular matrix (ECM) protein synthesis, and antioxidant status (Chularojmontri et al., 2013; Kumar et al., 2008; Sumitra et al., 2009). *Phyllanthus emblica* was discovered to have strong antioxidant and anti-inflammatory properties in previous investigations. Polyphenols, flavonoids, tannins, and vitamins are the main active phytochemicals typically found in *Phyllanthus emblica*, particularly the fruits. Polyphenols, including gallic acid, ellagic acid, and

chebulagic acid, are thought to be the main active ingredients behind *Phyllanthus emblica*'s anti-inflammatory and antioxidant properties (Li, W. et al., 2020; Yang et al., 2014). Polyphenols have been shown to be potent antioxidizing agents (Muthuraman et al., 2011; Saha et al., 2015) due to their abilities to donate/accept electrons and delocalize the unpaired electron inside their aromatic structure. For wound healing, antioxidants are thought to help with tissue repair by assisting in the defense against oxidative stress and inflammation (Catanzano et al., 2015). To have a long-lasting effect of antioxidants, the incorporation of antioxidants into sustained-release polymeric systems is one of the recognized approaches for extending antioxidant effects.

Polyvinylpyrrolidone (PVP) is a water-soluble and biodegradable polymer. PVP has various distinct physical and chemical characteristics including being chemically inert, colorless, temperature-resistant, and pH-stable (Franco et al., 2020). It also has excellent solubility in solvents of various polarities and superior adhesive qualities. As it is non-toxic and biocompatible, PVP is used extensively in the pharmaceutical and biomedical industries (Teodorescu et al., 2015). It has been employed in the development of a variety of drug delivery systems, including oral, ophthalmic, topical, and transdermal delivery in a variety of formats, such as particles, hydrogels, and fibers (Franco & De Marco, 2020; Waleka et al., 2021). PVP can also be used to create clear, thin, and well-distributed films (Umar et al., 2021). Since PVP keeps skin moist and prevents scab development, it can be applied as the core element in topical coverings for skin regeneration and wound dressings (Rogero et al., 2003; Yoo et al., 2008). PVP is also acknowledged as one of the most effective polymeric stabilizers for silver nanoparticles (de Lima et al., 2018).

Accordingly, this study set out to create a simple methodology for manufacturing a spray-on wound dressing with silver nanoparticles as a broad-spectrum antibacterial agent and *Phyllanthus emblica* extract as antioxidants to promote wound healing. The spray-on solution was made in one pot using a natural fruit extract made from *Phyllanthus emblica* for the green synthesis of silver nanoparticles (**Figure 1**). The adhesive hydrogel film will form quickly after being sprayed and act as a protective layer to keep moisture in the wound bed and keep germs out. Furthermore, the produced gel film serves as a polymeric-based matrix in

this scenario, controlling the discharge of the fruit extract and extending its antioxidant effects. A variety of procedures were used to characterize the prepared dressing. To illustrate the possibility of using the synthesized materials as wound dressings, in vitro release, antioxidant, antibacterial, and cytotoxicity tests were conducted.

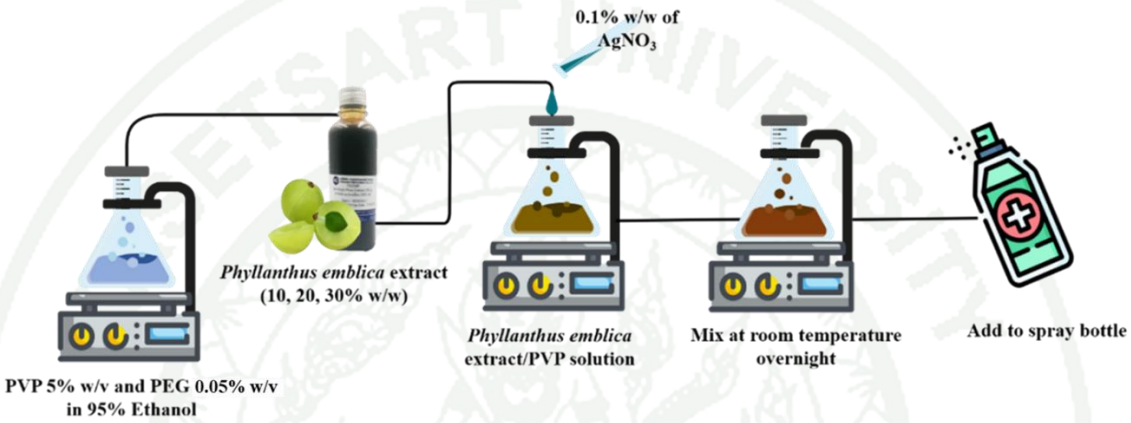


Figure 1 Schematic illustration of the one-pot synthesis of *Phyllanthus emblica* extract/silver nanoparticles/polyvinylpyrrolidone spray-on wound dressing

OBJECTIVES

1. To develop a prototype of antibacterial spray-on wound dressing using PVP as an adhesive film-forming component, silver nanoparticles as a broad-spectrum antimicrobial agent, and *Phyllanthus emblica* extract as a natural antioxidant.

2. To *in vitro* study the release of the fruit extract, antioxidant and antibacterial activities, and cytotoxicity of the prepared spray-on dressing.

LITERATURE REVIEW

Green synthesis of silver nanoparticles using plant extract

Singla et al. reported a protocol for the preparation of biocomposites from bamboo cellulose nanocrystals and AgNPs. In this work, AgNPs were synthesized in situ using *Syzygium cumini* leaf extract as a green reducing agent in the present of bamboo cellulose nanocrystals. The prepared biocomposites were successfully used to treat diabetic wound in the streptozotocin induced diabetic mice model. The results showed full recovery of wound within 18 days which is in contrast to the control group (**Figure 2**). The levels of proinflammatory cytokines IL-6 and TNF-α were reduced after the treatment, while the expression of collagen and growth factors were found to notably increased (Singla et al., 2017).

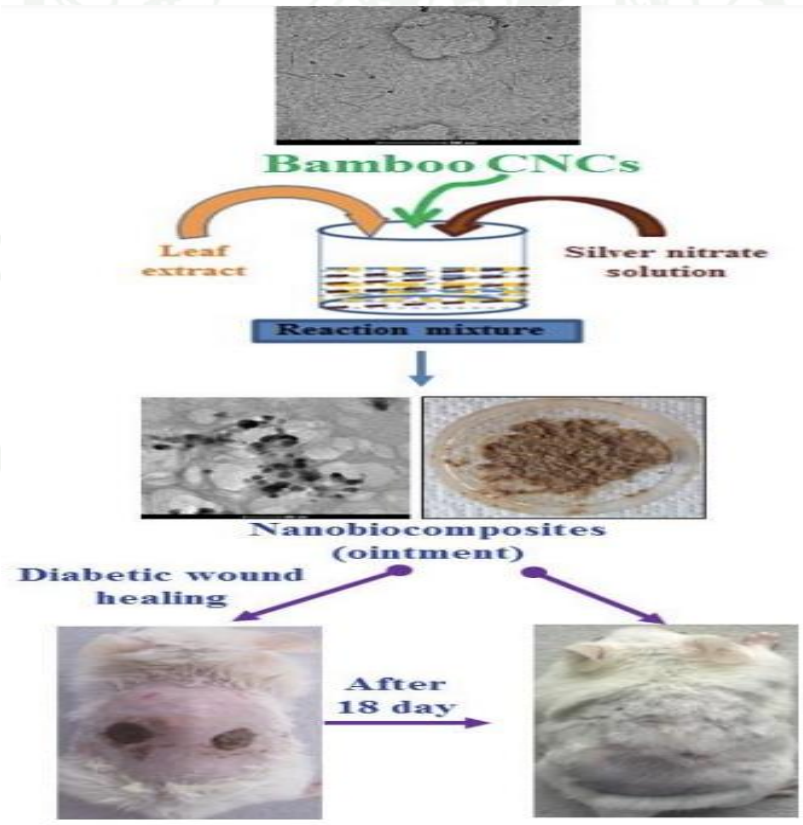


Figure 2 Preparation of bamboo cellulose nanocrystals/AgNPs biocomposite ointments for diabetic wound healing.

Source: Singla et al. (2017)

Jadhav et al. also demonstrated a green protocol to synthesize AgNPs using an aqueous extract of *Ammania baccifera* (**Figure 3**). The as-synthesized particles were thoroughly characterized by various analytical tools. A strong SPR absorption peak was observed at around 440 nm and the particle size was found to be around 113 nm using a Zetasizer. XRD and TEM confirmed the presence of crystalline AgNPs. The prepared AgNPs also exhibited excellent antibacterial properties against *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and methicillin resistant *Staphylococcus aureus* (MRSA) which are as efficient as Silverex™, the available formulation in the market (Jadhav et al., 2016).

