
FOOD
BIOSCIENCE

Editors-in-Chief

Jian Chen, PhD
Jiangnan University, Wuxi, Jiangsu, China

Receiving Editor

Bo Jiang
Jiangnan University, Wuxi, Jiangsu, China

Associate Editors

Katia Ciaffoni, PhD
University of Tuscia, Viterbo, Italy

John Muyonga
Makerere University, Kampala, Uganda

Fatih Özogul, PhD

Youling Xiong
University of Kentucky, Lexington, Kentucky, United States of America

Peng Zhou, PhD
Jiangnan University, Wuxi, Jiangsu, China

Managing Editor

Yue Zhou
State Key Laboratory of Food Science & Technology, Wuxi, China

Editorial Board Members

Polytechnic Institute of Bragança, Bragança, Portugal

Feng Chen
Clemson University, Clemson, South Carolina, United States of America

Hongda Chen
US Department of Agriculture, Washington, District of Columbia, United States of America

Steve Cui
Agriculture and Agri-Food Canada Guelph Research and Development Centre, Guelph, Ontario, Canada

Joshua Gong

Theodore P. Labuza
University of Minnesota Department of Food Science and Nutrition, Saint Paul, Minnesota, United States of America

Byong Lee
McGill University, Montreal, Quebec, Canada

Lin Li
South China University of Technology, Guangzhou, China

Yoshinori Mine
University of Guelph, Guelph, Ontario, Canada

Arjan Narbad
Quadram Institute Bioscience, Norwich, United Kingdom

George-John Nychas

Tanaboon Sajjaanantakul
Kasetsart University, Bangkok, Thailand

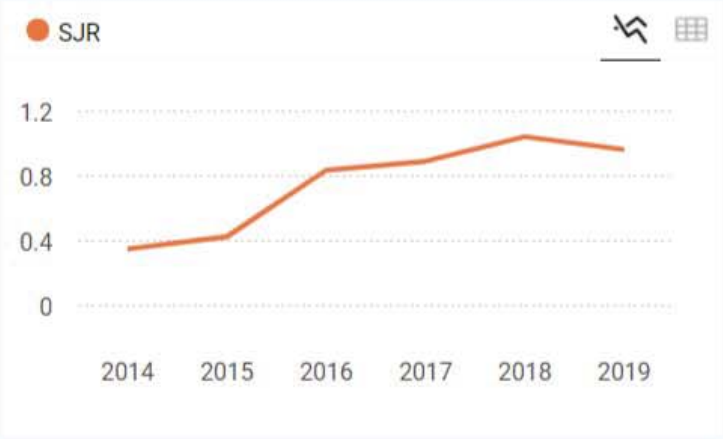
Anderson Sant'Ana
State University of Campinas, Campinas, São Paulo, Brazil

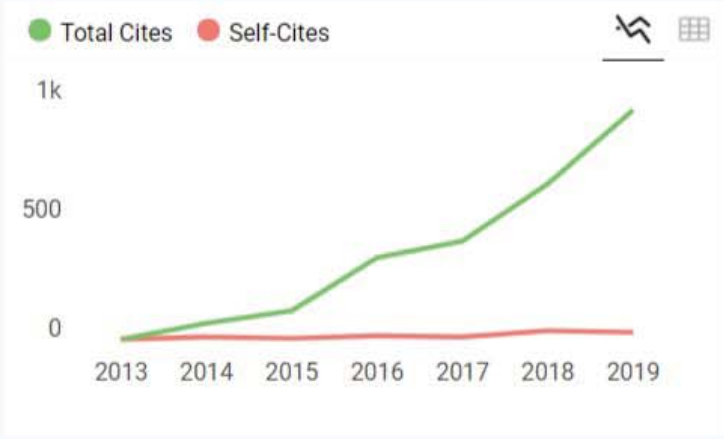
Fereidoon Shahidi
Memorial University of Newfoundland Department of Biochemistry, St John's,

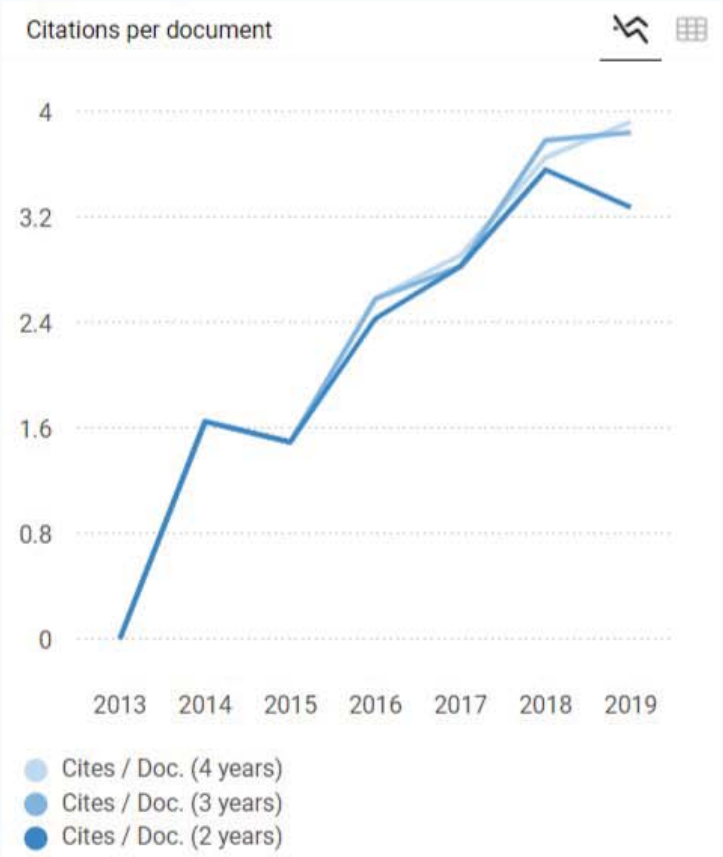
Minghu Wang
University of Hong Kong School of Biological Sciences, Hong Kong, Hong Kong

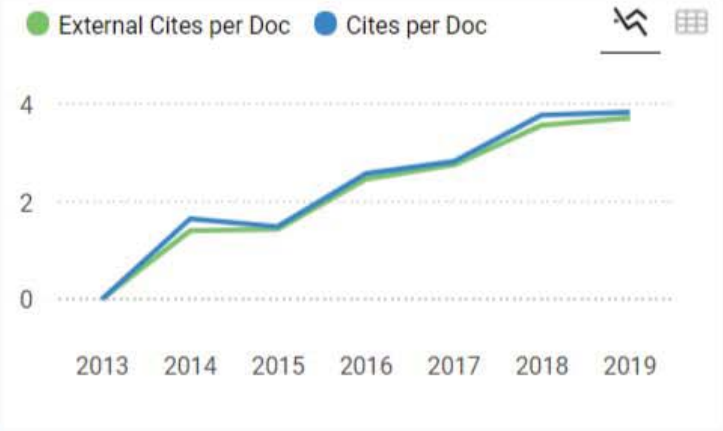
Hang Xiao
University of Massachusetts Amherst Department of Food Science, Amherst, Massachusetts, United States of America

