Clinical Plasma Medicine 15 (2019) 100090

Contents lists available at ScienceDirect

Clinical Plasma Medicine

journal homepage: www.elsevier.com/locate/cpme

Original research article

Ethanolic extract of the natural product of *Daun sirih* (*Piper betle*) leaves may impede the effectiveness of the plasma jet contact style for acute wounds

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ARTICLE INFO

Keywords:
Plasma medicine
ROS
RNS
PAW
Natural product
Wound

ABSTRACT

Purpose: An investigation was carried out to determine the effect of an ethanolic extract of the natural product of *Daun sirih* or *Piper betle* leaves on the effectiveness of plasma jet treatment for cutaneous acute wound healing in a small animal model mimicking a clinical setting.

Method: An atmospheric plasma jet using medical grade argon gas as a carrier gas was developed. The ethanolic extract of *Piper betle* leaf (EPB) was formulated. Optical emission spectroscopy and chemical methods were applied to evaluate the presence of reactive oxygen species (ROS) and reactive nitrogen species (RNS) in the gas phase and in aqueous and ethanolic media. Small animals were classified into 5 groups, namely, Control (C), Plasma jet (P), Ethanolic extract of *Piper betle* leaf (EPB), Plasma jet followed by EPB (P-EPB) and EPB followed by plasma jet (EPB-P). The contact and meander styles of plasma jet treatment for wounds were applied daily on acute wounds for 1 min, either alone or before or after EPB treatments. Visual evaluation of wounds was conducted for 14 days. Microscopic evaluation was conducted on days 7, 11 and 14. General staining, namely, haematoxylin-eosin and Azan staining, was conducted to evaluate neoepithelialisation and new collagen formation.

Results: This research showed that wound healing in the P group was faster than that in the other groups, while that in groups containing EPB was the same as that in C. In the P group, the number of days to reach peak inflammation was the fewest. On day 7, neoepithelialisation and new collagen formation in P were significantly higher than those in other groups.

Conclusion: Plasma jet treatment alone is able to promote inflammation, neoepithelialisation and new collagen formation to accelerate acute wound healing; however, its admixture with EPB may impede such effectiveness. Based on the characterization of the ROS and RNS results, the ethanol solvent may play a primary role in impeding its effectiveness.

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https://doi.org/10.1016/j.cpme.2019.100090
Received 9 October 2018; Received in revised form 18 May 2019; Accepted 12 June 2019

1. Introduction

It is well known that there are two main types of wounds, namely, acute and chronic wounds. Conceptually, optimal healing of acute wounds has been divided into several chronologically overlapping phases, namely, inflammation, proliferation, and remodeling [1]. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) play a pivotal role in the orchestration of such processes [2,3]. To date, there have been a number of therapeutic strategies based on modulation of ROS function, namely, topical H_2O_2 (or related ROS intermediates [4–10], recombinant glucose oxidase [11], honey [12–17], galvanic particles [18,19], recombinant PDGF [20–23] and recombinant galectin-1 [24]. Those strategies, unfortunately, have drawbacks. Sen [25] reported that the uncontrolled kinetics of ROS/RNS release is the primary barrier to using ROS/RNS-donating compounds for topical applications. The development of plasma medicine technology producing pharmacologically appropriate doses of ROS/RNS would open a new window of opportunity in new clinical treatment methods [26].

Plasma medicine is a multidisciplinary science involving plasma science, life sciences, pharmacy, biomedical and other health sciences aimed at applying physical plasma in the field of human health therapy. The plasma referred to in this context is not blood plasma but physical plasma as a phase of the fourth state of matter, in addition to solid, liquid and gas. Plasma is commonly known as ionized gas because in the plasma phase, there are stable parts (gases) and reactive parts (ions, energetic and radical particles). Conceptually, the medical aspects of plasma are related to the ability of plasmas to generate biological molecules, namely, reactive oxygen species (ROS) and reactive nitrogen species (RNS), which if carefully controlled and in the right doses can be efficacious for health therapy [27–29]. A recent study involving a multidisciplinary team of researchers and lawyers reported that the style of action of a plasma medicine source, specifically cold atmospheric pressure plasmas, is pharmacological and not physical in nature [30].

A primary goal in medical plasma research is to gain insight into how to optimize medical plasma as a health therapy while minimizing its side effects. One approach that now attracts substantial attention from medical plasma researchers is the combination of medical plasma with compounds in the liquid phase (solution or liquid), which is known as the plasma-activated water (PAW) approach. Furthermore, Jablonowski et al. [31] emphasized that the concept of plasma-liquid interaction is a determining factor for the application of plasma in the medical field.