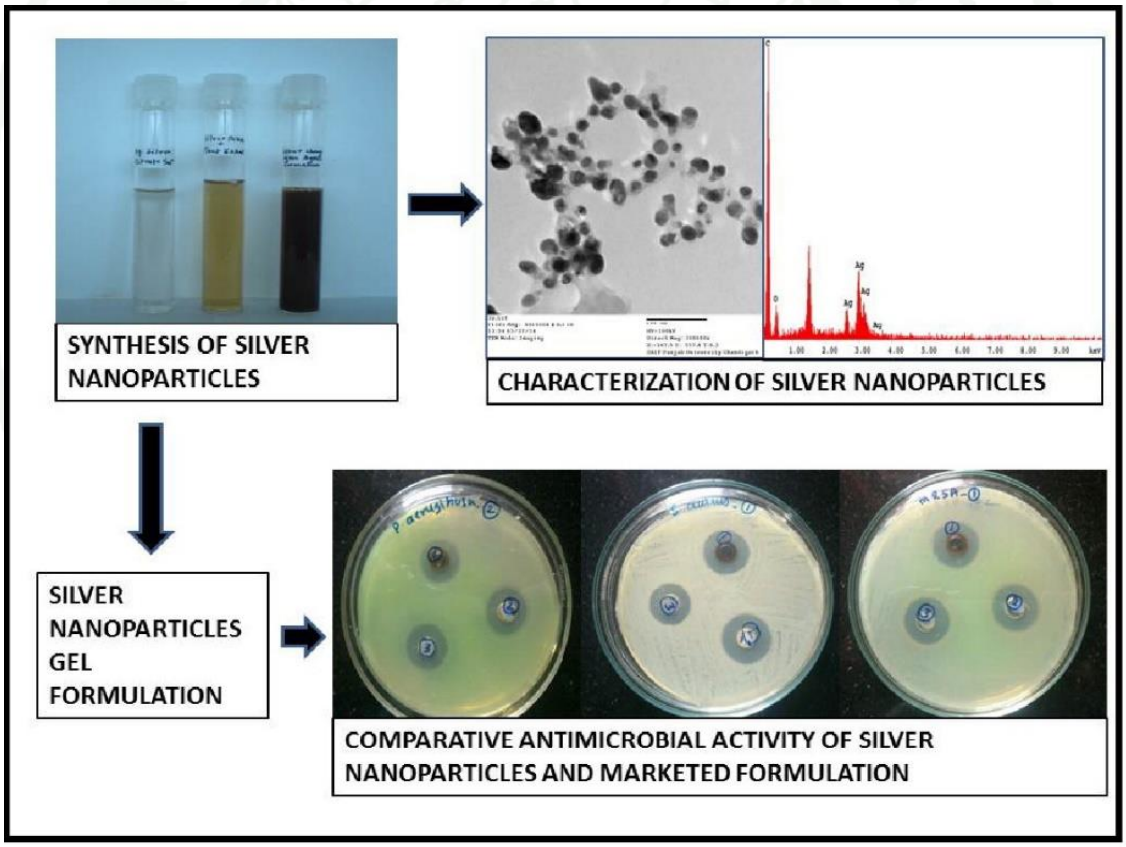


Figure 3 Silver nanoparticles (AgNPs) synthesized by simple treatment of silver nitrate with aqueous extract of *Ammania baccifera* and their antibacterial activities
Source: Jadhav et al. (2016)

Bharadwaj et al. demonstrated a successful green syntheis of AgNPs using the fruit extract of *Diospyros malabarica* (**Figure 4**). The obtained particles have size around 17.4 nm and showed good antibacterial activity against *Staphylococcus aureus* and *Escherichia coli*. Moreover, the obtained particles also exhibited good anticancer effect toward the U87-MG (human primary glioblastoma) cell line with the IC50 value of around 59 µg/mL. Additionally, the obtained particles also exhibited catalytic activity toward the reduction nitrophenols, pollutants as stated by the United States Environmental Protection Agency (USEPA) (Bharadwaj et al., 2021).

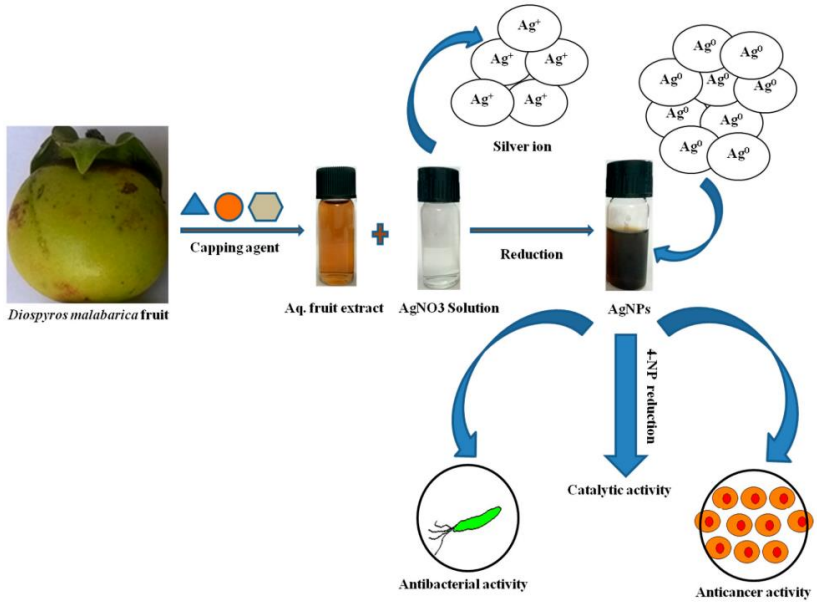


Figure 4 Schematic representation of the green synthesis of silver nanoparticles (AgNPs) by *Diospyros malabarica* fruit extract and their applications.
Source: Bharadwaj et al. (2021)

Besides the well-known antibacterial properties of AgNPs, potent antifungal properties of AgNPs synthesized by green reduction of $AgNO_3$ with *Glycosmis pentaphylla* extract has been demonstrated by Dutta et al. (**Figure 5**). The obtained AgNPs exhibited strong antifungal activities against *Alternaria alternata*, *Colletotrichum lindemuthianum*, *Fusarium moniliforme*, and *Candida glabrata*) and antibacterial properties against *Bacillus subtilis*, *Streptococcus mutans*, *Escherichia coli*, and *Salmonella enterica* (Dutta et al., 2022).

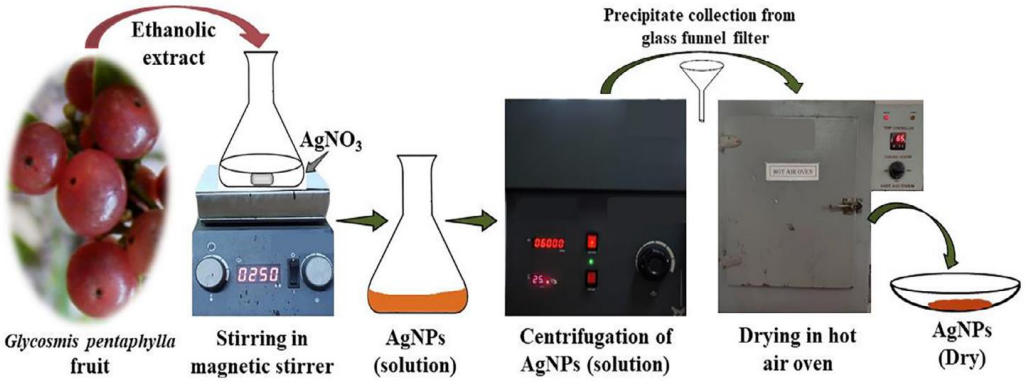


Figure 5 Green synthesis of AgNPs using *Glycosmis pentaphylla* extract
Source: Dutta et al., (2022)

Recently, Meena et al. have demonstrated the successful synthesis of AgNPs using the fruit extract of *Phyllanthus emblica* (**Figure 6**) (Meena et al., 2020). Singh et al. also reported the use of *Phyllanthus emblica* extract to synthesize AgNPs. Further investigation on the biological activities of the prepared AgNPs revealed that the prepared particles showed a chemoprotective potential in the prevention and intervention of hepatocellular carcinoma (Singh et al., 2019).

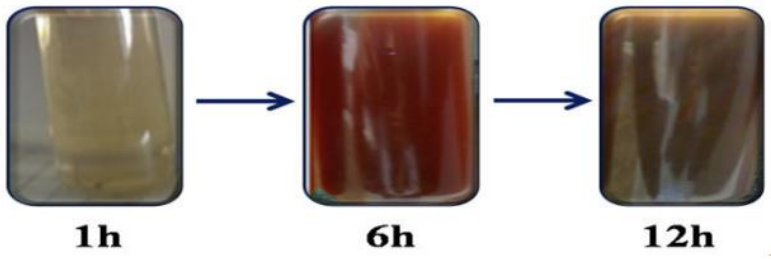


Figure 6 *Phyllanthus emblica* fruit extract after the addition of silver salt
Source: Meena et al. (2020)

Silver nanoparticles impregnated wound dressings

Alippilakkotte et al. prepared AgNPs by the in situ reduction of $AgNO_3$ using the bitter gourd extract in the diphase medium containing PLA. After that, the preformed solution was electrospun to obtain the AgNPs impregnated PLA nanofibers (**Figure 7**). The prepared PLA/Ag nanofiber mat was tested against *Escherichia coli* and *Staphylococcus aureus* and the result revealed good antibacterial properties.

Further in vitro cytotoxicity study also revealed that the nanofiber mat exhibited no toxicity toward fibroblasts and did not impair cell growth, indicating good potential for this material for wound dressing application (Alippilakkotte et al., 2017).

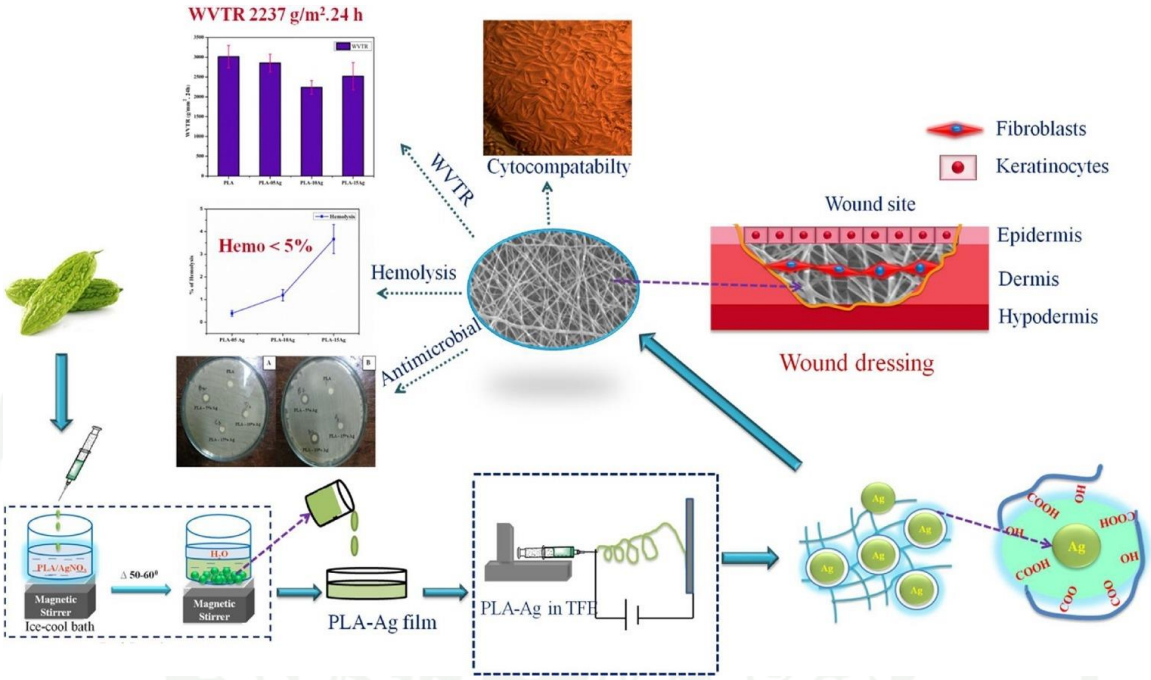


Figure 7 Preparation of electrospun PLA nanofibers containing stabilized AgNPs
Source: Alippilakkotte et al. (2017)

Khampieng et al. developed AgNPs/PVP hydrogels as antibacterial wound dressings using γ-irradiation. The prepared hydrogels exhibited strong antibacterial activity and showed no toxicity toward the tested mouse fibroblasts (Khampieng et al., 2014).