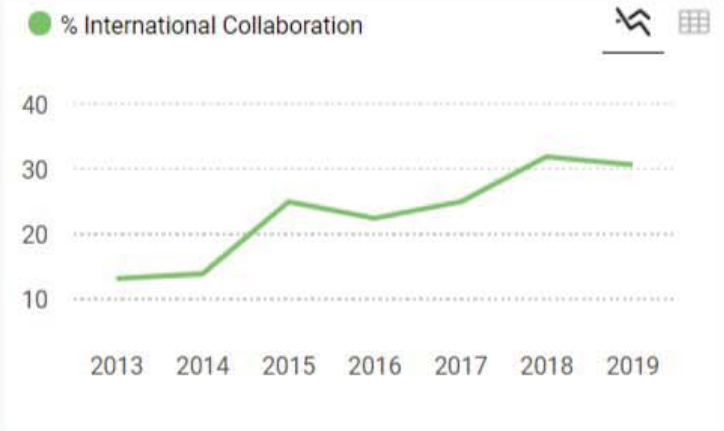
Mingyong Xie

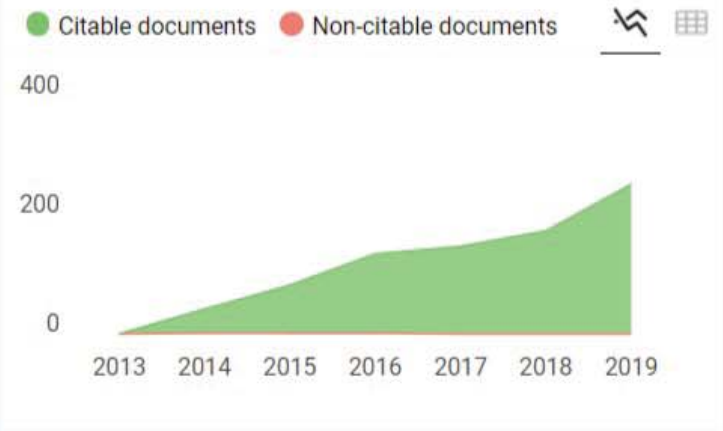


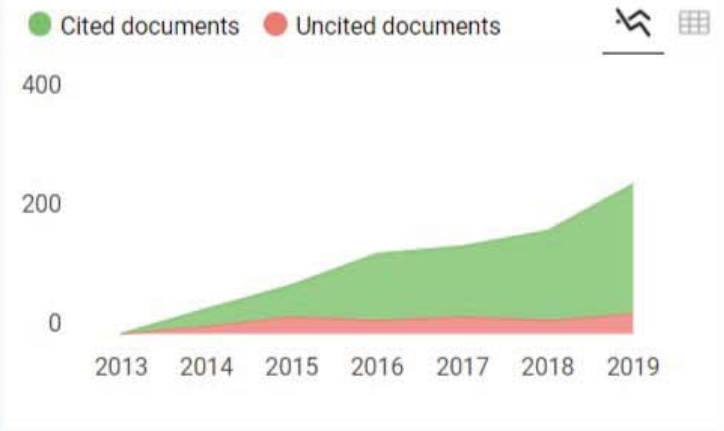












← Show this widget in your own website

Just copy the code below and paste within your html code:

```
<a href="https://www.scimag
```

Food Bioscience 41 (2021) 100937

Contents lists available at ScienceDirect

Food Bioscience

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

Wound healing effects of *Plantago major* extract and its chemical compounds in hyperglycemic rats

Kartini Kartini [a,*], Nina Wati [a], Rabbindra Gustav [a], Risa Wahyuni [a], Yosua Fernaldi Anggada [a], Risna Hidayani [a], Antoni Raharjo [a], Ridho Islamie [b], Sulistyo Emantoko Dwi Putra [c]

[a] Department of Pharmaceutical Biology, Faculty of Pharmacy, University of Surabaya, Surabaya, 60293, Indonesia
[b] Department of Clinical and Community Pharmacy, Faculty of Pharmacy, University of Surabaya, Surabaya, 60293, Indonesia
[c] Department of Biology, Faculty of Biotechnology, University of Surabaya, Surabaya, 60293, Indonesia

ARTICLE INFO

Keywords:
Plantago major
Greater plantain
Ursolic acid
Oleanolic acid
Diabetic wound

ABSTRACT

Impaired wound healing is among the serious complications of diabetes that can lead to amputation and even death. *Plantago major* has been used empirically to improve wound healing. The main bioactive compounds of *P. major* extracts, ursolic acid (UA) and oleanolic acid (OA), have also been studied for their benefits with non-hyperglycemic wounds. This study was done to examine the *in vivo* wound healing effects of *P. major* leaf extracts (PMLE), UA, and OA in hyperglycemic rats, to evaluate their *in vitro* diabetic wound healing activity, and to observe possible dermal irritation after topical application. Wound closure, duration of epithelialization, and histopathological profiles of healed tissue were observed in the hyperglycemic rats with excision wounds for 21 days. An anti-inflammatory test using the NO inhibitory assay, a fibroblast proliferation assay, and a migration assay with high-glucose medium were done to investigate the mechanism of action of the tested samples in wound healing. The acute dermal irritation test followed the international guidelines. PMLE, UA, and OA increased the percentage of wound closure and accelerated wound healing time. PMLE activities were assessed for the inhibition of NO production in the inflammation phase and enhancement of fibroblast proliferation. UA may contribute to this wound healing process through inhibition of NO production, whereas OA through activation of migration of fibroblast cells. Topical applications of PMLE, UA, and OA did not cause acute dermal irritation. PMLE, UA, and OA have the potential to improve wound healing with diabetes conditions.

1. Introduction

Plantago major or greater plantain is the most widely used species of the genus Plantago and belongs to the Plantaginaceae family. In many parts of the world, *P. major* is ubiquitous and, therefore, has various local names, including *daun sendok* in Indonesia (Adom et al., 2017; Gonçalves & Romano, 2016; Kartini et al., 2017; Samuelsen, 2000). They are wild plants that have good adaptability to diverse conditions in the environment. Nevertheless, to minimize the exploitation of natural resources, prior studies have attempted to optimize its cultivation, harvest, and postharvest processing (Prakash et al., 2011; Zubair et al., 2011). These practices can promote sustainable and efficient production now that there is an increasingly high demand for *P. major,* both in the nutraceutical and pharmaceutical sectors.