Based on previous research, it was reported that the jet type of medical plasma can accelerate the healing of acute full-thickness wounds in an in vivo setting by promoting inflammation, neoepithelialisation and wound contraction [32]. Efforts to optimize medical plasma performance for wound healing using a combination of plasma and liquid have previously been performed [33–35]. Nasruddin et al. [33] reported that a combination of plasma jet and water in a microlitre volume was able to significantly accelerate the healing of acute wounds by promoting wound contraction, compared to plasma treatment alone. Nasruddin et al. [34] also studied the effects of medical plasma treatment combined with low-concentration honey solutions supported by microwell dressings for wound healing, but the result was not significant. Wahyuningtyas et al. [35] reported that a combination of plasma jet and Manuka honey is more effective than a combination of plasma jet and Indonesian honey for the improvement of acute wound healing.

The effectiveness of natural products, such as hormones [36–38] and honey [39], as complementary and alternative medicine to support wound healing has been reported, while the leaves of the *Piper betle* plant (locally known as *Daun Sirih*) have long been in use for the relief of pain in Indonesian indigenous medicine. *Piper betle* leaves contain active compounds, such as alkaloids and tannins, that have antibacterial and anti-inflammatory effects [40]. The effectiveness of the ethanolic extract of the leaves to improve ulcer healing was previously reported [41]. It was hypothesized that the antioxidant or free radical scavenging activity of the plant extract may be correlated with its healing action. The present study was conducted to evaluate the effectiveness of a combination treatment of plasma jet and ethanolic extract of *Piper betle* leaves for acute wound healing using an experimental design mimicking a clinical setting in an animal model. The contact style of plasma jet treatment was applied in this investigation.

2. Methods and materials

2.1. Atmospheric pressure plasma jet system

An atmospheric pressure plasma jet system that was developed based on Teschke et al. [42]. and described previously [34] was applied in this experiment. A modification of the dimensions of the capillary quartz tube was conducted. In this experiment, the inner and outer diameters of the tube were to be 0.65 mm and 1.55 mm, respectively. This material was fabricated by Beijing Zhong Cheng Quartz Glass Co., Ltd (Beijing, China). Two electrodes from an aluminum foil ring were applied around the quartz tube for this system. Non-conductive material was applied to isolate those two electrodes.

Electrical and optical emission characterizations were conducted at the Research Center for Sustainable Energy and Technology, Institute of Science and Engineering, Kanazawa University, Japan. The discharge voltage and discharge current were measured with a high-voltage probe (P6015A; Tektronix, Inc., Tokyo, Japan) and a current probe (8585C; Pearson Electronics, Palo Alto, CA, USA). When argon gas at a flow rate of 1 standard litre per minute (slm) was allowed to flow from one end of the quartz tube, a low-frequency (~ 18.32 kHz)high AC voltage with a peak-to-peak voltage of 9.58 kV and current of 55.2 mA was measured at the upper ring electrode. Hospital grade ultra-pure argon gas (99.999% purity) (Samator Company, Indonesia) was used as a carrier gas.

Optical emission spectroscopy (OES) measurements taken approximately 10 mm under the nozzle showed the emissions of the OH (A-X transition) transition near 309 nm, N2 (C-B transition) (band head maximum at 337 nm) [43] and Ar I (maximum 763 nm).This measurement showed the presence of both hydroxyl radical (OH) and nitrogen-based reactive species in the gas phase during its generation (Fig. 1).

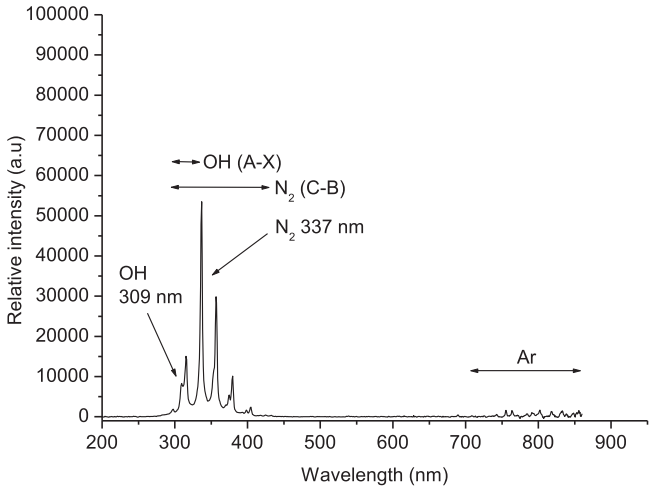

Fig. 1. Optical emission spectroscopy (OES) identification of atmospheric plasma jet near 10 mm under the nozzle of the plasma jet reactor without mouse. OH and nitrogen-based reactive species were detected.