Archana et al. also prepared a wound dressing film based on a composite of chitosan, PVP, and AgNPs. The prepared material showed potent antibacterial activity arising from synergistic effect between chitosan and AgNPs. The dressing film also exhibited no toxicity toward L929 cell lines and showed improved wound healing properties on the adult male albino rats in comparison to the cotton gauge and other chitosan-based dressings (Archana et al., 2015).

Samadi et al. developed chitosan/PVA based hydrogel impregnated with AgNPs as antibacterial agent and sildenafil as an angiogenesis stimulant (**Figure 8**). The proposed wound dressing exhibited not only good antibacterial properties, but also significant would healing ability in comparison to the other control tests (Samadi et al., 2020).

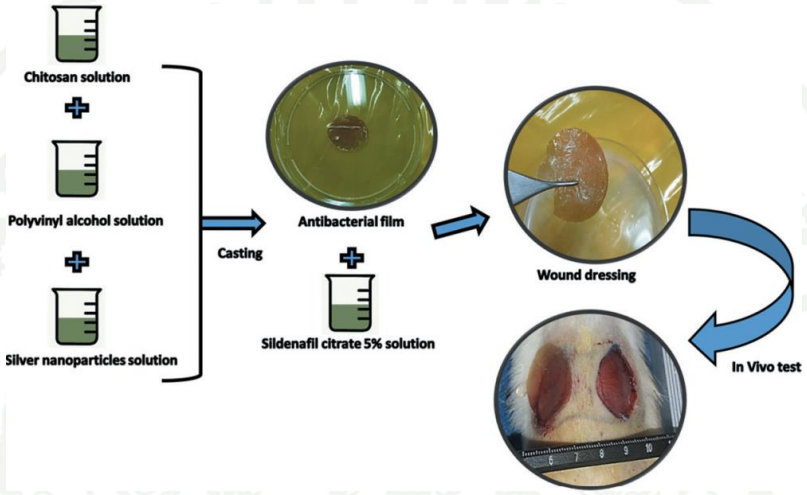


Figure 8 Fabrication of chitosan/PVA hydrogel films impregnated with AgNPs and sildenafil citrate
Source: Samadi et al. (2020)

Liu et al. developed a composite film of sericin (SS) and agar coated with AgNPs impregnated polydopamine (DPA) layer as a wound dressing material. SS and agar solutions were mixed, casted and dried at 60°C to obtain the composite film base. Afterward, the preformed film was soaked into the PDA solution and subsequently $AgNO_3$ was added. AgNPs were formed through a green reduction with PDA. The obtained AgNPs-PDA- SS/Agar film showed excellent and long-lasting antibacterial activity but no toxic toward the fibroblast NIH/3T3 cells (Liu et al., 2018).

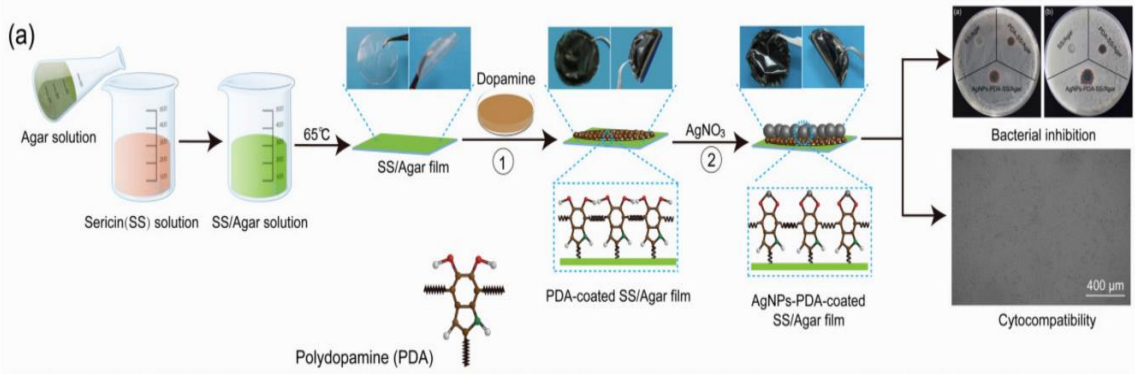


Figure 9 Preparation of antibacterial AgNPs-PDA-SS/Agar film
Source: Liu et al. (2018)

Spray-on wound dressing

Catanzano et al. developed a spray-on dressing using alginate as a film forming agent. Tea tree oil microemulsion was also loaded into the spray formulation aiming to promote the antibacterial properties of the dressing. After being sprayed with the chitosan solution, an ionic crosslinker solution was sprayed directly on top to produce a hydrogel film of crosslinked chitosan (**Figure 10**). As expected the spray dressing showed good antibacterial activity against the tested strain (Catanzano et al., 2015).

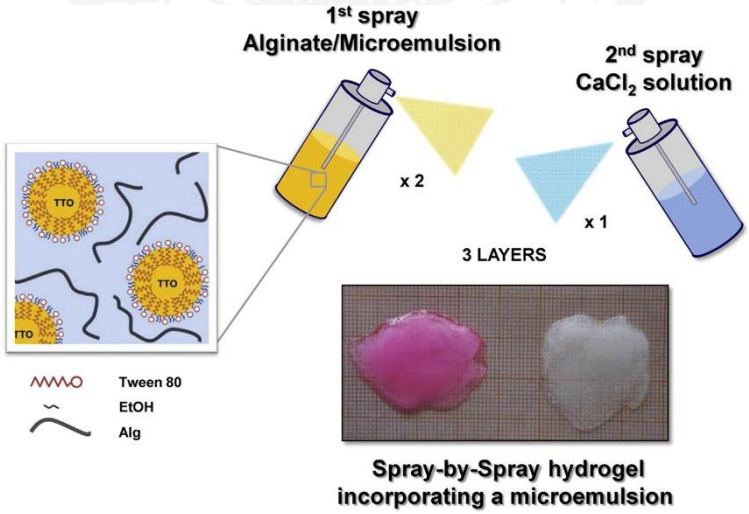


Figure 10 The spray-by-spray hydrogel containing tea tree oil microemulsion
Source: Catanzano et al. (2015)

Another spray-on dressing was prepared by Sritharadol et al. using Eudragit E100 as a film former and mupirocin as a potent antibacterial agent. The spray was simply prepared by dissolving mupirocin in the Eudragit solution and then the spray solution was loaded inside the spray canister. Release study revealed that up to 90% of mupirocin was discharged within 2 h. The spray also had a potent antibacterial activity against *S. aureus* and *S. epidermidis* and safe to keratinocytes, fibroblasts and monocytes. The spray also did not stimulate the production of NO and inflammatory cytokines (IL-1b and TNF-a) (Sritharadol et al., 2017).

Boonmak et al. successfully developed a poly(vinyl acetate) spray-on antibacterial wound dressing loading with mangosteen extract (**Figure 11**). The antibacterial test showed successful bactericidal activities against various bacterial strains. In vitro cytotoxicity test also revealed no toxicity toward L929 mouse fibroblasts, human keratinocytes, and human fibroblasts. Using poly(vinyl acetate) as a film forming agent, the dressing can extend the release of the extract as long as 60 h (Boonmak et al., 2018).

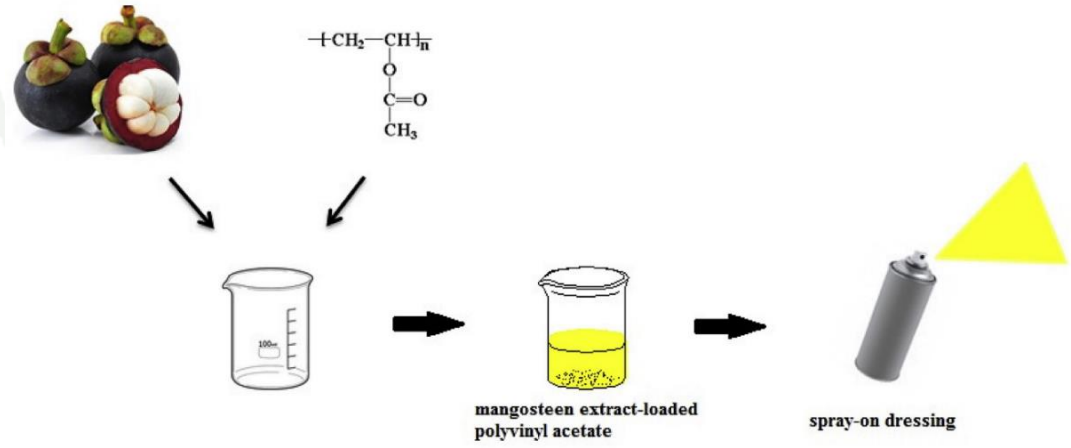


Figure 11 Preparation scheme for mangosteen extract-loaded poly(vinyl acetate) spray-on dressing

Source: Boonmak et al. (2018)

A spray-on dressing of water-soluble chitosan containing liposomes loading with human epidermal growth factor (hEGF) was developed by Umar et al. hEGF was integrated into the spray formulation to promote wound healing, while liposome was

selected as carrier platform to protect hEGF from enzymatic degradation and to prevent the immune response. Chitosan acts in this work as a film forming agent and a matrix to control the release of hEGF-liposome. hEGF-liposome was prepared using a hydration film technique. The obtained liposomes have size of around 219.3 nm and encapsulation efficiency as high as 99.87%. A wound healing test revealed an accelerated wound closure within 6 days (Umar et al., 2021).

Grip et al. also developed a spray-on wound dressing containing β-1,3/1,6-glucan (βG) as a bioactive ingredient to promote wound healing (**Figure 12**). Carboxymethyl cellulose was used in this report as a film forming agent to generate film after spray and to stabilize βG. The prepared spray showed no toxicity toward keratinocytes and exhibited good wound healing ability comparable to the existing commercially available βG-gel (Grip et al., 2021).

