Hydrogel patch from liquid smoke and vitamin K Collard Greens extract for wound healing applications

S Saputra1*, G Wibisono1, E Ramadhani2, T Dewi3

1Department of Dental Medicine, Universitas Diponegoro, Semarang, Indonesia
2Department of Chemistry, Universitas Diponegoro, Semarang, Indonesia
3Department of Nutrition Sciences, Universitas Diponegoro, Semarang, Indonesia

*Email: aasutadisaputra@alumni.undip.ac.id

Abstract. Wound care by using wound dressing has been evolved available in various physical forms including hydrogels with the highest global use reaching 43%. Nowadays, hydrogels are available at high cost because produced from synthetic materials, so natural materials are needed to obtain antibacterial wound dressing. This study aims to analyze the hydrogel patch PVA/chitosan/starch infused liquid smoke and vitamin K collard greens extract which are antibacterial effects and accelerates wound healing. This study was a laboratory experiment. Collard greens were extracted by using the Direct Solvent Extraction II method. The membrane characteristic tested using SEM test. The parallel streak method (AATCC 147-2004) was used for antibacterial testing. The hydrogel patch application was carried out on 28 male Rattus norvegicus strain wistar rats. They were given incisions wound and treated by a control group and 3 treatment groups and conducted wound healing analysis on the 4th, 7th, 11th and 14th day observation. The antibacterial test showed the hydrogel patch infused liquid smoke with a concentration of 12% was the optimum concentration inhibiting S. aureus. In conclusion, the hydrogel patch has a smooth and porous surface, can be inhibited the growth of S. aureus, and proven to completely heal the animal wound.

1. Introduction
Daily activity can inflict a possibility of injury happen within the body. Wound is a discontinued or partial damaged body tissue that happens due to burns, surgery, diabetes, trauma, or other causes. Currently, the treatment of injury using wound dressing is already overgrowing. According to the usage, the wound dressing is divided into primary dressing (contact with the injury) and secondary dressing (cover the primary dressing) [1]. Primary dressing usually used is gauze, plaster, natural or synthetic bandage, and cotton with dry characteristics does not offer a humid environment, and tends to stick with the wound, making it painful and cause bleeding when removed, which damaged the new epithelium [2].

Hydrogel can be produced from natural or synthetic polymer or adding both to create the best characteristic [3]. Nowadays, hydrogel wound dressing made from PVA, chitosan, and starch are already developed [4]. PVA is the most often used synthetic polymer as the material of hydrogel [2]. Furthermore, chitosan is a natural-polysaccharides with biodegradable, biocompatible, nontoxic and film-forming characteristics [5]. In addition, starch is a natural polymer with a large cross- linking ability through the hydroxyl group (OH) [6].
Hydrogel is presently developed as antibacterial transport media to be used for infection injury [2], [7]. As compared to commercial antibiotics, the natural antibacterial agents have less side effects and more effective [8]. One of the natural antibacterial which has the potential for wound healing materials is liquid smoke [9]. Liquid smoke is produced from the pyrolysis and condensation of smoke from the burning process of coconut shell [10,11]. It is known to have a considerably high compound of polyphenol. Besides acting as antioxidant, polyphenol also has antibacterial properties [11]. On the other hand, Vitamin K1 or phylloquinone from collard greens leaves (Brassica oleracea var. Acephala) can be used as an agent to accelerate wound healing because it is needed for the synthesis of clotting factors II, VII, IX and X. Collard greens leaves contain the largest phylloquinone reaching 440 mg/100 g [12]. Based on these descriptions, the researchers are motivated to conduct research in the wound dressing membranes development of liquid smoke and vitamin k, preventing bacterial contamination and accelerating wound healing.

2. Material and methods

This research was a true experimental designs research with post-test only control groups design plan. This research was conducted in May-December 2019 which was carried out in five laboratories, namely UNDIP Integrated Laboratory (CENURE), Faculty of Medicine UNDIP Experimental Animal Laboratory, Anatomical Pathology Laboratory of Diponegoro National Hospital, Faculty of Medicine UNDIP Microbiology Laboratory, and SEM of Mechanical Engineering FTI ITS Laboratory. Ethical clearance was obtained from the Health Research Ethics Commission, Faculty of Medicine UNDIP with No.52 / EC / H / KEPK / FK- UNDIP / V / 2019.