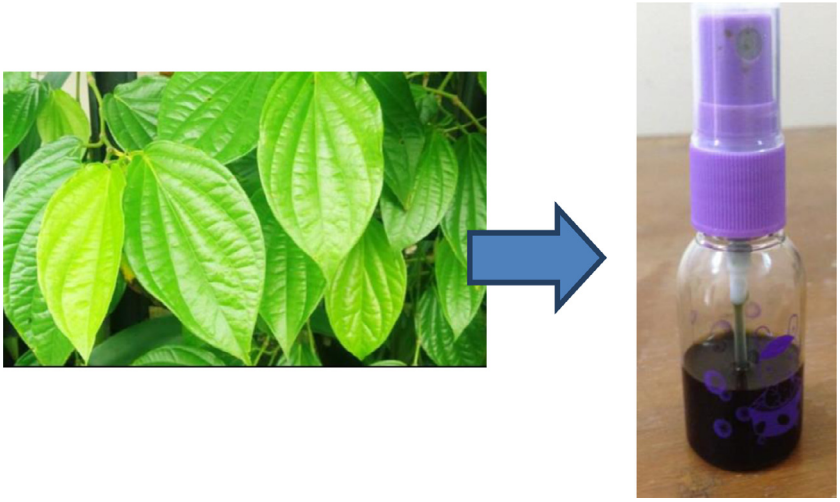

Fig. 2. *Left: Piper betle* leaves. *Right*: Ethanolic extract of *Piper betle* leaves ready to be used.

2.2. Preparation of an ethanolic extract of Piper betle leaf and determination of its H_2O_2 and its NO_2

2.2.1. Preparation of ethanolic extract of Piper betle leaves

Fresh *Piper betle* leaves were collected locally and authenticated by the Laboratory of Pharmacy, Universitas Muhammadiyah Magelang, Indonesia. After being air dried and powdered in a hand crusher, the dried, powdered leaves (600 g) were extracted three times with absolute ethanol (1800 mL each time) and the resultant filtrate was solvent-recovered by distillation in a Soxhlet apparatus [44]. The concentrated extract, referred to as ethanolic extract of *Piper betle* leaf (EPB), was used as a stock solution for this experiment (Fig. 2).

2.2.2. Determination of H_2O_2 and NO_2

Concentrations of H_2O_2 and NO_2 in pure water (A), plasma jet-treated pure water (A-P), EPB diluted in pure water (EPB-A), plasma jet-treated EPB-A (EPB-A-P), ethanol (E), plasma jet-treated ethanol (E-P), ethanolic extract of *Piper betle* leaves diluted in ethanol (EPB-E) and plasma jet-treated EPB-E (EPB-E-P) were analyzed with a method employing a peroxidase enzyme for $H_2O_2$and a naphthylethylenediamine visual colorimetric for NO_2 using commercial reagent (Kyoritsu Chemical-Check Lab., Japan; Model WAK-H2O2, range: 0.05–5.0 mg/L and ModelWAK-NO2, range: 0.02–1.00 mg/L) after plasma jet treatment. This method was also previously used by other researchers [33]. EPB-A was 1 ml EPB diluted in 299 ml pure water. E was 96%ethanol. EPB-E was 1 ml EPB diluted in 299 ml ethanol 96%. Ten millilitres of A,EPB-A, E and EPB-E in 10-ml beakers were treated by plasma jet for 2 min, perpendicularly. Before treatment, the distances between the nozzle of the plasma jet reactor and the surfaces of the objects were approximately 10 mm. Digital packtest devices (Kyoritsu Chemical-Check Lab., Japan; Model DPM-H2O2 and Model DPM-NO2) were used to measure the concentrations of H_2O_2 and NO_2.

2.3. Animals and investigation protocol

The investigation protocol and animal care were conducted based on the Guidelines for the Care and Use of Laboratory Animals at Laboratorium Penelitian dan Pengujian Terpadu/Integrated Research and Testing Laboratory (LPPT UGM), Gadjah Mada University, Yogyakarta, Indonesia (certificate number:00004/04/LPPT/III/2018). This institution works under the standard of the ISO/IEC 17,025 and the National Accreditation Committee of Indonesia (Komite Akreditasi Nasional/KAN, Indonesia). Forty-five Balb/c male mice aged 8 weeks and weighing 21.0–24.0 g purchased from Histology Laboratory, Universitas Sebelas Maret, Surakarta, Indonesia were used. The mice were housed individually in a room with a temperature of 28.0 ± 2.0 °C and light from 09:00 to 21:00 h. They were allowed *ad libitum* access to feed and water.

2.4. Wound healing model and plasma treatment

The experimental animals, namely, Balb cmice, were anaesthetized via the peritoneal cavity by injection of 50 mg/kg ketamine (K) and 5 mg/kgxylazine (X) [45]. The protocol to make an acute wound on a small animal as described previously [34] was adopted. This protocol was applied to create 2 disc-shaped (4 mm in diameter), full-thickness skin wounds, including the panniculus, on both sides of the mouse dorsum using a disposable 4 mm biopsy punch.

In this experiment, wound samples were distributed in five groups:

a Hydrocolloid dressing alone (Control group or C)
b Extract of *Piper betle* alone (EPB)
c Plasma alone (P).
d Plasma pursued by extract of *Piper betle* (P-EPB)
e Extract of *Piper betle* pursued by Plasma (EPB-P)

The wounds of the control group animals were covered with a hydrocolloid dressing (HD). Ethanolic extract of *Piper betle* was applied daily on animals in the experimental groups using a sprayer. The contact style of plasma jet used daily to treat the wound for 1 min. The wound surface was placed approximately 10 mm under the nozzle tip. To ensure that all areas of the wound surfaces were exposed in the plasma jet-treated groups, the meander style of plasma jet treatment was implemented. The experimental protocol is shown in Fig. 3.