Plantago major contains high concentrations of mucilaginous carbohydrates that are active as immunostimulants and antioxidants and are important as excipients in food processing, tablets and emulsions formulations (Akbari et al., 2016; Lukova et al., 2017; Niknam et al., 2020). Its mucilaginous property underlies the use of *P. major* as a nutraceutical, especially to improve intestinal health (Gonçalves & Romano, 2016; Samuelsen, 2000; Samuelsen et al., 1995). In some regions, fresh leaves of *P. major* are also used as ingredients in salads and soups, and the seeds can also be processed into snacks, cakes, and breads (Gonçalves & Romano, 2016). The medicinal properties of *P. major* are attributable to its various biologically active compounds such as terpenoids, phenolic acids, flavonoids, alkaloids, and iridoids. It is traditionally used in the treatment of a number of diseases, such as wounds and other skin diseases, infectious diseases, problems concerning the digestive and respiratory organs, reproduction and circulation issues, and tumors, and for pain relief and fever reduction (Adom et al., 2017; Gonçalves & Romano, 2016; Najafian et al., 2018; Samuelsen, 2000). Many studies have been carried out to confirm these practical applications (Chiang et al., 2002, 2003; Kartini et al., 2014; Kolak et al., 2011; Mansor et al., 2014;

* Corresponding author.
E-mail address: kartini@staff.ubaya.ac.id (K. Kartini).

https://doi.org/10.1016/j.fbio.2021.100937
Received 24 June 2020; Received in revised form 21 February 2021; Accepted 22 February 2021
Available online 27 February 2021

Abbreviations

DFU	diabetic foot ulcer
HPTLC	high performance thin layer chromatography
NO	nitric oxide
OA	oleanolic acid
OECD	Organisation for Economic Cooperation and Development
PMLE	*Plantago major* leaves extract
UA	ursolic acid

Ozaslan et al., 2007; Poor et al., 2017).

These lead to the conclusion that *P. major* may have promise for the management of various chronic clinical disorders, including diabetes mellitus and its complications, particularly diabetic foot ulcers (DFU). Abdulghani et al. (2014) found that *P. major* methanol extracts at 500 and 1000 mg/kg bw lowered blood glucose levels in streptozotocin-induced diabetic rats. The mechanism underlying this activity was studied by increasing the control of the glycemic mechanism of the remaining pancreatic cells in diabetic rats (Abdulghani et al., 2014; Adom et al., 2017). *P. major* also has a long history of wound healing. Many countries in Europe, Asia, and the Middle East (e.g., Iran) have long used this herb either for acute or chronic wounds (Adom et al., 2017; Gonçalves & Romano, 2016; Hosseinkhani et al., 2017; Jarić et al., 2018; Jivad et al., 2016). Moreover, both *in vitro* and *in vivo* tests have been done to show the wound healing effect of *P. major* and its constituents, such as UA, OA, and aucubin (Agra et al., 2015; Amini et al., 2010; Kartini et al., 2018a; Moura-Letts et al., 2006; Phipps & Mahmood, 2006; Velasco-Lezama et al., 2006; Zubair et al., 2012, 2016).

However, there has been no report on *P. major* activity for diabetic wound healing. The wound healing process involves a series of overlapping phases, namely, hemostasis, inflammation, proliferation, and remodeling, that may be inhibited by the presence of oxygen free radicals, microbial infection, and high blood glucose (Houghton et al., 2005; Patel et al., 2019). Chemical or herbal medicines that can modulate one or more of those phases can be examined as candidates for wound healing agents with normal or hyperglycemic conditions. Previous studies showed that *P. major* and its phytochemicals are active anti-inflammatory, antioxidant, and antimicrobial agents (Hussan et al., 2015; Ikeda et al., 2008; Kartini et al., 2014, 2017; Liu, 1995; Mazzutti et al., 2017; Shirley et al., 2017; Stenholm et al., 2013; Vasconcelos et al., 2006). Therefore, this study was intended to determine the *in vivo* wound healing effects of *P. major* extracts and chemical compounds, namely UA and OA, in hyperglycemic rats (i), to evaluate their *in vitro* diabetic wound healing activity through the anti-inflammatory process, fibroblast proliferation, and fibroblast migration (ii), and to determine possible dermal irritation after topical application (iii).

2. Materials and methods

2.1. Plant materials and chemicals

Wholes plants of *P. major* was collected from the cultivation area of the Balai Materia Medica, Batu City, Jawa Timur Province, Indonesia in September 2016 (Global Positioning System (GPS) coordinates 7°52′2.262″ S 112°31′11.719″ E; 875 m above sea level) and promptly transferred within 1 h to the laboratory. Authentication of the plant was done by the Center for Traditional Medicine Information and Development, University of Surabaya, Surabaya, Indonesia, with accession certificate number: 1212/D.T/IX/2016. UA, OA, alloxan monohydrate, methylthiazolyldiphenyl-tetrazolium bromide (MTT), and hematoxylin-eosin were obtained from Sigma Aldrich Co. (St. Louis, MO, USA), whereas absolute ethanol, toluene, acetone, formic acid,

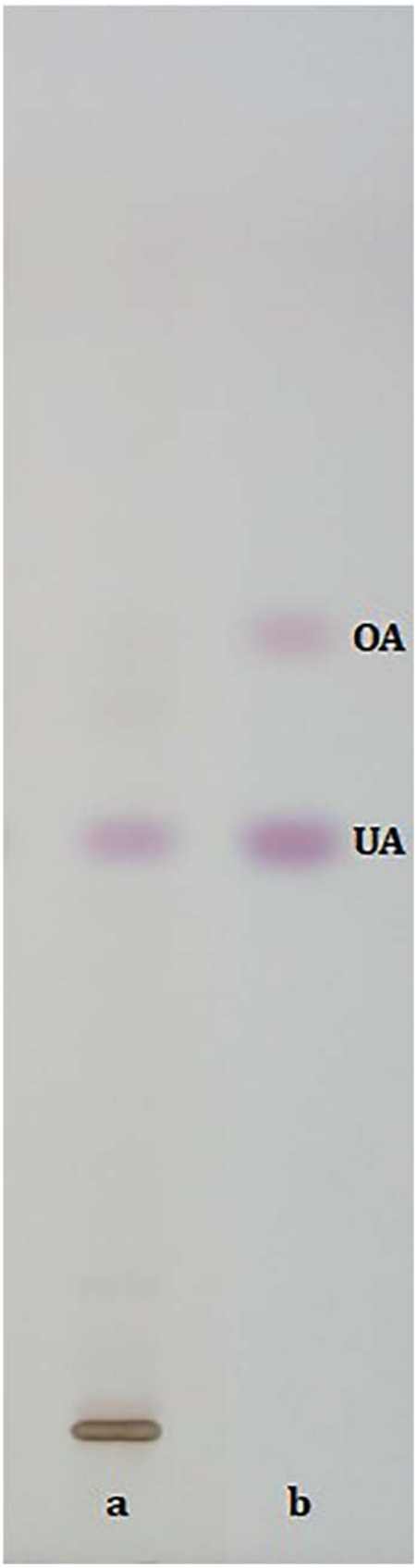


Fig. 1. High performance thin layer chromatography profile of PMLE. Stationary phase: silica gel 60 F_{254}, mobile phase: toluene:acetone:formic acid (78:22:0.15). a: PMLE, b: ursolic acid (UA) - oleanolic acid (OA) standard.