2.1 Vitamin K Extraction of Collard greens

Vitamin K extract of collard greens leaf was obtained through Direct Solvent Extraction type II. A total of 1 mL concentrated extract of collard greens was measured in a beaker glass. Then extraction solvent (dichloromethane: methanol = 20 mL: 10 mL) was added to the sample and it was mixed using a homogenizer. The homogenous mixture was filtered through dehydrated sodium sulphate and transferred to a 50 mL volumetric flask, then it was diluted with methanol. A total of 2 mL of the sample is taken and then evaporated. The residue was dissolved again with hexane and purified along with that. It was then added a mixed solution (methanol: water = 9: 1, v / v, 8 mL) to the hexane extract and centrifuged for 5 minutes at 40 rpm. The hexane layer was removed and evaporated to dryness [13].

2.2 Preparation of Hydrogel Membranes

The wound dressing is made from the base hydrogel of the PVA, chitosan and starch. 1 mL acetic acid mixed with 100 mL distilled water to produce 1% acetic acid solution. Chitosan 3 gr was mixed with 1% acetic acid solution as much as 100 ml produce chitosan concentration of 3%. 5 gr starch were blended with 100 mL distilled water to produce 5% starch concentration. 3 gr PVA were fused with 100 mL distilled water to produce PVA concentration of 3%. Each ingredient was homogenized separately using a 70ºC temperature stirrer with a speed of 400 rpm for 2-3 hours (until it dissolves completely). Liquid smoke was diluted in a 10mL volumetric flask using distilled water to produce 25%, 50%, and 75% liquid smoke. According to the treatment formulation, all solutions were combined with chitosan content; PVA: chitosan: starch: liquid smoke: vitamin K (3: 2: 3: 1: 1). Homogeneous membrane material was poured on a petri dish and roasted at 60ºC for 6 hours. Then it was thawed for 2 hours at 25ºC. The membrane was dried at room temperature then it was cut into a square shape according to the shape and number of the sample. The membrane was applied with sterile tweezers [4].

2.3 Subject

The study subjects were divided into 4 groups, namely: group 1 (control, treatment with hydrogel base), group 2 (treatment with hydrogel base infused liquid smoke), group 3 (treatment with hydrogel base infused vitamin K), group 4 (treatment with hydrogel base infused liquid smoke and vitamin K).
2.4 Surface Morphology Test
Morphological testing of the surface of the hydrogel wound dressing was used the Scanning Electron Microscope (SEM) test. SEM characterization was applied to seeing the surface topography of a material. The control sample (group 1) and the best group sample from the experimental animal test (group 4) performed the SEM test to determine the surface morphology and pore structure contained in the membrane.

2.5 Antibacterial Test
Antibacterial testing was used a parallel streak method (AATCC 147-2004). The appearance of inhibited or clear zones along the line indicates the organism's sensitivity to the sample. One loopful of the subculture bacteria was streaked to Mueller Hinton Agar (MHA) plate. The test specimen was gently pressed transversely across the agar surface, then applied hydrogel membrane on the streak as many as 6 lines with a distance of 1 cm each which was incubated at 37°C for 20 h. The incubated plate was examined for the interruption of growth along with the streaks of inoculum beneath the specimen and a clear zone of inhibition beyond its edge. The average width of a zone of inhibition along a streak on either side of the test specimen is calculated using the following equation based on ISO AATCC 147-2004:

\[ W = \frac{T - D}{2} \]  

where W is the width of clear zone of inhibition in mm; T is the width of test specimen and clear zone in mm; and D is the width of test specimen in mm. The calculation results of the inhibitory measurements were made by three different observers and then the average was calculated.