2.5. Macroscopic evaluation of wound

The protocol for visual or macroscopic wound inspection described previously [34] was adopted in this experiment. These inspections were conducted for 14 days, with Day 0 being the day when the acute wounds were created. A digital camera (Panasonic, Lumix FH6) was used daily to document wound conditions.

2.6. Estimated day of healing

Using a graph of the ratio of the current wound area to the initial wound area, an estimated day of wound healing was determined from the y-axis when the trend of reduction of the wound size leveled off, as reported previously [34], at $y = 0.4$ (Fig. 6). From that point, line z was drawn, crossing the lines of reduction for each group. Wound healing

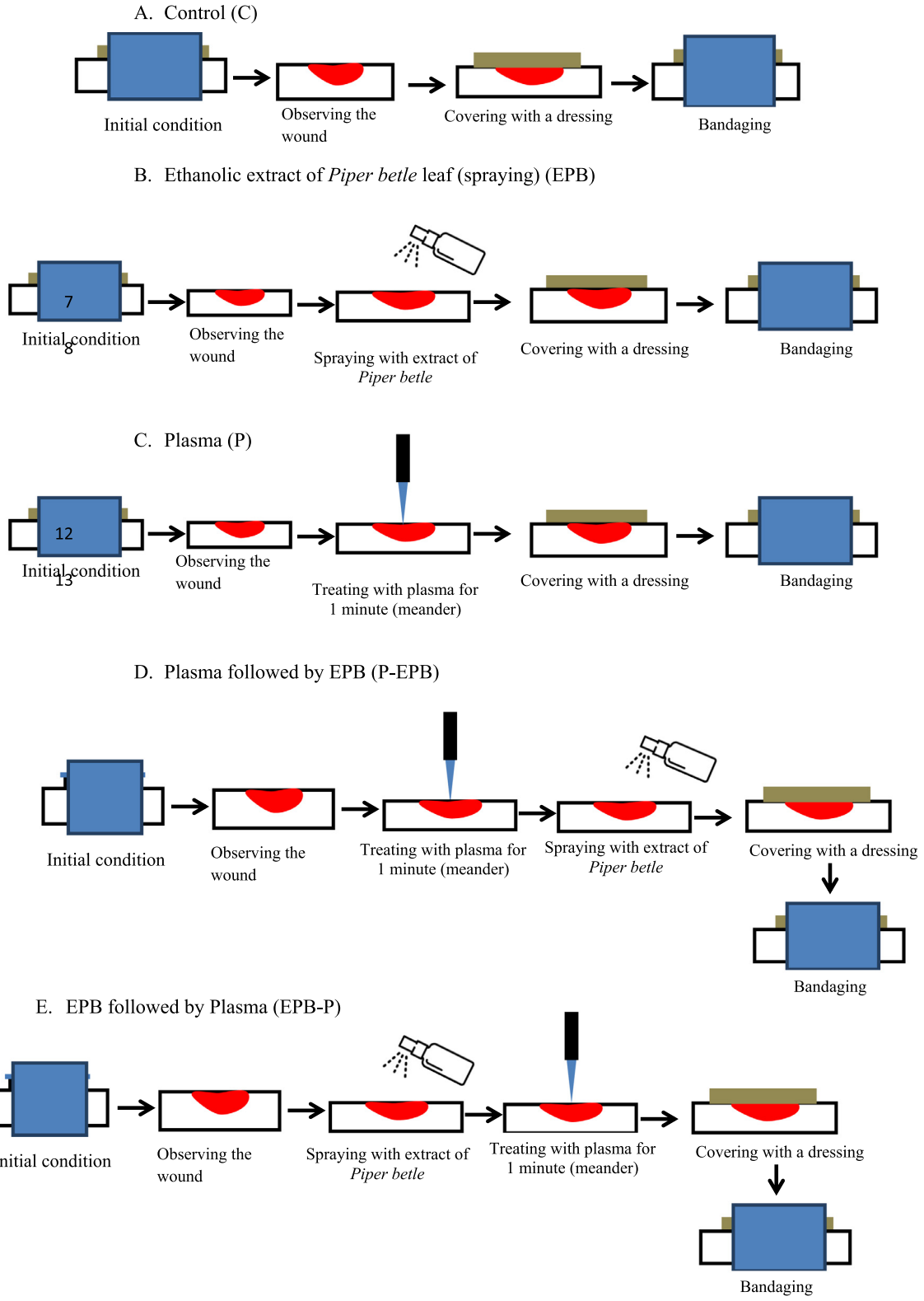

Fig. 3. Experimental protocol from day 0 until day13.

days at points c*, p*, epb*, p-epb* and epb-p* were calculated based on their points of intersection with the x-axis.