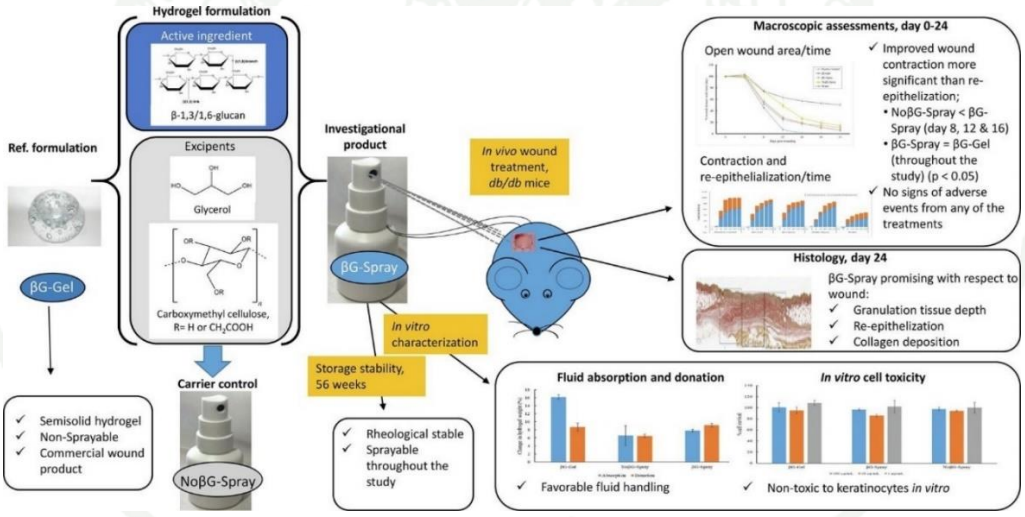


Figure 12 Preparation of a spray-on dressing containing beta-glucan and wound healing ability in a diabetic mouse model

Source: Grip et al. (2021)

Li et al. developed a novel sprayable wound dressing containing curcumin-loaded chitosan nanoparticles modified with epidermal growth factor (EGF) (**Figure 13**). The in vitro cytotoxicity test revealed that the formulated spray exhibited low toxicity toward the tested cells. To test the efficacy of the prepared formulation on wound healing, Wismar rats with full thickness dermal defect were chosen and the results showed complete skin restoration within 12 days (Li, Y. et al., 2021).

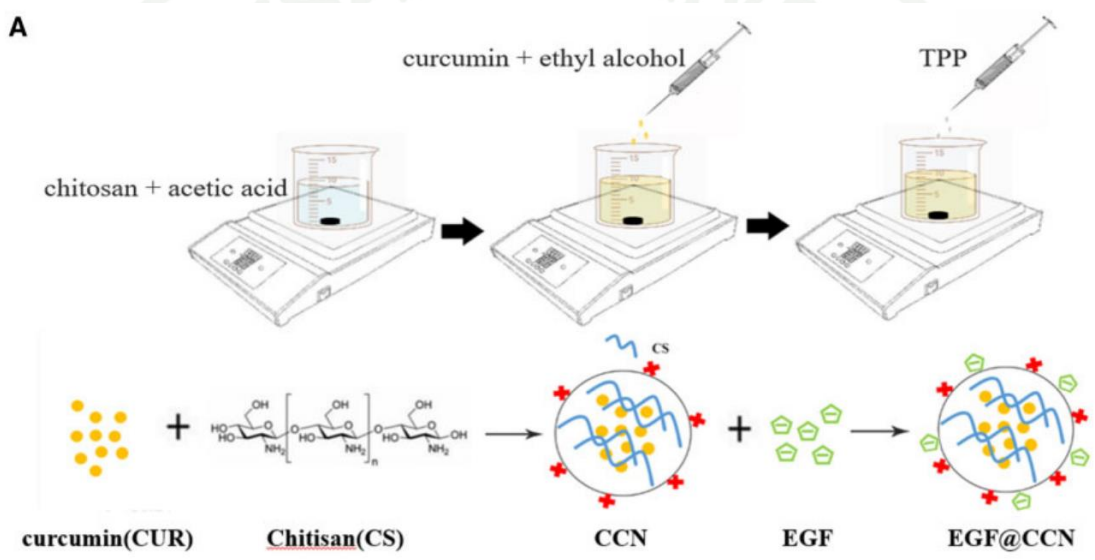


Figure 13 Preparation of a novel sprayable wound dressing containing curcumin-loaded chitosan nanoparticles modified with epidermal growth factor (EGF)
Source: Li et al. (2021)

Islam et al. successfully developed a gelatin-based spray-on dressing for the first aid use (**Figure 14**). Diethyl ether was incorporated into the formulation to ensure fast film forming. After being sprayed, a very quick gel was formed within only 3s. Cytotoxicity test showed low toxicity toward the tested cells, while antibacterial assay revealed moderate bactericidal activity of the prepared spray (Islam et al., 2021).

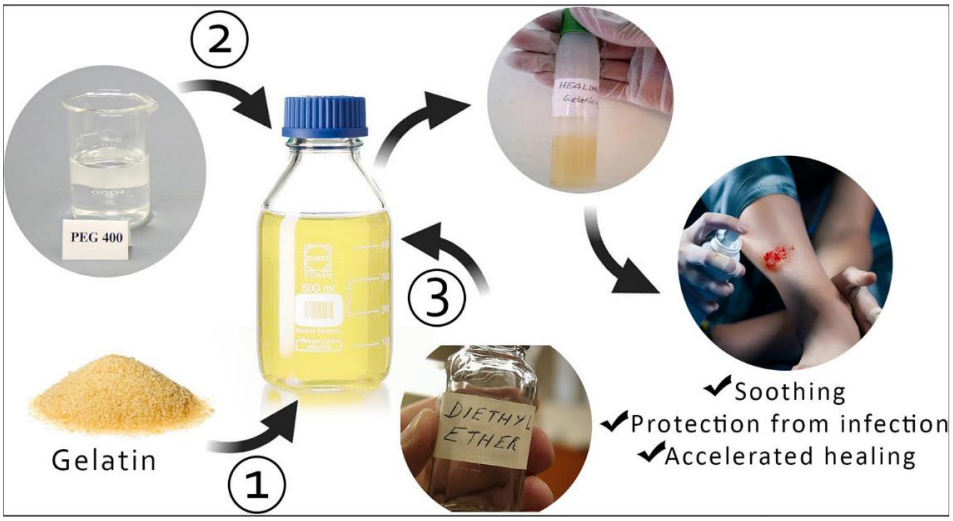


Figure 14 Preparation of a gelatin-based spray-on dressing with fast film forming
Source: Islam et al. (2021)

MATERIALS AND METHODS

Materials

1. Apparatus

1.1 Scanning Electron Microscope, SEM (Quanta 450, FEI, the Netherlands)

1.2 Sputter coater (Polaron Range SC7620, Quorum Technology Ltd., UK)

1.3 X-Ray Diffractometer, XRD (Bruker D8 ADVANCE, Germany)

1.4 Franz diffusion cell (diffusion area 2.54 cm^{-2}, volume 12 mL)

1.5 Microplate reader

1.6 UV/Vis spectrophotometer (UV-2600i, Shimadzu Corp., Japan)

1.7 Field-emission transmission electron microscope (FE-TEM, JEM-3100F, JEOL, Japan)

2. Reagent

2.1 *Phyllanthus emblica* fruit extract (Chemipan Corporation Co., Ltd. (Thailand)

2.2 Polyvinylpyrrolidone (MW~60,000) from TCI chemicals (Japan)

2.3 PEG (Mw ~60,000) from TCI chemicals (Japan)

2.4 Silver nitrate from Alfa Aesar

2.5 Buffer salts and organic solvents, Merck

2.6 Dulbecco's modified Eagle's medium (DMEM, Gibco®) with 10% fetal bovine serum (FBS, Gibco®), Life Technologies

2.7 1 mM sodium pyruvate (Gibco®), Life Technologies

2.8 Penicillin/streptomycin (Gibco®), Life Technologies

2.9 (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt from Promega Corporation.

Methods

1. Preparation of *Phyllanthus emblica* extract/silver nanoparticle/polyvinylpyrrolidone solution

The polymer solution was prepared by dissolving polyvinylpyrrolidone (5% w/v) and polyethylene glycol (0.05% w/v) in 95% ethanol. *Phyllanthus emblica* extract was added into the polymeric solution at 10%, 20%, 30% (w/w to the polymer content), and the solution was adjusted to pH 8. $AgNO_3$ (0.1% w/w to the polymer content) was slowly added to the *Phyllanthus emblica* extract/polymer solution and thoroughly mixed at room temperature overnight.

2. Characterization of the spray-on solution

2.1 Evaporation time

The evaporation time of each spray formulation was evaluated in terms of weight loss percentage over time. The experiment was carried out at a temperature of 25±1°C and relative humidity of 20% by spraying the solution onto a glass Petri dish. Then, the real-time change in weight was monitored using a computer-controlled electronic analytical balance (ME204, Mettler Toledo) with an accuracy of 0.0001 g.

2.2 UV/Vis spectra analysis

The absorption spectrum of the spray solution was obtained using a UV/Vis spectrophotometer (UV-2600i, Shimadzu Corp., Japan). The spectra were recorded between 320 and 700 nm.

2.3 XRD analysis

The X-ray diffractometer (Bruker D8 ADVANCE, Germany) was used to identify the crystalline phase of silver nanoparticles in the sample. The measurement was carried out at an applied current of 30 mA and an accelerating voltage of 30 kV using Ni filtered CuK_{α} radiation (λ = 1.5406 Å). The diffraction data were recorded from 5° to 80° at a scan rate of 5°/min.

2.4 Particle size analysis

The particle size of silver nanoparticles was determined by a field-emission transmission electron microscope (FE-TEM, JEM-3100F, JEOL, Japan) operated at the voltage of 300 kV. The distribution and average of silver nanoparticles diameters were evaluated by analysis of 100 particles from the TEM micrographs, using ImageJ software (NIH).

2.5 Film morphology analysis

The sample was cast onto a clean glass slide and attached to a sample holder stub with two-sided adhesive tape. The morphology of the film was examined with a scanning electron microscope (Quanta 450, FEI, the Netherlands) at 15 kV accelerating voltage.