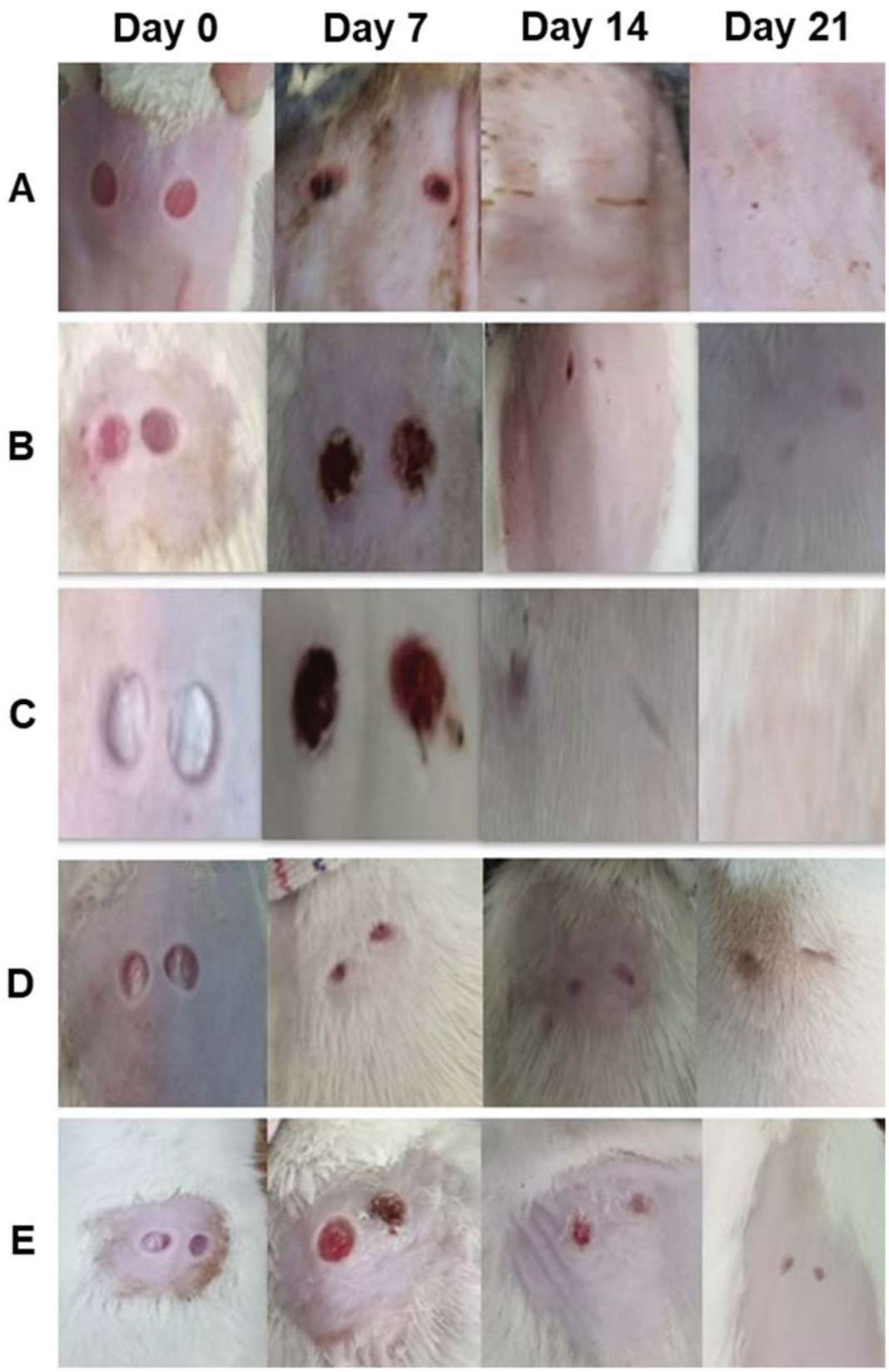


Fig. 2. Wound morphology of hyperglycemic rats from Day 0–21. A: PMLE, B: UA, C: OA, D: Positive control, E: Negative control.

sulfuric acid, methanol, chloroform, iodine, and DMSO were from Merck KGaA (Darmstadt, Germany). Carbomer, sodium hydroxide, methylparaben, propylparaben, and propylene glycol were pharmaceutical grade and obtained from Tristar Chemical Co. (Surabaya, Jawa Timur, Indonesia).

Table 1
Effect of gels on wound closure and time of epithelialization.

Group	Percentage of wound closure			Time of epithelialization (day)
	Day 7	Day 14	Day 21	
A	69 ± 3	98 ± 1	100 ± 0	16 ± 1*
B	70 ± 3	99 ± 1*	100 ± 0	15 ± 1*
C	84 ± 8*	100 ± 0*	100 ± 0	12 ± 1*
D	81 ± 6*	99 ± 2	100 ± 0	15 ± 2*
E	67 ± 2	95 ± 4	100 ± 0	19 ± 1

Group A: PMLE, B: UA, C: OA, D: Positive control (Mebo®), E: Negative control. Data represent means ± SD (n = 5), *p < 0.05 against negative control (Kruskal-Wallis, followed by Mann-Whitney test).

2.2. Preparation of *P. major* extract and determination of UA and OA

The leaves of *P. major* were separated from the other parts of the plant, then air-dried for 120 h, powdered into 2 mm particles using a household Panasonic MX-J1G blender (Panasonic Corp., Kadoma, Osaka, Japan), and stored at room temperature (31 ± 2 °C) for a maximum of 12 wk until used. Three hundreds g of these leaves were macerated with ethanol (3 × 800 ml, 24 h) at room temperature. The extracts were filtered using Whatman® qualitative filter paper grade 1 (Merck) and evaporated under vacuum using a rotavapor R-200 (BÜCHI Labortechnik AG, Flawil, Switzerland) to yield viscous extracts. High performance thin layer chromatography (HPTLC) was done to determine OA and UA as described previously (Kartini et al., 2014). Chromatography was done on a pre-coated HPTLC plate with silica gel 60 F_{254}, 0.20 mm layer thickness (Merck), using toluene:acetone:formic acid (78:22:0.15) as the mobile phase. PMLE and a mixture of OA and UA (1:1 w/w) were spotted as 5 mm bands using a Linomat 5 sample applicator (Camag, Muttenz, Switzerland) and then prederivatized using iodine vapor (1% iodine in chloroform). Development was then carried out using 10 ml of the mobile phase in a twin trough chamber (Camag) previously equilibrated with the mobile phase for 20 min at room temperature. Following development, the plate was dried in a fume hood and subjected to derivatization by spraying the plate with 5% sulfuric acid in methanol and then heating at 120 °C for 3 min. The chromatogram was obtained using Reprostar 3 documentation densitometry (Camag) with a VH-C20 3CCD color video camera (Hitachi Denshi Ltd., Shinjuku City, Tokyo, Japan).