2.7. Tissue processing and histological analysis

On days 7, 11 and 14 after wound creation, the experimental animals were massively euthanized by injection with ketamine-xylazine, administered via IP injection.Tissue surgery was conducted on the wound and its surrounding skin. Tissue processing was then conducted by means of a previously described technique [34].The harvested wound tissue was stapled onto polypropylene sheets to prevent hypercontraction of the samples and then fixed for approximately 24 h in

neutral-buffered 10% formalin solution at pH 7.4.Tissue bisection was carried out at the wound center. Subsequently, sections were dehydrated in an alcohol series, cleaned in xylene, and embedded in paraffin to prepare serial 5 μm sections. The sections containing the wound center were stained with haematoxylin-eosin (HE) and Azan for general histological analysis. On the basis of the results of haematoxylin-eosin staining from histological samples on days 7, 11 and 14 the percentage of neoepithelialisation was calculated using the following formula [46]:

$$Neo-epithelialisation\ (\%) = \frac{length\ of\ new\ epithelium}{length\ of\ wound\ between\ wound\ edges} \times 100\%$$

Using the results of Azan staining from histological samples on days 7 and 11, the percentage of new collagen formation was calculated using the following formula:

$$New\ collagen\ formation\ (\%) = \frac{portion\ of\ new\ collagen}{portion\ of\ wound\ between\ wound\ edges} \times 100\%$$

2.8. Statistical analysis

Data were subjected to statistical analyses using SPSS 16.0. The ratios of the average wound area to the original wound area, neoepithelialisation and new collagen formation were evaluated by ANOVA followed by the Tukey-Kramer method; P values <0.05 were considered significant.

3. Results

3.1. Determination of H_2O_2 and NO_2

In non-plasma-treated aqueous sample EPB-A and the plasma-treated aqueous samples (A-P and EPB-A-P), NO_2 was identified with different concentrations, while in all ethanolic samples, NO_2 was almost unidentified. While $NO_2$was detected in EPB-A, its concentration was almost the same as that in A-P.H_2O_2 was identified in all ethanolic samples, but its rates varied. The H_2O_2 levels in the plasma-treated ethanolic samples (E-P and EPB-E-P) were higher than those in non-plasma-treated ethanolic samples (E and EPB-E). H_2O_2 in EPB-E-P, however, was lower than that in E-P. Finally, two points were hypothesized. First, in the ethanolic extract of *Piper betle* samples, plasma jet treatment increasesH_2O_2 concentrations, but the extract of *Piper betle* may suppress them. At the same time, ethanol may reduce or eliminate the RNS produced by the plasma jet. Second, in the ethanolic extract of *Piper betle* diluted in pure water, plasma jet treatment decreasesH_2O_2 but increases RNS. Water may act as a mediator of plasma jet treatment's production of RNS.

3.2. Macroscopic results

Fig. 5 shows the observed wounds on days 0, 3, 7, 11 and 14 for every group. The healing in all groups has a similar pattern, in which the wounds increased in size during the inflammation phase and then decreased gradually during the proliferative and maturation phases. The wound size on day 11 in the P group is smaller than that in the other groups. The yellowish color shown in the EPB, EPB-P and P-EPB images consists of residue of the ethanolic extract of *Piper betle* leaf.

3.3. Wound area reduction and estimated day of healing

All groups healed with a similar pattern. During the inflammation phase (from days 0 until 3,4, or 5), the wounds increased dramatically, and then they decreased gradually until day 14. The peak inflammation days for the P group, the groups with ethanolic extract of *Piper betle* (EPB, P-EPB and EPB-P) and the C group were days 3, 4 and 5, respectively. The wound area on the peak inflammation day in the P group (see A with a dash-lined circle) was larger than that in the groups treated with ethanolic extract of *Piper betle* (see B with a dash-lined circle), while the wound area in the P and EBP-treated groups was larger than that in the C group (see C with dash-lined circle). From days 3 to 14, the observed wound area in the P group was significantly smaller than those in the C group on days 6 and 9; those in the P group were significantly smaller than those in the P-EBP group on days 7, 8 and 9; those in the P group were significantly smaller than those in the EPB group on days 8 and 9; and finally, those in the P group were significantly smaller than those in the EBP-P group.

The day of wound healing for the P (p*) group was estimated on Day 10, whilst those for the C group (c*) and the groups treated with ethanolic extract of *Piper betle* (epb*, p-epb* and epb-p*) were estimated to occur on the same day: Day 12.The prediction showed that the day of wound healing in the P group was earlier compared with those of the other groups. Finally, it was indicated that the ethanolic extract of *Piper betle* leaf may reduce the effectiveness of atmospheric plasma jet to accelerate acute wound healing.