3. Determination of total phenolic content in *Phyllanthus emblica* extract

The total phenolic content of the spray-on dressing solutions was determined using the Folin–Ciocalteu method with slight modifications. The total phenolic content was expressed as mg of gallic acid equivalence per gram of sample (mg GAE/g sample). The test was carried out in triplicate and reported as the mean ± standard deviation.

4. In vitro antioxidant test

4.1 DPPH radical scavenging activity

DPPH radical scavenging activity of the spray-on dressing solutions was determined using the protocol previously described by Das and Goyal with slight modifications (Das et al., 2015). Briefly, 100 µL of DPPH solution (0.4 mM) in methanol and 100 µL of test samples were mixed, and incubated at 37°C for 30 min in the dark. The scavenging activity was evaluated by measuring the absorbance at 517 nm using a microplate reader. Then, the percentage of DPPH radical scavenging activity was calculated using the following equation:

$$DPPH\ radical\ scavenging\ activity\ (\%) = \frac{[A\ control - A\ sample\ ]}{[A\ control]} \times 100$$

In this equation, A sample and A control refer to the absorbance of DPPH solution with and without sample, respectively.

4.2 ABTS radical scavenging activity

ABTS radical scavenging activity of the spray-on dressing solutions was determined using the protocol previously described by Tsai et al. with slight modifications (Tsai et al., 2013). Briefly, the ABTS radical cation (ABTS·+) solution was prepared by mixing 7.4 mM ABTS in methanol and 2.6 mM potassium persulfate in water for 16 h in the dark. The mixture was diluted in methanol to obtain the

absorbance of 0.70 ± 0.02 at 734 nm. Then, 10 µL of the test sample and 190 µL of the ABTS·+ solution were mixed and incubated for 5 min in the dark. The scavenging activity was evaluated by measuring the absorbance at 734 nm using a microplate reader. Then, the percentage of ABTS radical scavenging activity was calculated using the following equation:

$$ABTS\ radical\ scavenging\ activity\ (\%) = \frac{[A\ control - A\ sample\ ]}{[A\ control]} \times 100$$

In this equation, A sample and A control refer to the absorbance of ABTS solution with and without sample, respectively.

5. In vitro release

In vitro release of *Phyllanthus emblica* extract from the dressing film was tested using static Franz diffusion cells (diffusion area 2.54 cm^2, volume 12 mL). The temperature of the cells was kept at 32 ± 0.5ºC to mimic skin surface temperature. The precast spray-on dressing film was mounted onto a cellulose acetate membrane and then placed between the receptor and donor compartments. The phosphate buffer solution (pH 7.4) as a receptor medium was stirred constantly throughout the experiment. At the predetermined times of 5, 10, 15, 20, 25, 30, 60, 90, 120, 240, 360, and 480 minutes, 1 mL of receptor medium was collected and immediately recharged with a fresh receptor medium of an equal amount. The samples were subsequently analyzed by UV/vis spectrophotometer. The in vitro release studies were carried out in triplicate and the cumulative release of *Phyllanthus emblica* extract was calculated. To explain the release mechanism, the initial 60% of the cumulative release data were fitted to the following Ritger–Peppas kinetic equation.

$$M_t/M_\infty = kt^n$$

In this equation, M_t/M_∞ is the cumulative release of the extract at time t, k is the kinetic constant, and n is the diffusion exponent, which classifies the release mechanism. When n is equal to or less than 0.5, the release mechanism follows a

Fickian diffusion (diffusion through a non-swollen matrix). When n lies between 0.5 and 1, the release mechanism is considered an anomalous non-Fickian transport (release through both diffusion and dissolution/swelling of the matrix). When n is equal to 1.0, the release mechanism is the case II transport (release through dissolution/swelling of the matrix) (Ritger et al., 1987).

6. In vitro antibacterial test

The antibacterial activity was determined by the disk diffusion test. Four representative bacterial strains often found in wound infections including *Staphylococcus aureus* (*S. aureus*, TISTR 517), *Methicillin-resistant Staphylococcus aureus* (MRSA, TISTR 142), *Pseudomonas aeruginosa* (*P. aeruginosa*, TISTR 1467), and *Escherichia coli* (*E. coli*, TISTR 887) were incubated on the Mueller-Hinton agar (MHA) at 37℃ for 24 h. The bacterial suspension with a turbidity equivalent to the 0.5 McFarland standard was prepared and spread over the Mueller–Hinton agar plate. The test samples were prepared by impregnation of the spray solutions onto the 6 mm disks of Whatman filter paper and then placed on the agar plates and incubated at 37℃ for 24 h. The antibacterial efficacy was afterward determined by measuring the zone of inhibition (mm). The tests were carried out in triplicate and reported as the mean diameter ± standard deviation.

7. In vitro cytotoxicity test

In vitro cytotoxicity on human dermal fibroblast (HDFa) (ATCC® PCS-201-012™) and immortalized human keratinocyte (HaCat) (ATCC® Number PCS-200-011™, 300493, CLS, Germany) was carried out using the (3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-7sulfophenyl)-2H-tetrazolium) (MTS) assay. HDFa and HaCat cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco®, Life Technologies, USA) with 10% fetal bovine serum (FBS, Gibco®), 1 mM sodium pyruvate (Gibco®), 100 U/mL penicillin and 100 µg/mL streptomycin at 37 ℃ and 5% CO_2. One day before the experiment, the cultured cells were seeded into the 96-well plate (10,000 cells/well for fibroblast and 8,000 cells/well for keratinocyte) and incubated for 24 h. After that, the medium was

exchanged with the test substances at various doses (0.63, 1.25, and 2.5 mg/mL) and the cells were incubated for another 24 h. The test sample was removed and the MTS reagent was added and incubated at 37°C for 3 h. The solution containing soluble formazan product was collected and the optical density (OD) of the obtained solution was measured at 490 nm. The percentage of cell viability was then calculated using the following equation.

$$\%\ cell\ viability = \frac{[OD\ sample]}{[OD\ negative\ control]} \times 100$$

RESULTS AND DISCUSSION

1. Characterization of *Phyllanthus emblica* extract/silver nanoparticles /polyvinylpyrrolidone spray-on solution

   1.1 Evaporation rate

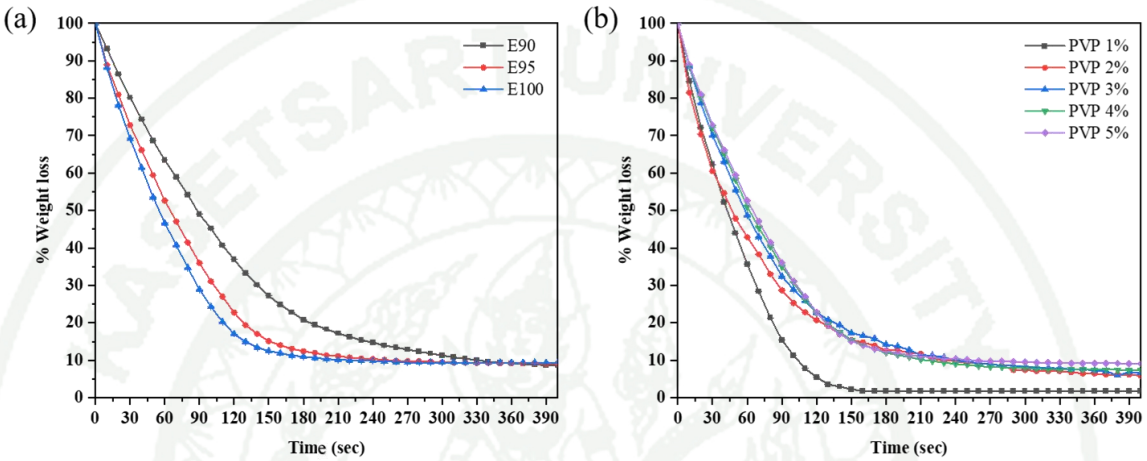


Figure 15 Evaporation rates of PVP solutions at different (a) solvent ratios and (b) polymer concentrations.

To formulate the spray-on dressing, the evaporation rate is an important factor to be concerned with to verify how fast the film forms after being sprayed. The evaporation rate can be evaluated in terms of the time required for the solvent to evaporate off and the film to form. The evaporation rate was assessed in this work by monitoring the weight loss percentage over time. This can be done by spraying the dressing solution onto a glass Petri dish and then the real-time change in weight was monitored using a computer-controlled electronic analytical balance. The effects of solvent ratio and polymer concentration on the evaporation rate were evaluated in this work. Ethanol and water were selected as carrier solvents for the spray formulations because they are safe and generally used in pharmaceutical preparations. The solvent system that fully dissolves the polymer and simultaneously provides quick evaporation was optimized by varying the mixing ratio of ethanol and water. To verify the effect of solvent ratio on the evaporation rate, spray solutions of 5% (w/v) PVP in three different solvent systems (90%, 95%, and 100% ethanol) were tested. The percentage of weight loss of the sprayed solution was monitored and plotted as a

function of time. From the plot, the evaporation rate can be simply identified by the steepness of the curve. In **Figure 15(a)**, the weight loss declines rapidly in the beginning and then gradually decreases until reaching a plateau. It means that the rate of solvent evaporation is very high at the beginning due to the massive loss of the volatile organic solvent and then becomes slower when the polymeric gel starts to set and begins to slow down solvent evaporation. As shown, the spray solution prepared using 100% ethanol exhibited the steepest decay when compared to the other two. Although it provided the fastest evaporation rate, the obtained film was rather too dry and showed a certain degree of brittleness possibly due to the lack of water in the solvent system. As the water content in the binary solvent system increased, the evaporation rate became lower. This is because water is less volatile than ethanol. With 5% of water in the solvent, the spray evaporated as quickly as the one prepared in 100% ethanol, but the obtained gel film was clear, wet, and soft. Therefore, 95% ethanol was considered the most appropriate solvent system and was further used to prepare the spray. Besides the solvent ratio, the polymer concentration was also varied to obtain the optimal amount of polymer that rapidly forms a quick gel film. The polymer concentration was ranging from 1% to 5% w/v in 95% ethanol. At all the polymer concentrations, the obtained solutions were all clear, meaning that the polymer can be completely dissolved. As depicted in **Figure 15(b)**, when the concentration of polymer increases from 1% to 2%, the rate of evaporation apparently decreases. That means the polymer concentration exerts a certain effect on the evaporation rate. However, this effect seems less obvious when the concentration of polymer is higher than 2%. This effect can be ascribed as a result of the polymeric gel formed after being sprayed. When the polymer concentration is too low, the spray droplets are relatively too small. Thus, they could fail to spread over the substrate and could dry rapidly. At a higher concentration of polymer, the obtained film gets more uniform and serves as a better barrier to maintain moisture within the gel. As a result, the evaporation rate is lower (Boonmak et al., 2018). Since the spray containing 5% w/v of PVP gave a more uniform film with a comparable evaporation rate to the other sprays with less PVP content, it was chosen and further used to prepare the spray-on dressing containing silver nanoparticles and the *Phyllanthus emblica* extract.