2.6 Wound Healing Animal Test
Test on experimental animals was used male Rattus norvegicus Wistar strain rats aged 3-4 months weighing 120-170 gr. Criteria for healthy mice is characterized by active motion, clean fur, clear eyes and has never received prior treatment. Prior to the experiment, each rat was acclimatized for 7 days in an individual cage to prevent infection due to other rat bites. Then we made incision wounds with a wound size of 15x10 mm² using a sterile surgical scissors that was anesthetized first using ketamine 0,1/110grBB. The hydrogel wound dressing was done every two days which is coated with non-woven plaster so the hydrogel wound dressing cannot be separated. Observations were made on days 4, 7, 11 and 14. Wound incision wounds were measured using a ruler and were observed by three different observers and the average result was calculated. On day 14 all mice were terminated using ether and their skin tissue was taken. Furthermore, histological preparations were made using Haematoxylin-Eosin (HE) staining to analysed the number of fibroblast cell distribution.

3. Results

3.1 Scan Electron Microscopic (SEM)
SEM test is carried out to analysed the surface, texture, shape and size of the hydrogel wound dressing membrane. SEM test results in Figure 1 (A-D) showed that the surface morphology of the control group (hydrogel base) was uneven and there were no pores. Figure 1 (E-H) in treatment group 3 (hydrogel base with the addition of liquid smoke and vitamin K), the surface morphology looked almost flat, smooth, and porous. The presence of these pores showed that the addition of liquid smoke and vitamin K resulted in a greater surface area than the control group. In addition, the presence of pores indicated that the hydrogel wound membrane contains active compounds from liquid smoke and vitamin K which will be transported by the hydrogel to the wound tissue. Also, porous hydrogel wound caps are capable of
absorbing excess exudate in the wound. However, more than 3000x magnification was needed to get clearer and more detailed surface morphology.

![Figure 1](image)

**Figure 1.** SEM test results of hydrogel wound dressing control group PVA/Chitosan/Starch with magnification 1000x (A) 1500x (B) 2500x (C) 3000x (D), treatment group 3 PVA/Chitosan/Starch/liquid smoke/vitamin K with magnification 1000x (E) 1500x (F) 2500x (G) 3000x (H).

### 3.2 Antibacterial Test

The effects of hydrogel membranes infused liquid smoke on the growth inhibition of *S. aureus* studied were illustrated by the average of growth inhibition width of *S. aureus*. The results of the inhibition width at a concentration of 16% (1,625 mm) which was greater than at concentrations of 12% (1,191 mm), 8% (0,416 mm) and 0% (0,013 mm) as shown in Table 1. Based on descriptive analysis, it can be stated that the growth inhibition width of *S. aureus* was significant with the increase of liquid smoke concentration.

**Table 1.** The average inhibition width of hydrogel membranes on the *S. aureus* growth.

| Groups     | n  | Mean (mm) | SD (mm) |
|------------|----|-----------|---------|
| Control    | 6  | 0.013     | 0.033   |
| I          | 6  | 0.416     | 0.513   |
| II         | 6  | 1.191     | 1.328   |
| III        | 6  | 1.625     | 0.332   |

Note:
A. Control group (hydrogel base membrane)
B. Treatment I group (hydrogel base membrane infused liquid smoke 8%)
C. Treatment II group (hydrogel base membrane infused liquid smoke 12%)
D. Treatment III group (hydrogel base membrane infused liquid smoke 16%)

The reliability test with Cronbach’s Alpha was to determine the level of observer’s confidence of inhibitory width measurement. The value from 3 observers was 0.932 (0>0.60) so it can be concluded that the observational data was reliable or consistent. In order to evaluate the difference between control and treatment groups, Kruskal-Wallis test was showed the results of which indicated a significant difference in the inhibition width of *S. aureus* bacteria growth among all treatment groups with a p value of 0.001 (p<0.05). Furthermore, the Mann Whitney test was conducted to determine the differences between two treatment groups within the entire research samples.

The data analysis results indicated that the hydrogel membrane without infused liquid smoke and infused liquid smoke 8% did not have antibacterial properties. By contrast, the hydrogel membrane
infused liquid smoke 12% and 16% had antimicrobial activity. From the recapitulation of the antibacterial activity test of the treatment groups, it can be revealed that the hydrogel membrane infused liquid smoke with a concentration of 12% was the lowest concentration inhibiting S. aureus.