3.4. Neo-epithelialization

On days 7, 11 and 14 after wound creation, the percentages of neoepithelialisation for every group were calculated (Fig. 7). On day 7, the superiority of the neoepithelialisation percentage in the P group was apparent. The neoepithelialisation percentage in the P group was significantly higher than those of the other groups (P vs $C = P < 0.05$; P vs EPB $= P < 0.05$; P vs P-EPB $= P < 0.01$; P vs EPB-$P = P < 0.05$), while those in groups containing ethanolic extract of *Piper betle* leaf were not significantly different from the Control (C vs EPB $= P > 0.05$; C vs P-EPB $= P > 0.01$; P vs EPB-$P = P > 0.05$). From days 7 to 11, the neoepithelialisation percentages for all groups increased dramatically; however, on day 11, those for all groups did not differ significantly. On day 14, the wounds in the C, EPB and P groups were completely covered by neo epithelium, whilst those in the P-EPB and EPB-P groups were more than 90% covered by neoepithelium. The neoepithelialisation percentages for all groups on that day, however, did not differ significantly.

3.5. Collagen formation

On days 7 and 11 after wound creation, the percentages of collagen formation for every group were calculated (Fig. 8**).** On day 7, the percentage of new collagen formation in the P group was significantly higher than those in other groups (P vs $C = P < 0.05$; P vs EPB $= P < 0.01$; P vs P-EPB $= P < 0.01$; P vs EPB-$P = P < 0.01$), while those in groups containing ethanolic extract of *Piper Betle* leaf were not significantly different from the Control (C vs EPB $= P > 0.05$; C vs P-EPB $= P > 0.01$; P vs EPB-$P = P > 0.05$). On day 11, the percentage of new collagen formation for the P group was significantly higher than that for the P-EPB group, but those for the C, P and EPB-P groups did not differ significantly.

4. Discussion

It has been reported that there is an intimate connection between parameters and styles of atmospheric plasma jet treatment and its healing effectiveness [34]. Although plasma-activated water (PAW) has recently gained a great deal of attention in the study of plasma medicine, well-tested models of PAW applied in animal or human clinical settings are still rare to date. To the best of our knowledge, this is the first report to study the effectiveness of combinatory treatment of atmospheric plasma jet and ethanolic extract of natural product *Piper betle* leaf for acute wound healing, using an animal model mimicking a modern clinical setting and supported by hydrocolloid dressing as a

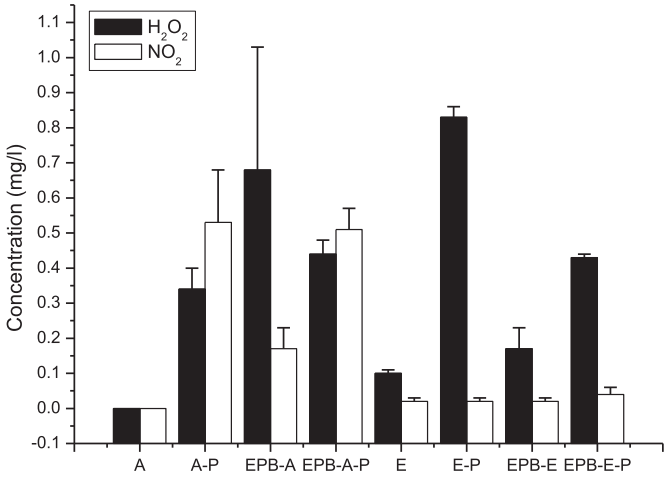

Fig. 4. Concentrations of H_2O_2 and NO_2 in pure water (A), plasma jet-treated pure water (A-P), ethanolic extract of *Piper betle* diluted in pure water (EPB-A), plasma jet-treated EPB-A (EPB-A-P), ethanol (E), plasma jet-treated ethanol (E-P), ethanolic extract of *Piper betle* leaf diluted in ethanol (EPB-E) and plasma jet-treated EPB-E (EPB-E-P).

means of keeping the wound environment moist.

Indications regarding the contact style of plasma treatment for burn wound were investigated previously [47]. Our prior research, however, has shown that plasma jet treatment employing the spot and contact styles with a duration of 5 min causes detrimental effects on the normal skin of a mouse, which was previously marked by an increase of more than 4 °Celsius in the local temperature, as shown by an infrared thermal camera [33,34]. It was also reported that the use of the contact style of plasma jet at a distance of 5 mm for 3 min impedes wound healing [48].

In the present study, the contact style of plasma treatment was also applied, alone and before/after EPB, but in order to reduce its detrimental effect, the meander style of treatment was applied. Weltmann et al. [49] reported that plasma jet treatment using a moving style was able to significantly minimize the temperature elevation. This investigation showed that there was no significantly different effect between the two styles of plasma treatment combined with EPB, namely, plasma followed by extract of *Piper betle* (P-EPB) and extract of *Piper betle* followed by plasma (EPB-P). This investigation, however, indicated that plasma jet alone accelerates wound healing more effectively than either combination. Neo-epithelialization and new collagen formation was significantly enhanced in the plasma jet alone group,