2.3. Preparation of the gel dosage form

P. major leaf extract (PMLE), UA, and OA were formulated into gel dosage forms as described previously using carbomer, sodium hydroxide, methylparaben, propylparaben, and propylene glycol (Kartini, et al., 2018). Carbomer (2 g) was dispersed over 40 ml water, stirred (1000 rpm) using a high shear mixer-HSM 2003 SV/DV (CKL Multimix (M) Sdn Bhd, Puchong, Selangor Darul Ehsan, Malaysia) while adding 1% sodium hydroxide gradually until it formed a gel. PMLE (5 g), UA (15 mg), or OA (15 mg) were dissolved in 2 ml ethanol. Methyl paraben (180 mg) and propyl paraben (20 mg) were mixed and dissolved in propylene glycol (16 g). These two solutions were then mixed and poured into the gel with 100 g water and stirred until homogeneous. The gels were administered up to 0.5 g/test animals. Every 0.5 g PMLE gel contained 25 mg extract, whereas 0.5 g UA or OA gels contained 75 μg of the respective active compounds.

2.4. In vivo wound healing assay

Male Wistar rats (180–200 g) were from the Integrated Research and Testing Laboratory, Gadjah Mada University, Yogyakarta, Indonesia and housed at room temperature, 70% RH, and a 12 h light-dark cycle. These test animals were put in plastic cages (1 rat/cage) and fed with a standard diet (water max. 13%, crude protein 21–23%, fat min. 5%, fiber max. 5%, ash max. 7%, calcium min. 0.9%, and phosphorus min 0.6%), given water *ad libitum,* and acclimatized for 2 wk before the experiment. All handling procedures were according to the institutional rules on animal experiments (approval No: 721-KE; dated June 15, 2017). Before the induction of hyperglycemia, test animals were weighed, their blood was collected from the tail vein, and the fasting blood glucose levels were measured. Then, they were intraperitoneally injected with a single dose of alloxan monohydrate (160 mg/kg bw) in normal saline. Two days later, the fasting blood glucose levels were measured to confirm their hyperglycemic status. Hyperglycemia was indicated if the random blood glucose levels were ≥200 mg/dl.

Hyperglycemic rats were intraperitoneally anesthetized with ketamine HCl (Ketalar®, Pfizer Indonesia, Jakarta, Indonesia) at 100 mg/kg bw. An excision wound was induced on the dorsal area of the animals using a biopsy punch from Medax Srl Unipersonale (San Possidonio, MO, Italy) with a diameter of 5 mm. Test animals were then divided into 5 groups, each consisting of 5 rats. Three groups were treated with PMLE, UA, and OA gels, respectively; whereas two other groups were treated with positive and negative control, respectively. A commercially available gel product containing Copitidis Rhizoma, *Phellodendri chinensis* Cortex, and Scutellariae Radix was used as a positive control (Mebo®, from Shantou MEBO Pharmaceutical Co., Ltd. (Shantou, Guangdong, China)), while the gel without active ingredient was applied as a negative control. All treatments were carried out topically once daily. Parameters of the *in vivo* wound healing were the percentage and time of wound closure. Wound diameter was measured using a digital caliper (resolution 0.01 mm) on Day 0, 7, 14, and 21. The percentage of wound closure was determined using the following equation:

$$((D_1 - D_2) / D_1) \times 100 \quad (1)$$

where D_1 = the largest wound diameter (on Day 0), and D_2 = wound diameter on the day of observation. Healing time was measured as the

Table 2
Effect of gels on wound healing phase on Day 21.

Group	Healing score
A	2.2 ± 0.3
B	2.7 ± 0.0*
C	3.0 ± 0.7*
D	3.3 ± 0.1*
E	2.0 ± 0.0

Group A: PMLE, B: UA, C: OA, D: Positive control (Mebo®), E: Negative control. Data represent the means ± SD (n = 3), *p < 0.05 against negative control (Kruskal-Wallis, followed by Mann-Whitney test).

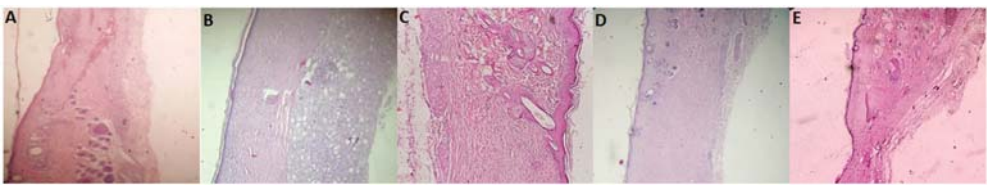


Fig. 3. Histological profiles of healing tissue on Day 21. A: PMLE, B: UA, C: OA, D: Positive control, E: Negative control.