It is worth mentioning that, the evaporation rate on the actual skin may be faster than the test carried out on the plain glass plate because the skin is covered by a large number of skin pores whereby the solvent can be absorbed through. Additionally, the increase in skin temperature also promotes solvent evaporation, thereby lowering the drying time of the film.

1.2 UV/Vis spectra analysis

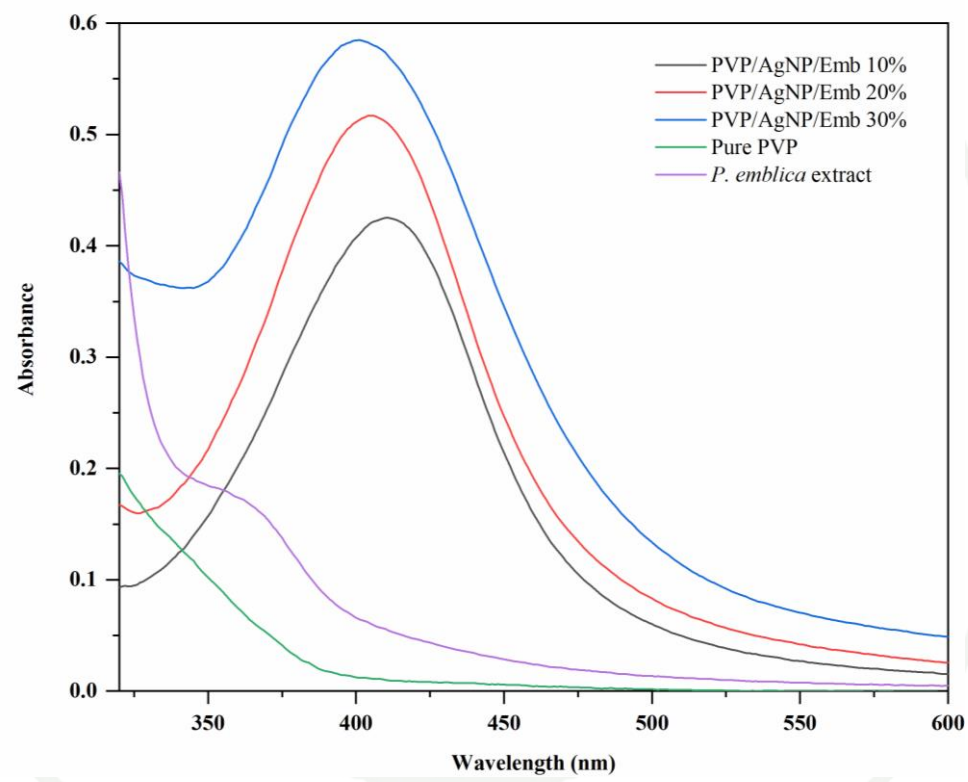


Figure 16 UV/VIS absorption spectra of PVP, *Phyllanthus emblica* extract, and dressing spray containing different amounts of *Phyllanthus emblica* extract.

Silver nanoparticles were synthesized in this work by a green chemical method using the *Phyllanthus emblica* extract. The mechanism for the synthesis has been previously proposed by Kannaujia et al. (Kannaujia et al., 2019). Ascorbic acid as one of the major components in the *Phyllanthus emblica* fruit extract behaves in this case as a green reducing agent to convert silver ions into metallic silver atoms, which subsequently agglomerate to form silver nanoparticles. After this reduction process, ascorbic acid will turn itself into dehydroascorbic acid.

A successful formation of silver nanoparticles can be confirmed by UV/VIS spectrophotometry. As depicted in the absorption spectra **(Figure 16)**, the dressing spray containing different amounts of *Phyllanthus emblica* extract shows characteristic broad absorption bands ranging from 320 to 600 nm due to the intense surface plasmon resonance (SPR) of the colloidal silver nanoparticles. Since the absorption maxima do not exceed 500 nm, the size of the obtained particles is likely to be smaller than 100 nm. As the extract loading amount increases, the peak intensity increases. This increment in the absorbance of the SPR peaks implies an increase in the number of silver nanoparticles upon the increment of the biogenic reducing agent in the *Phyllanthus emblica* extract. Besides, the increase in the extract loading amount also leads to a blue shift of the maximum absorption peaks towards shorter wavelengths due to the decrease in particle size. This is possibly because of the increase in the nanoparticle nucleation seeds, which in turn leads to the formation of smaller particle sizes (Bardania et al., 2020).

1.3 XRD analysis

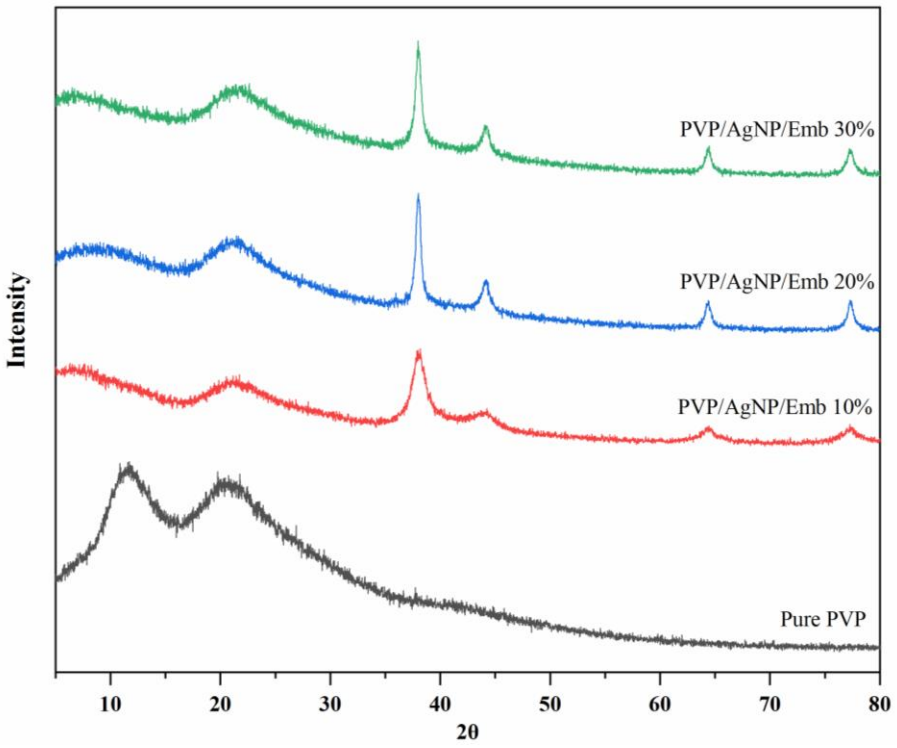


Figure 17 X-ray diffraction patterns of spray-on dressing films loaded with different amounts of *Phyllanthus emblica extract* and pure PVP film.

X-ray diffraction was used to further confirm the existence of silver nanoparticles. As depicted in **Figure 17**, the diffraction pattern of the neat PVP showed typical broad peaks of amorphous structure at 2θ of 11.7° and 21.3°, whereas the spray-on dressing displayed a broad peak of PVP at around 21° and the other four distinct diffraction peaks at 38.2°, 44.2°, 64.3°, and 77.2°, which correspond to the (111), (200), (220), and (311) planes of the face-centered cubic crystal structure of silver. This clearly confirmed that silver nanoparticles can be successfully synthesized via the proposed protocol. As the loading amount of the extract increases, the peak intensity of silver becomes higher because of the increment in the number of silver nanoparticles as previously described in the case of the UV/VIS absorption spectra.

1.4 Particle size analysis

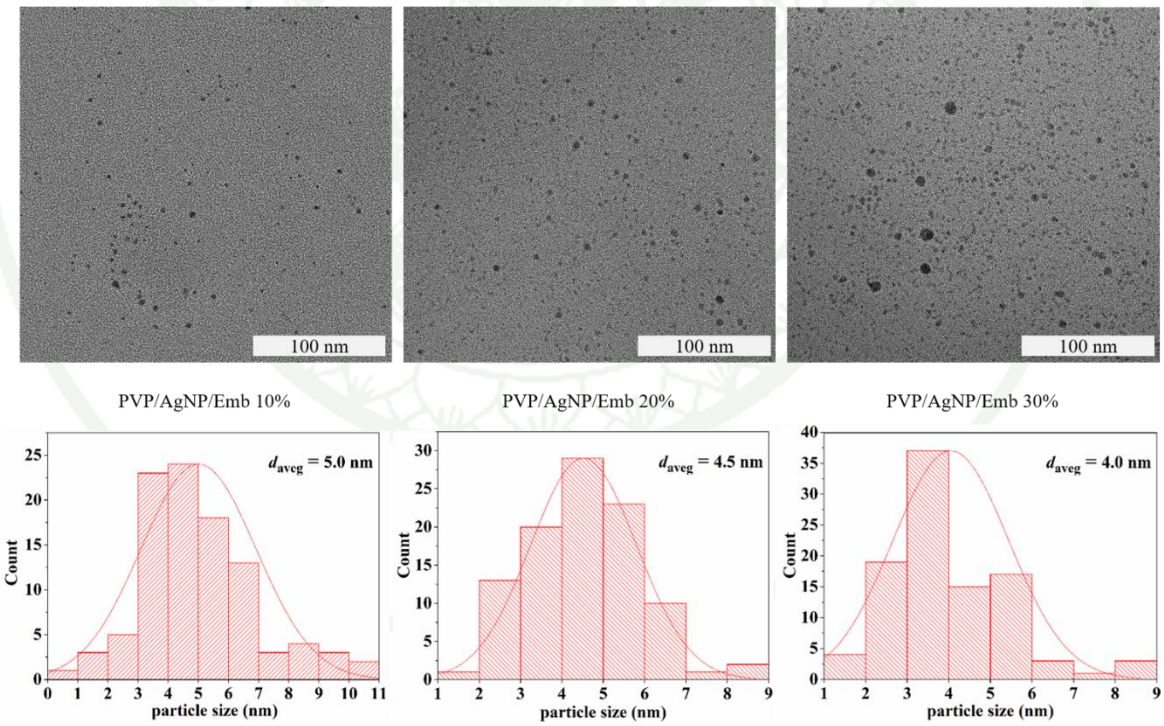


Figure 18 TEM images of silver nanoparticles presented in three different formulations of the dressing spray at ×100,000 magnification and the particle size histograms.