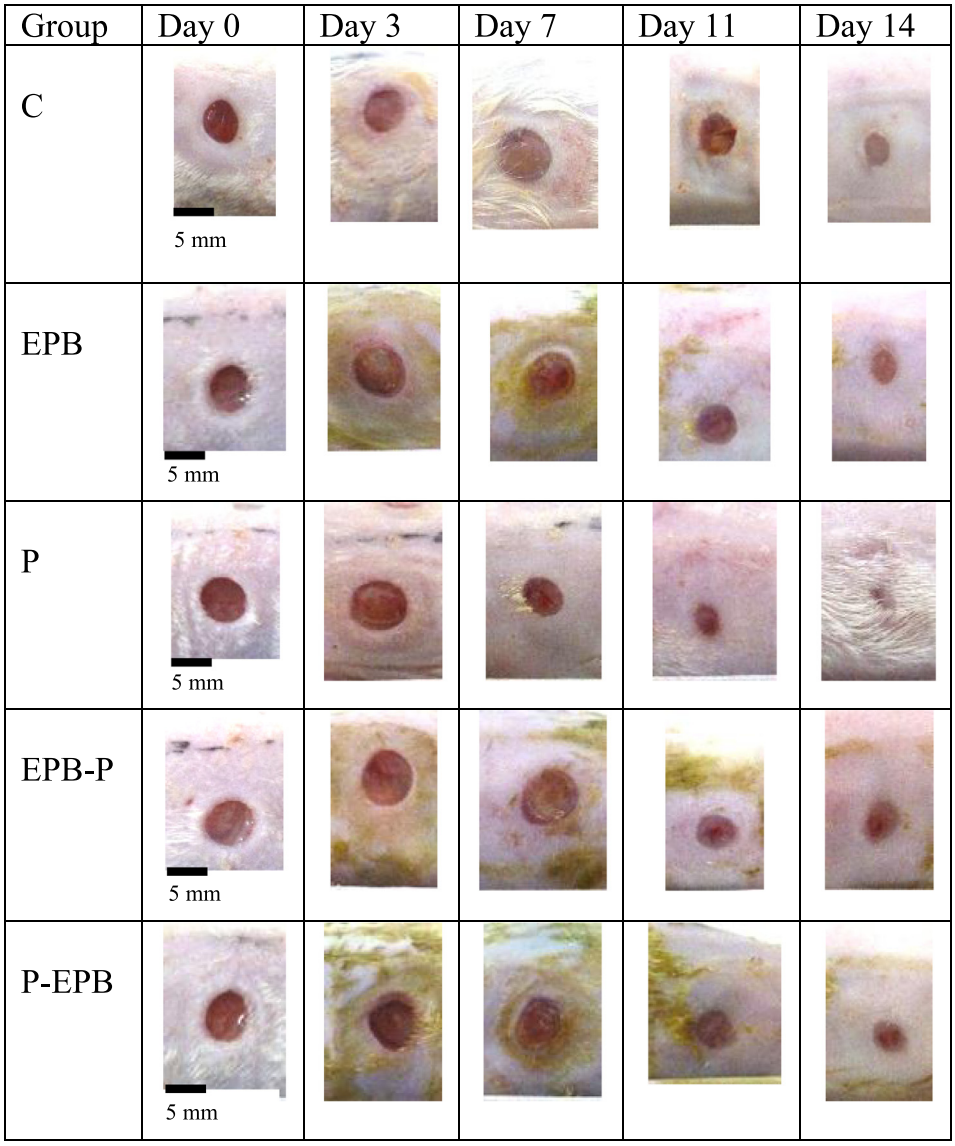

Fig. 5. Macroscopically observed wounds on days 0, 3, 7, 11, and 14.

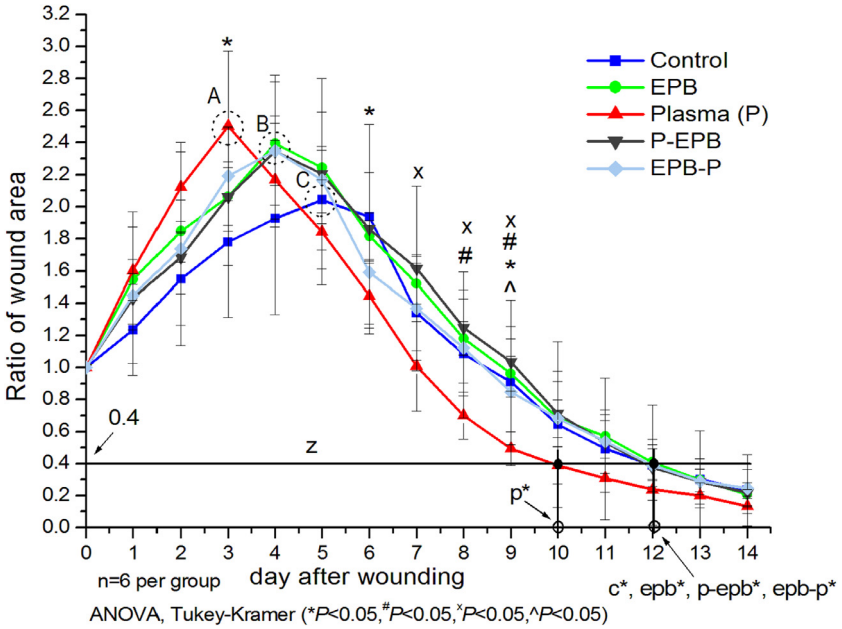

Fig. 6. Graph of ratio of wound area to initial wound area and estimated days of healing.