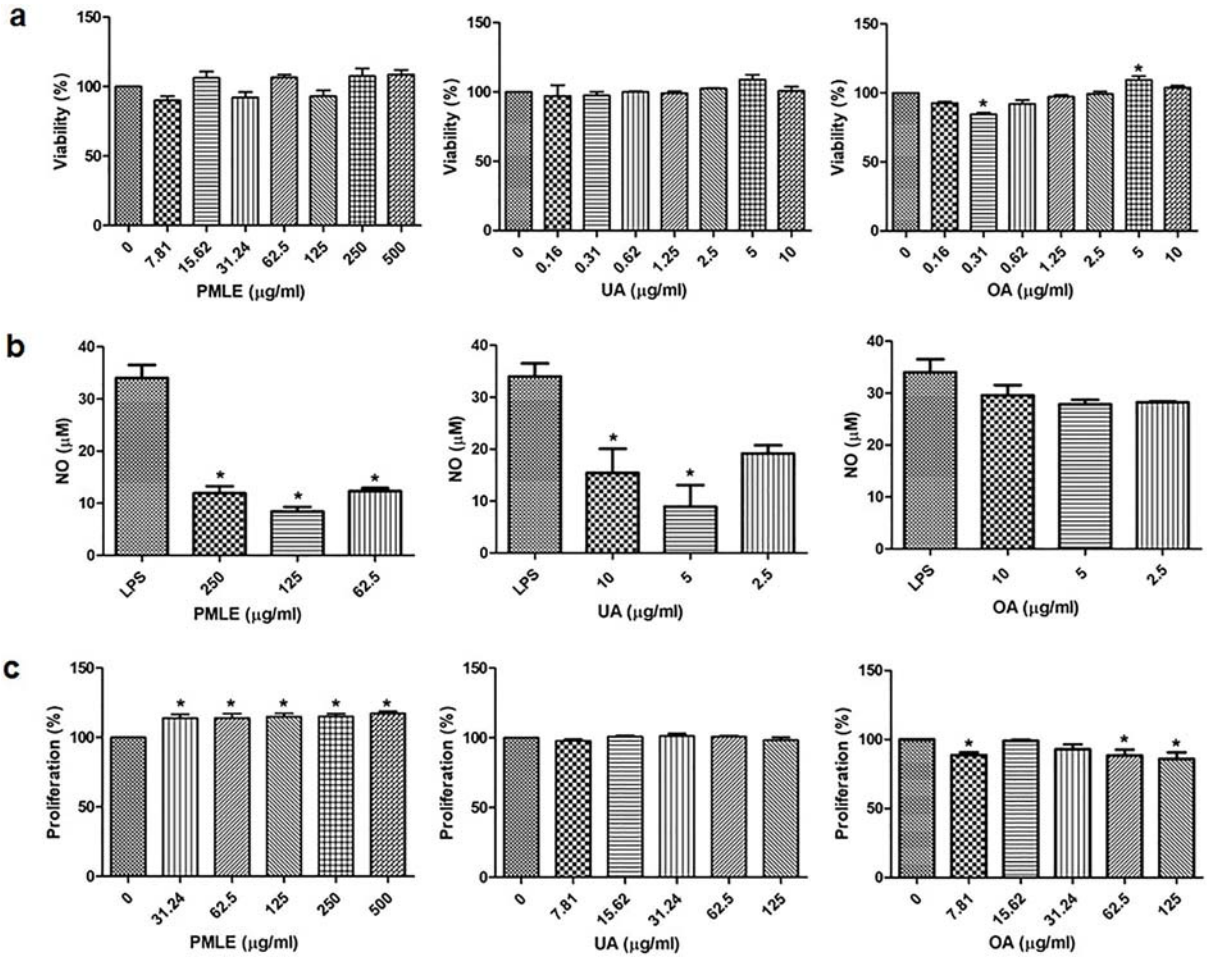


Fig. 4. The effects of PMLE, UA, and OA on the viability of the RAW 264.7 cell line (a), NO production of RAW 264.7 cell line (b), and proliferation of NIH/3T3 fibroblast cells. Data are expressed as mean ± SD (n = 3), *p < 0.05 against control (one-way ANOVA, followed by Tukey's multiple comparison test).

day when the wound closed fully. Also, healing tissues were collected for a histopathological study on Day 21, in which skin tissues were fixed in 10% formalin, sliced using an automatic rotary microtome (Thermo Fisher Scientific, Waltham, MA, USA) and prepared on a glass slide, stained with hematoxylin-eosin, and then observed under a light microscope (Olympus Corp., Shinjuku City, Tokyo, Japan). Histopathological study was done to evaluate the condition of healed tissue by observing the presence of inflammatory cells, angiogenesis, fibrosis, fibroblasts, and collagen, on which a score (0–4) of each sample was based. Score 0 means the wound was still open. Score 1 indicated the presence of bleeding (++), inflammation (++), angiogenesis (++), and epithelialization (-); while at score 2, there were signs of inflammation (+), angiogenesis (+), epithelialization (+), and fibrosis (+). Score 3 or early remodeling was represented by the presence of fibroblasts and collagen (+), as well as epithelialization (+); and score 4 or the complete remodeling phase was the formation of collagen (++) and epithelialization (++).

2.5. In vitro wound healing assay

2.5.1. Nitric oxide (NO) inhibitory assay

The *in vitro* anti-inflammatory test was done following the method used in a previous study (Tam et al., 2011). RAW264.7 cells (mouse macrophage, ATCC® TIB-71™, 4×10^5/well, counted using a counting chamber with v-slash (Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany)) were seeded in a 24-well plate overnight. The cells were provided by the Parasitology Laboratory, Medical Faculty, Gadjah Mada University. PMLE, UA, or OA were added at various concentrations together with 0.1 μg LPS/ml of the medium and incubated for 24 h. The cell culture supernatant was taken out and added into Griess Reagent (Promega Corp., Madison, WI, USA) at a 1:1 ratio in a 96-well plate, followed by incubation in the dark for 10 min. The absorbance was measured at 540 nm using a Bio-Rad Benchmark microplate reader (Bio-Rad Laboratories Inc., Hercules, CA, USA). The $NaNO_2$ was used to prepare a standard curve and linear regression equation.

2.5.2. Fibroblast proliferation assay

The procedures used for this assay were according to the method described previously, with a slight adjustment (Tam et al., 2011). NIH/3T3 cells (mouse fibroblasts, ATCC® CRL-1658™; provided by the same parasitology laboratory) were seeded at 10^3 cells/well in a 96-well plate in high-glucose DMEM (4500 mg/l; Fisher Scientific International Inc., Waltham, MA, USA). Then, the cells were exposed to different concentrations of PMLE, UA, and OA for 48 h at 37 °C. MTT solution (5 mg/ml) in PBS (Fisher Scientific) was added to the medium in each well, and the plate was then incubated at 37 °C for 4 h. All medium was then removed and replaced with DMSO. The optical density was measured at 540 nm to determine the relative amounts of viable cells and expressed as the percentage of control samples without treatment.

2.5.3. Migration assay

This test, which followed Tam et al. (2011) with adjustment for the type of cell (Tam et al., 2011), was started by seeding rat gingival