A transmission electron microscope was used to confirm the existence of silver nanoparticles and also to analyze their sizes. As depicted in **Figure 18**, the synthesized nanoparticles were spherical in shape for all the spray formulations. The distribution and average diameters of silver nanoparticles were assessed by analysis of 100 particles from the TEM micrographs and the results are shown in the histograms. The average diameters of the particles present in the spray-on dressing loading with 10%, 20%, and 30% of the *Phyllanthus emblica* extract were found to be 5.0 ± 0.6, 4.5 ± 0.6, and 4.0 ± 0.8 nm, respectively. The particle size of around 5 nm has been previously reported to exhibit the best and fastest bactericidal performance in comparison to the silver nanoparticles with a larger size (Agnihotri et al., 2014).

1.5 Film morphology analysis

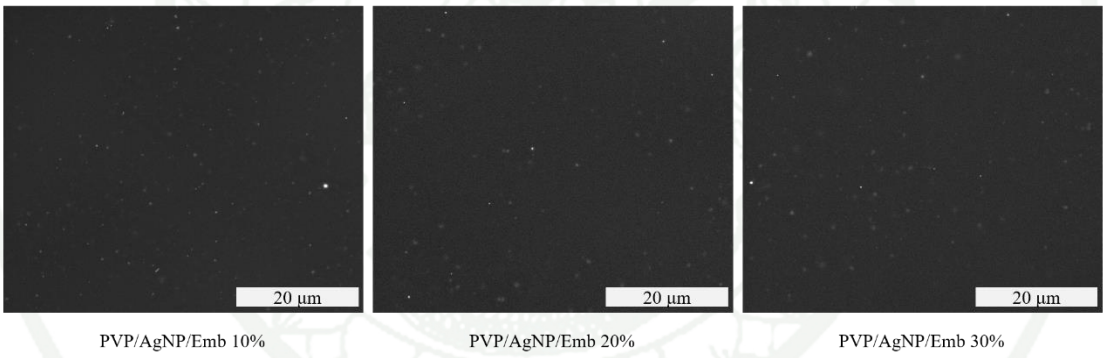


Figure 19 Surface morphology of spray-on dressing films loaded with different amounts of *Phyllanthus emblica* extract at ×2,000 magnification.

Surface morphology of the spray-on dressing films loaded with different amounts of *Phyllanthus emblica* extract was visualized using a scanning electron microscope (SEM). As depicted in **Figure 19**, all the spray-on dressing films have smooth and uniform surfaces without any sign of aggregation, demonstrating that silver nanoparticles and the *Phyllanthus emblica* extract were homogeneously dispersed and distributed throughout the PVP films.

2. In vitro release study

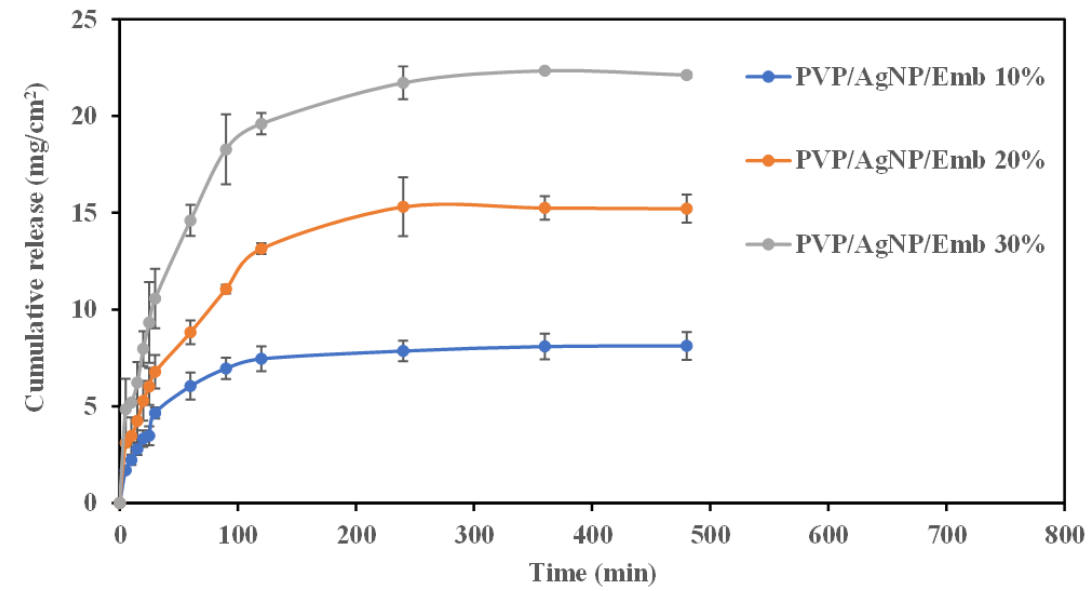


Figure 20 Cumulative release of *Phyllanthus emblica* extract from spray-on dressing films loaded with different amounts of *Phyllanthus emblica* extract.

The in vitro release of the *Phyllanthus emblica* extract from the spray-on dressing films was investigated using in vitro Franz diffusion experiments. The cumulative release of the fruit extract from the films loading with three different amounts of *Phyllanthus emblica* extract is illustrated in **Figure 20**. As the loading amount increases, a higher release of the extract was observed. In all cases, the dressing films showed a fast release at the initial step stemming from the burst release of the fruit extract loosely bound or entrapped close to the dressing film surfaces. After the initial state of fast release, the extract embedded within the polymeric gel film was gradually released in a controlled and sustained manner until reaching the plateau at around 4 h. This means that the sprayed gel film would provide immediate and efficient delivery of the extract to the wound bed to promote the healing process.

The Ritger-Peppas model which is commonly used for the polymeric matrix-based system was selected to describe the release mechanism of the tested system because it considers more than one mechanism at the same time, such as diffusion, matrix swelling and dissolution (Uhljar et al., 2021). After fitting the release data with

the Ritger-Peppas model, the coefficients of determination (R^2) were found to be 0.9924, 0.9939, and 0.9919 for the dressing films loading with 10%, 20%, and 30% of the *Phyllanthus emblica* extract, respectively, signifying that the release kinetics were very well fitted to the chosen model. The diffusion exponent (n) was used to identify the mechanism involving in the release process. When $n \leq 0.5$, the release mechanism follows a Fickian diffusion. The drug in this case diffuses through a non-swollen matrix where the release relies on the drug concentration gradient between the polymeric matrix and the release media (Sinsup et al., 2021). When $n = 1.00$, the release mechanism is defined as the case II transport. The release is in this instance caused by the dissolution or swelling of the polymer film. When $0.50 < n < 1.00$, the release mechanism is considered an anomalous non-Fickian transport. The drug release in this case is governed by both diffusion and dissolution/swelling of the polymer matrix. In this work, the exponent n values were 0.61, 0.53, and 0.54 for the dressing films loading with 10%, 20%, and 30% of the extract, respectively. These values lie between 0.50 and 1.00, indicating that the release is governed by a combination of both diffusion-controlled and matrix swelling-controlled mechanisms.

3. Total phenolic content and antioxidant activity

Reactive oxygen species (ROS) are highly reactive oxidizing agents from molecular oxygen molecules mainly produced in mitochondria. ROS are found to involve in various stages of wound healing. A low level of these active species has been demonstrated to be beneficial in protecting cells from infection. However, the presence of excess amounts may produce oxidative stress by exerting adverse effects on the tissues, thereby slowing down the wound healing process (Comino-Sanz et al., 2021). Antioxidants are chemical compounds that inhibit free radicals or reactive oxygen species through the prevention of propagation of the radical chain reactions. Therefore, antioxidants are postulated to promote the wound healing process by helping defend against wound oxidative stress and inflammation (Bardania et al., 2020).

Polyphenolic compounds derived from plants have been reported to show potent antioxidant activities both *in vitro* and *in vivo*. In the *Phyllanthus emblica* fruit extract, these compounds have been found as major components (Li, Y. et al., 2019;

Sawant et al., 2011; Wu et al., 2022). As a result, they are believed to have a major contribution to the antioxidant activity of the present dressing sprays. The total phenolic content and the antioxidant activities of the dressing spray containing *Phyllanthus emblica* fruit extract were evaluated and the results were displayed in **Table 1**. The total phenolic contents found in the sprays containing 10%, 20%, and 30% of the fruit extract were 1.56 ± 0.13, 2.73 ± 0.11, and 3.86 ± 0.17 GAE mg/g of extract, respectively. This result reveals that the total phenolic content increases with the increasing loading amount of the extract. The free radical scavenging activities of the spray formulations were tested using the DPPH and ABTS assays. Similar to the phenolic content, as the loading amount of the *Phyllanthus emblica* fruit extract increases, the percentage of scavenging activity increases from 49.02 ± 3.35% to 64.56 ± 2.44%, and 69.22 ± 0.26% for the DPPH and from 20.00 ± 2.44% to 24.18 ± 1.03%, and 67.47 ± 5.04% for the ABTS assay. This suggests that the *Phyllanthus emblica* extract is responsible for the antioxidant activities of the dressing sprays.