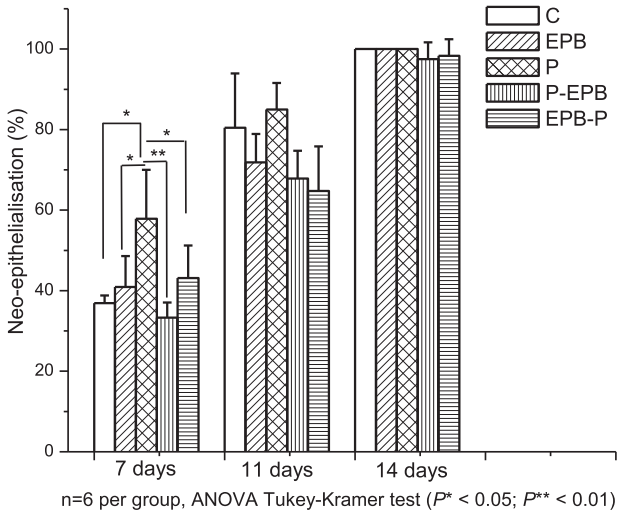

Fig. 7. Neo-epithelialisation percentage on days 7, 11 and 14 after wound creation. On day 7, the superiority of neoepithelialisation percentage in P was observed.

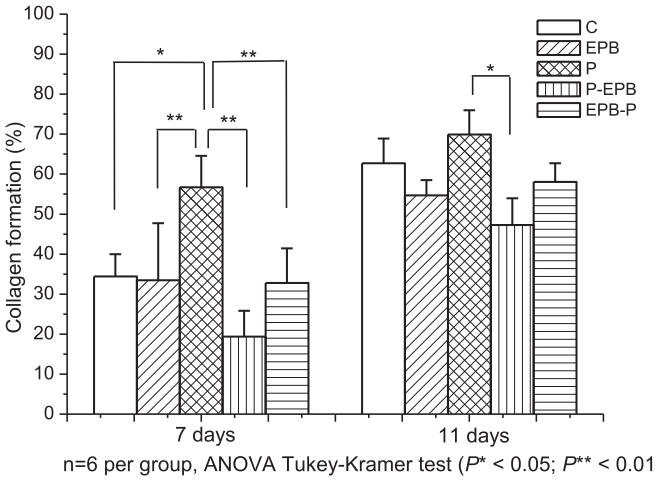

Fig. 8. Percentage of new collagen formation.

while plasma jet treatment combined with EPB tended to be similar to the control group. Based on this finding, it is hypothesized that plasma jet with parameters as applied in this research design is effective in improving wound healing, but such effectiveness can possibly be impeded by the use of ethanolic extract of *Piper betle* leaf. In this context, however, the ethanol solvent may be acritical factor in the reduction in effectiveness. As shown in the results (Fig. 4), ethanol solvent reduces RNS and increases ROS excessively. It was reported that low concentrations of RNS [28] and ROS [3] have efficacy for wound healing, while high levels of ROS clearly have the potential to be a complicating factor in the regeneration and remodeling of nascent tissue. Detrimental effects on wound healing from the exposure to acute ethanol were also studied previously. Radek et al. [50] reported that one of the most significant effects of ethanol exposure on wound healing occurs during the inflammatory response, and altered cytokine production is a main component. Ethanol exposure also disturbs the proliferative response during healing, causing delays in neoepithelialisation, collagen formation, and angiogenesis.

This experiment also revealed that plasma jet treatment alone decreases the time that it takes a wound to reach its peak day in the inflammatory phase. In the context of the wound area, however, the shortness of the time required to reach the peak inflammatory day in the P group was followed by the highest level of expansion of the wound area. This phenomenon may be caused by the effect of plasma jet treatment on macrophage and fibroblast cells. It is well known that these two types of cells are critical to acute wound healing. Macrophage cells are considered as biomarkers for the late inflammatory phase, whilst fibroblasts play a main role in the proliferative phase, being especially associated with new collagen and myofibroblast cells formations. It was reported that plasma jet treatment has the ability to enhance phagocytotic activity of macrophages [51] and to increase the proliferation of fibroblasts. Liu et al. reported that argon plasma jets have the ability to promote fibroblasts by activating the NF-κB pathway and increasing the expression of cyclinD1 [52]. In summary, this investigation showed that topical treatment consisting of plasma jet alone has the ability to accelerate acute wound healing via the promotion of inflammation, neoepithelialisation and new collagen formation; however, its admixture with EPB may impede such effectiveness. High concentrations of ethanol solvent may play a role as an obstructive

factor.

Conflicts of interest

None

Acknowledgment

This research was supported by the research grant program Penelitian Dasar Unggulan Perguruan Tinggi (PDUPT) the Ministry of Research, Technology and Higher Education, Republic of Indonesia (contract number: 004/K6/KM/SP2H/PENELITIAN/2018).

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.cpme.2019.100090.

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