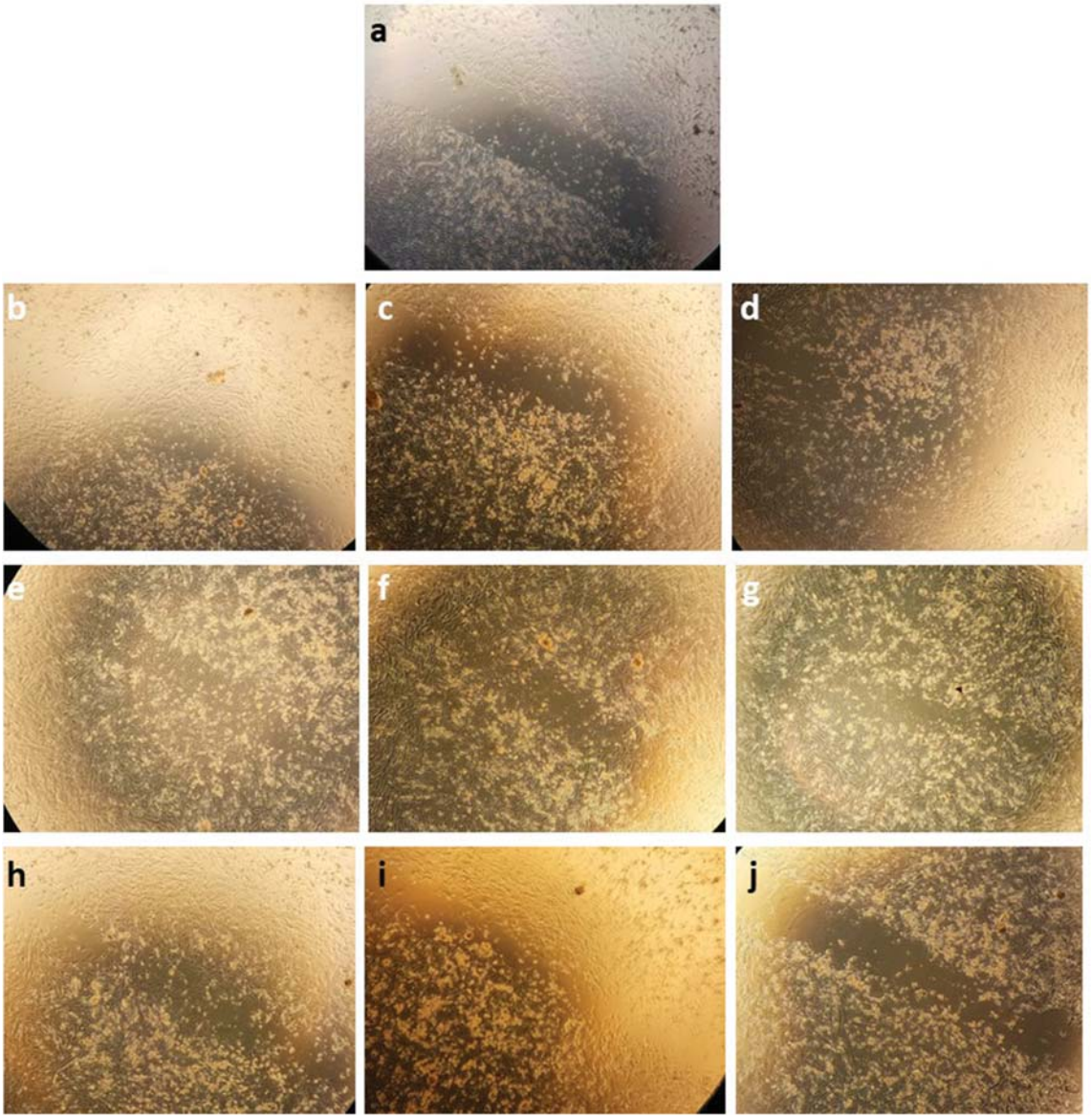


Fig. 5. Representative microphotograph of gingival fibroblast cells before the addition of the test samples (a) and after 24 h of incubation. b, c, d were induced with PMLE 250, 500, 1000 μg/ml, respectively; e, f, g with UA 25, 50, 100 μg/ml, respectively; h, i, j with OA 25, 50, 100 μg/ml, respectively.

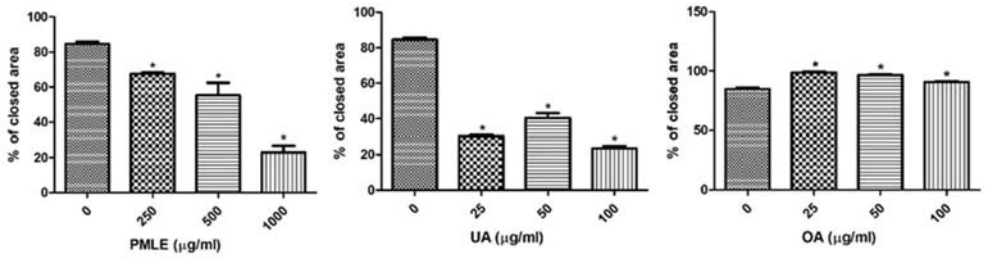


Fig. 6. The effects of PMLE, UA, and OA on closed areas observed in the gingival fibroblast migration test on rats. Data are expressed as mean ± SD (n = 3), *p < 0.05 against control (one-way ANOVA, followed by Tukey's multiple comparison test).

fibroblast cells at 10^5 cells/well in a 24-well plate in high-glucose DMEM. These cells were obtained from the Institute of Tropical Disease, Airlangga University (Surabaya, Indonesia). Cells were incubated for 24 h at 37 °C in a 5% CO_2 humidified incubator. Cells were then observed under a microscope to ensure that they had adhered to the well and achieved a confluent state. Fibroblast cells in the well were then scratched in one direction using a 1000 μl pipette tip, then observed under a light microscope, and photographs were taken. A series of concentrations of PMLE, UA, or OA solutions were added into each well, then incubated overnight. The results were observed under a light microscope and photographed. The photographs were compared and analyzed using the TScratch program (CSElab, Zurich, Switzerland) to