The first concentration of liquid smoke used in this study was 50%, 75% and 100% with the last concentration of liquid smoke when becoming a hydrogel membrane was 8%, 12% and 16% respectively. The results of the difference test subsequently revealed there to be a significant difference between the treatment groups (p<0.05). Based on the results of the study, this data was linear with previous research that the higher concentration of liquid smoke positively effects on the growth of S. aureus. This result is consistent Kailaku et al., an increase concentration of liquid smoke from coconut shells of 25%, 50% and 75% affect the increase growth inhibition of S. aureus [11]. According to Adhiasari, the value of the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) of liquid smoke against S. aureus was 25% and 50% respectively [14]. Another related research by Anisah revealed that the application of liquid smoke against S. aureus bacteria caused the surface of the bacterial cell wall to become more rough and uneven [15]. Liquid smoke contained antibacterial active compound such as phenols, acidic acids, and carbonyls [16].

Polyphenols are bioactive molecules related to the structures of the molecules; by their hydroxyl groups or by the phenolic ring, phenolic compounds can link with proteins and bacterial membranes to form complexes [17]. Phenolic compounds, like phenol, 2-methoxyphenol (guaiaicol), 3,4 dimethoxyl phenols, and 2-methoxy-4-methyl phenol are prominent in coconut shells liquid smoke and play a major contribution to antibacterial activity [18].

The phenols agent of liquid smoke is able to inhibit the growth of gram negative and gram positive bacteria [19]. S. aureus is gram-positive bacteria that tend more thinner because they do not have an outer membrane making it easier for hydrophobic compound to penetrate into cells [20]. Phenols and their derivatives are bacteriostatic because they are able to coagulate amine functional group (-NH) and sulfhydryl (-SH) cell wall of bacterial proteins [15]. Phenols interact with bacterial cells through absorption, resulting in hydrogen linking. At low levels, the protein is formed with weak links and immediately decomposes followed by penetration of phenols into the cell causing protein denaturation. On the other hand, phenol causes protein coagulation and membrane lysis [20].

The acidic of liquid smoke also has antimicrobial properties. The antibacterial activity of phenols will increase when together with acidic compounds [15]. The principle of bacteria inhibition by organic acids is that the non-dissociated acid will penetrate into the bacterial cell wall to disrupt the normal cell physiology [21]. Furthermore, the mechanism of carbonyl is penetration through bacterial cell membranes and inactivating enzymes found in the cytoplasm of bacteria. This will affect the overall pH and form an acid that is not dissociated resulting in disturbed metabolism [16].

3.3 Wound Healing Animal Test

Data normality test was done using the Saphiro-Wilk test because the number of samples were less than 50 samples. Normally distributed data were analysed using the One-way ANOVA Test and Tukey HSD Post-Hoc Test while non-normally distributed data were analysed using the Kruskal Wallis Test and the Mann- Whitney Test. The results showed in table 2 that there was a significant difference between the control group and the treatment group on observations of days 4 and 7 so it can be seen that the hydrogel membrane is effectively given in the first 10 days after injury. Macroscopic observations are calculated to determine the average wound and the percentage of healing in figures 2 A and B. The results showed the best treatment group was treatment group 3 (treatment on the basis of PVA hydrogel, chitosan, starch with the addition of liquid smoke and vitamin K) with an average wound area on the 14th day of 2.6 mm2 and the percentage of wound healing by, 98.3%. Experimental animal wounds in treatment group 3 healed on the 10th day of observation. Treatment group 3 healed on the 10th day of observation.
Table 2. Results of Macroscopic Observation of Experimental Wound Data Analysis.

| Group Type | Average ± SD | Day 4     | Day 7    | Day 11   | Day 14   |
|------------|--------------|-----------|----------|----------|----------|
| Control    | 130 ± 42.85a| 54.4 ± 19.7a| 13.2 ± 5.9| 3.7 ± 2.13|
| Treatment 1| 64.8 ± 26.75b| 37 ± 12.95ab| 9.9 ± 3.95| 3 ± 2.58  |
| Treatment 2| 81.75 ± 41.19abc| 37.97 ± 16.24abc| 10 ± 6.28| 3.01 ± 3.68|
| Treatment 3| 48.66 ± 14.3abc| 24.55 ± 18.52abc| 6.9 ± 8.25| 0.96 ± 1.9  |

| P value    | 0.002*       | 0.040**    | 0.409*    | 0.196**   |

Note: Numbers followed by different superscript letters (a, b, c, d) show real differences.
*Testing with One-way ANOVA; ** Testing with Kruskal-Wallis

Figure 2. Average Wound Area (A) and Percentage of Wound Healing (B).