Table 1 Antioxidant activities and total phenolic content of neat PVP and dressing spray containing different amounts of *Phyllanthus emblica* extract

Sample	Total phenolic content (mg GAE/g extract)	Antioxidant activities	
		DPPH scavenging (%)	ABTS scavenging (%)
PVP	0	0	0
PVP/AgNP/Emb 10%	1.56 ± 0.13	49.02 ± 3.35	20.00 ± 2.44
PVP/AgNP/Emb 20%	2.73 ± 0.11	64.56 ± 2.44	24.18 ± 1.03
PVP/AgNP/Emb 30%	3.86 ± 0.17	69.22 ± 0.26	67.47 ± 5.04

4. Antibacterial test

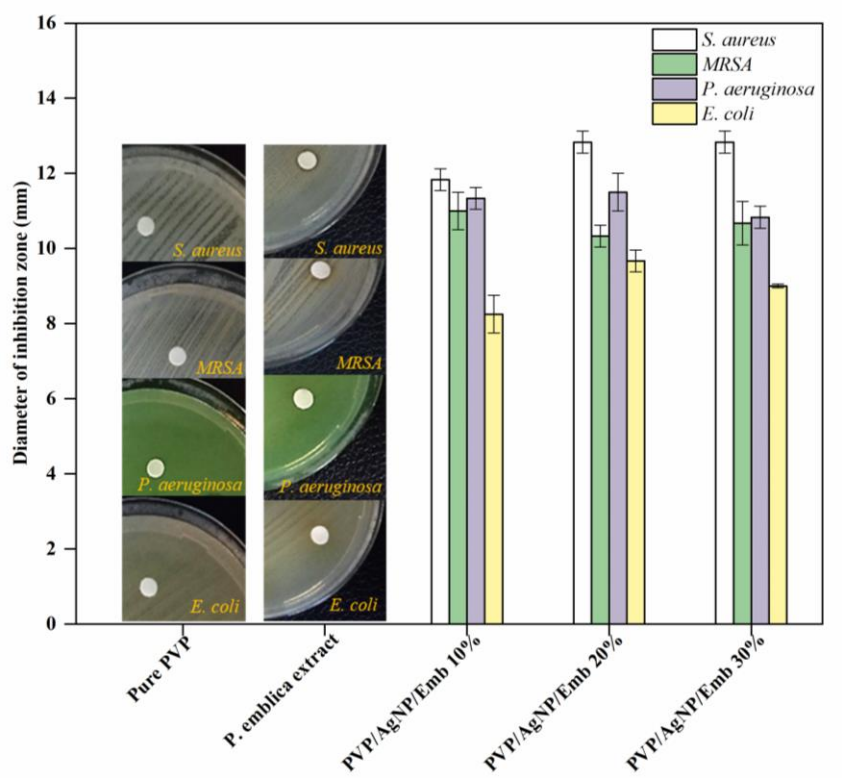


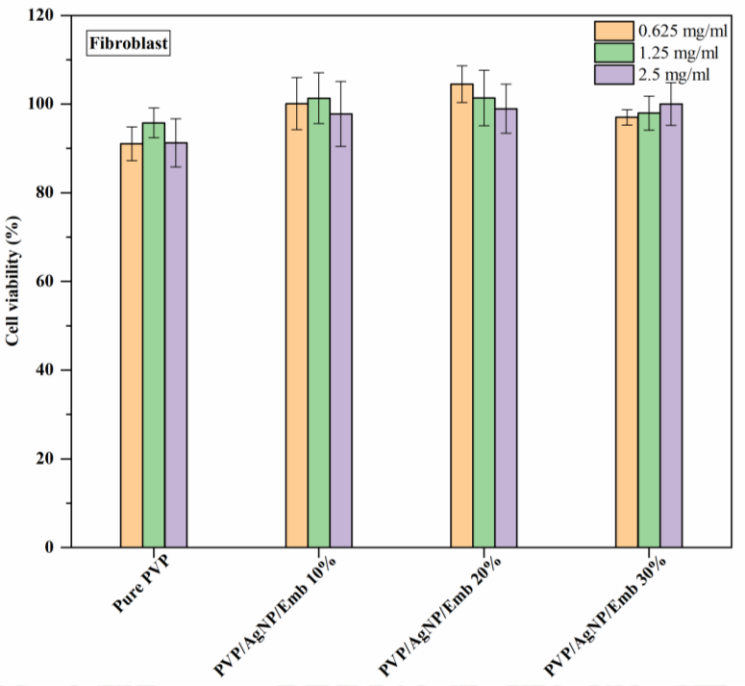
Figure 21 Antibacterial activity of PVP, *Phyllanthus emblica* extract, and *Phyllanthus emblica* extract loaded spray-on dressing films against *S. aureus*, MRSA, *P. aeruginosa*, and *E. coli*

In vitro antibacterial activity of *Phyllanthus emblica* extract/silver nanoparticle/ polyvinylpyrrolidone spray-on dressing was assessed using the disk diffusion method. Four representative gram-negative and gram-positive bacterial strains commonly involved in wound infections including *P. aeruginosa*, *E. coli*, *S. aureus*, and the resistant MRSA were selected for the test. Five sample films from three different spray-on dressing formulations, neat PVP spray, and the *Phyllanthus emblica* extract were placed on the MHA plates for 24 h. After that, the clear inhibition zones were examined and the results are depicted in **Figure 21**. As the control, the PVP spray and the *Phyllanthus emblica* extract exhibited no clear inhibition zones against all the tested strains, suggesting that PVP and the extract themselves have no antibacterial activity. This is in contrast to the spray-on dressing,

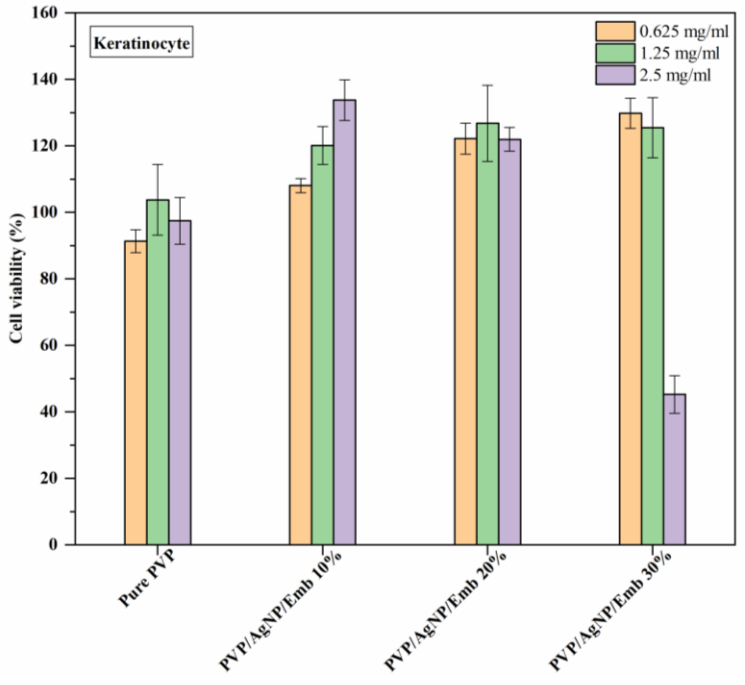
where all the tested formulations containing different loading amounts of the fruit extract exhibited obvious clear inhibition zones against all the bacterial strains. This clearly indicates that silver nanoparticles are responsible for the inhibition of bacterial growth. However, no significant differences in the diameter of the inhibition zone were observed among each strain, implying that the amount of *Phyllanthus emblica* extract has no obvious impact on the antibacterial activity of the present spray-on dressing.

5. In vitro cytotoxicity

The in vitro cytotoxicity of the *Phyllanthus emblica* extract/silver nanoparticle/polyvinylpyrrolidone spray-on dressing was determined using the MTS assay on human dermal fibroblast (HDFa) and keratinocyte (HaCat) cells. Three spray-on dressing formulations containing different loading amounts of the *Phyllanthus emblica* extract and the neat PVP spray were examined in this study. The tested cells were incubated with the test samples at three different doses (0.63, 1.25, and 2.5 mg/mL) and the cell viability was determined after 24 h of incubation **(Figure 22)**. Percentages of cell viability greater than 80% are considered non-toxic in this test. As depicted in **Figure 22(a)**, at every concentration of the test samples, the viability of the fibroblasts was higher than 90%, indicating that the present spray-on dressing is non-toxic to the fibroblast cells. Although the *Phyllanthus emblica* extract exhibits a potent antioxidant activity, the loading amount of the extract can exert negative effects on the viability of the tested cells, particularly on keratinocytes. This can be evident in **Figure 22(b)**. As the tested dose of the spray-on dressing containing 30% of the *Phyllanthus emblica* extract increases to 2.5 mg/ml, the viability of the keratinocyte decreases down to less than 50%. This suggests that the spray-on dressing is biocompatible and safe for use as a wound dressing material when the loading amount of the *Phyllanthus emblica* extract is not higher than 20%. It is worth mentioning that this loading amount already provides good antioxidant and antibacterial activities comparable to those of 30% loading. Therefore, 20% of the *Phyllanthus emblica* extract is considered the optimal loading amount in the spray-on dressing.



(a)



(b)

Figure 22 Cytotoxicity effects of PVP and dressing sprays at different loading amounts of *Phyllanthus emblica* extract on (a) human dermal fibroblast and (b) human keratinocyte. Each formulation was tested at three concentrations (0.625, 1.25, 2.5 mg/ml).

CONCLUSIONS

In this work, an antibacterial spray-on wound dressing was successfully prepared using PVP as an adhesive film-forming component, silver nanoparticles as a broad-spectrum antimicrobial agent, and *Phyllanthus emblica* extract as a natural antioxidant. The spray-on solution was facilely prepared in a one-pot using a natural fruit extract from *Phyllanthus emblica* for the green synthesis of silver nanoparticles. After being sprayed on the wound surface, the adhesive hydrogel film will be rapidly formed and serve as a protective barrier to maintain moisture and prevent the ingress of germs into the wound bed. Besides its role as a biogenic reducing agent, the *Phyllanthus emblica* extract also serves as an antioxidant to help defend against wound oxidative stress and modulate the inflammation, thereby promoting the wound healing process. As the extract was entrapped inside the PVP film, it was released from the polymer gel matrix in a controlled and sustained manner. The incorporation of silver nanoparticles into the spray also provided antibacterial activities against the representatives for gram-positive and gram-negative strains including *S. aureus*, *P. aeruginosa*, *E. coli* as well as the resistant ones such as MRSA. Further *in vitro* cytotoxicity test also revealed that the dressing film was biocompatible with the tested human dermal fibroblasts and human keratinocytes except for the formulation with high loading content of the *Phyllanthus emblica* extract (30%) at the highest dose of the tested sample (2.5 mg/ml). Based on the obtained results, the proposed spray-on dressing has a promising potential for use as antibacterial wound dressing, particularly for first-aid purposes.

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