In microscopic tests the number of fibroblast cells was calculated using a light microscope with a magnification of 40x. Fibroblasts were counted in 10 times the field of view by counting the number of fibroblast cells in the form of large, flat, branched cells, which from the side look fusiform in shape with one or two oval-shaped nuclei painted in purple on HE staining. The data normality test was done using the Saphiro-Wilk test because the samples were less than 50 samples. Normally distributed data were
analysed using the One-way ANOVA Test and the Tukey HSD Post-hoc Test. The results showed significant differences between the control group and the treatment group with a value of $p = 0.002$ ($p < 0.05$). The results of the calculation of the number of fibroblasts showed the best treatment group is treatment group 3 (PVA/chitosan/starch/liquid smoke/vitamin K) with an average number of fibroblasts as much as 90.60.

**Figure 3.** Histopathological picture of skin tissue affected by incision wounds per visual field in all treatment groups (arrows indicate fibroblasts with 40x magnification) Remarks: (A) Control group, (B) Treatment group 1, (C) Treatment group 2, (D) Treatment group 3.

Hydrogel wound dressing with the addition of liquid smoke of coconut shell level I and vitamin K extract of collard greens is able to accelerate the healing of incision wounds of experimental animals due to the presence of active compounds in it. Liquid smoke contains phenols as well as their inheritance, tannins and flavonoids which act as anti-oxidants, anti-microbial, and anti-inflammatory properties [22]. The anti-oxidant properties of liquid smoke phenols play a role in inhibiting fat oxidation, preventing lipid oxidation by stabilizing free radicals, as well as increasing blood flow to scarring and minimizing scars. Liquid smoke has a system to detoxify reactive oxygen species (ROS) in protecting against oxidative stress [23]. Several materials of liquid smoke that may stimulate fibroblasts proliferation are tannins and phytosterols. One study found that these materials accelerate the wound healing process by increasing capillary formation as well as fibroblast proliferation. These result in enhancement of the rate of epithelization. [24].

The increase in the percentage of wound healing is proportional to the increase in the number of fibroblast cells. Treatment group 3 (hydrogel base treatment with the addition of liquid smoke and vitamin K) was the best treatment group as evidenced by the average smallest wound width with the largest percentage of healing on the 14th day of observation and the highest number of fibroblasts distribution compared to other groups. This is in line with research conducted by Tarawan, the longer the administration of coconut shell liquid smoke, the greater the effect produced on increasing the number of fibroblasts [9].

In addition, the active compounds from liquid smoke, the vitamin K content of the collard greens also plays an important role in wound healing. Vitamin K acts as a coenzyme and is involved in the synthesis of a number of blood clotting proteins [25]. Vitamin K is a cofactor of carboxylation of glutamate residues in post-synthesis modification of proteins to form the amino acid γ-carboxyglutamate. Initially, vitamin K hydroquinone is oxidized to epoxide, which activates a glutamate residue in the protein substrate into a carbanion, then reacts non-enzymatically with CO2. The γ-carboxyglutamic amino acid can bind to calcium ions so that blood-forming proteins can bind to the membrane [26]. The dosage form of the hydrogel membrane wound dressing also influences wound healing. Hydrogel film can absorb and hold a certain amount of water when in contact with a wet wound [27]. Hydrogel effectively keeps wounds moist to prevent wound infections and stimulates skin cell regeneration. Hydrogel with porous surface morphology allows transport of scaffold cells to form new tissue [4].
4 Conclusion
Based on the results of research, the hydrogel base (Polyvinyl-Alcohol (PVA), Chitosan, Starch) wound dressing that infused with coconut shell liquid smoke grade I and extracted vitamin K from collard greens appears capable making a surface morphology that looks flat, smooth, and porous. Also, it can inhibit the growth of S. aureus bacteria. This wound dressing has strength to accelerate wound healing process. Animal incision wound can heal on the 11th day.

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