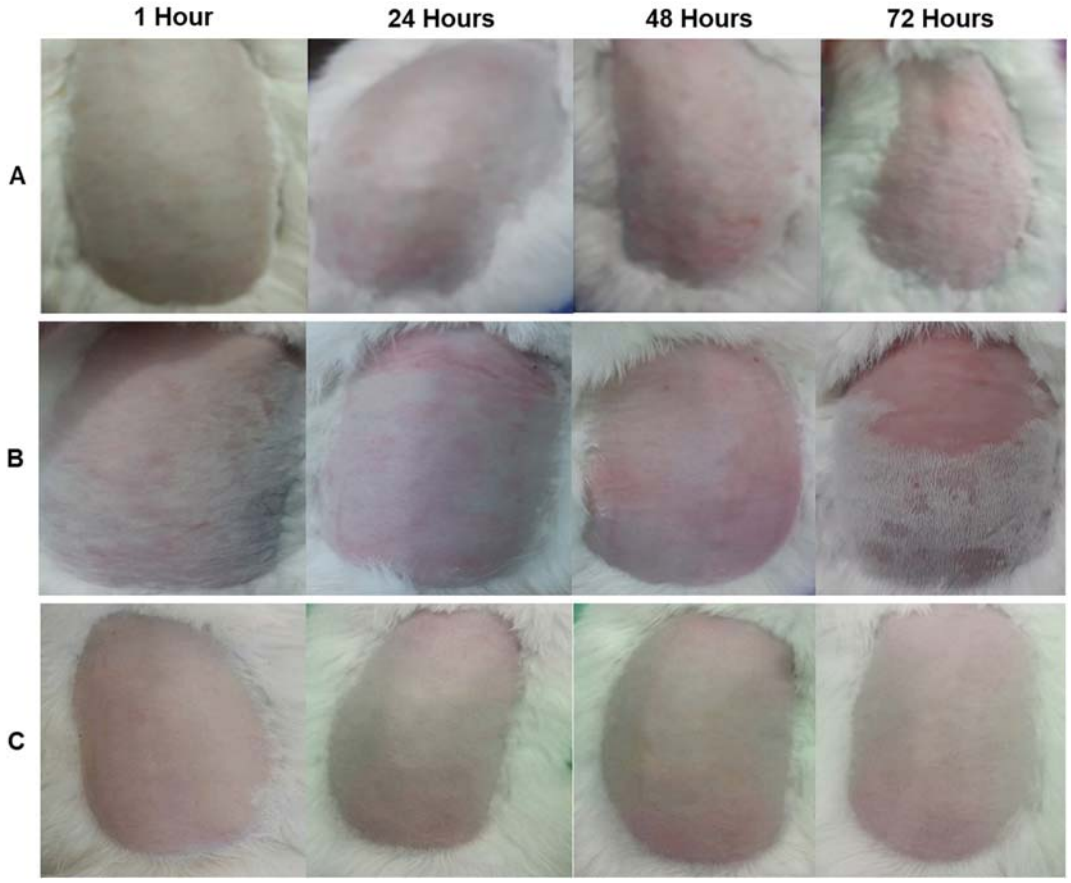


Fig. 7. The results of the 72-h acute irritation dermal test. A: PMLE, B: UA, C: OA.

determine the migration. This program measures open areas in the images. Percentage of the closed area before and after treatments were then calculated. An increase of the percentage of closed area indicated the migration.

2.6. Acute dermal irritation assay

The test followed the method recommended in the Organisation for Economic Cooperation and Development Guideline No. 404 (2015) (OECD, 2015). Healthy male albino rabbits aged 8–9 months (adult) and weighing around 1.8–2.3 kg were used as test animals. They were individually housed and acclimatized to the experimental conditions for 1 wk, fed with conventional laboratory diets (crude protein 18–19%, crude fiber 13%, fat 5–6%, calcium 0.9%, phosphorous 0.7%, water 11%, flumequine antibiotic and vitamins), and given *ad libitum* access to drinking water. Approximately 24 h before the test, fur was removed by carefully clipping the dorsal area of the trunk of the animals using an electrical hair clipper. The test samples were applied to a small area (±6 cm^2) of skin and covered with a gauze pad, which was held in place with non-irritating tape (Beiersdorf Indonesia, Malang, Jawa Timur, Indonesia). For the initial test, one animal was used. Three test patches were applied sequentially to the animal. The first patch was removed after 3 min. If there was no apparent serious skin reaction, a second patch was applied at a different site and removed after 1 h. If the observations at this stage indicated that exposure could humanely be allowed to extend to 4 h, a third patch was applied and removed after 4 h, and the response was graded. If a corrosive effect appeared after any of the three sequential exposures, the test was immediately terminated. However, if no corrosive effect (irritation) was formed after the last patch was removed, the animal was observed for 14 days, unless corrosion developed at an earlier point of time. If there was no serious skin reaction in the initial test, the irritant or negative response was confirmed using up to two additional animals, each with one patch, for an exposure period of 4 h. The animals were observed for 14 days after the removal of patches. All animals were examined for erythema and oedema, and the responses were scored at h 1, 24, 48, and 72 after patch removal. Score 0 was indicated by no erythema and no oedema; score 1 was very slight erythema and oedema; score 2 was well defined erythema and slight oedema; score 3 was moderate to severe erythema and moderate oedema; and score 4 was severe erythema and oedema.

2.7. Statistical analysis

All data were reported as mean ± SD, then analyzed by descriptive statistics, one-way ANOVA and Tukey's multiple comparison test in the statistical program, GraphPad Prism (GraphPad Software Inc., San Diego, CA, USA, Windows Version 5.01). If the data did not meet the requirements of a parametric statistical test, then they were processed with the Kruskal-Wallis test and the Mann-Whitney post hoc test. $p < 0.05$ distinguished a statistically significant difference.

3. Results and discussion

3.1. High performance thin layer chromatography of PMLE

The presence of UA and OA in the PMLE was identified using HPTLC (Fig. 1). The chromatogram showed that PMLE contains UA, but the presence of OA cannot be detected. This is consistent with the previous studies which showed that OA was present in the petiole, seed, and aerial parts, but not in the leaves and roots (Kartini et al., 2014, 2017).

3.2. In vivo wound healing

The wound healing efficacy of PMLE, UA, and OA was investigated from the wound closure, time of epithelialization, and gross examination for 21 days. Also, at the end of the test, the histopathological profile of healed tissue was documented. Wound closure and time of epithelialization were examined to determine the rate of reduction of the unhealed area during the healing process. A faster rate of wound closure indicates a better efficacy. The shrinking of the wound area in different groups of treated animals over 21 days due to PMLE, UA, and OA is shown in Fig. 2 and Table 1.

Overall, the topical application of PMLE and its chemical constituents (UA and OA) increased wound closure significantly ($p < 0.05$), particularly on Day 7 and 14. On Day 21, although complete wound closure was observed in all groups, no significant differences were detected. Table 1 also shows that time of epithelialization of the groups treated with PMLE and UA was not significantly different from that of the positive control (Mebo®). It suggested that PMLE and UA are as active as the positive control. Animals treated with OA gel showed the fastest epithelialization, indicating that OA had higher efficacy for wound healing with hyperglycemic conditions. A previous study on wound healing in mice with normal conditions confirmed that OA is 42.9% faster and more effective than the placebo, and at the dose of 40 μg/g bw, it produces the highest wound healing effect (Moura-Letts et al., 2006). Histological profiles of healing tissue (Fig. 3 and Table 2) show that on Day 21, animals treated with OA and positive control gels had started the remodeling phase, approaching complete remodeling. However, groups treated with PMLE, UA, and negative control were still in phase 2 and progressing to early remodeling, as characterized by ongoing angiogenesis and inflammation, and early development of fibrosis and epithelialization. These results were consistent with OA producing the best outcome in wound closure and the fastest epithelialization.

3.3. Effect of PMLE, UA, OA on NO production by RAW264.7 cells

Repair needs the coordination of various cells, growth factors, and cytokines. In diabetic patients, the normal wound healing process is interrupted by high blood glucose. Diabetic wounds show a persistent inflammatory phase resulting in the inhibited formation of mature granulation tissue and reduction in wound tensile strength, which may be caused by ischemic damage to blood vessels (Patel et al., 2019).

The anti-inflammatory activity of PMLE, UA, and OA was investigated using the NO inhibition test on RAW264.7 macrophage cells. The cells were cultured in high-glucose medium to mimic a diabetic condition. Apart from RAW264.7, macrophage-like cells such as THP-1 can also be used to evaluate *in vitro* anti-inflammatory activity. These monocytes will differentiate into macrophage after induction with phorbol 12-myristate 13-acetate (PMA) (Chanput et al., 2014). The use of RAW264.7 cells was preferred since this research did not need to differentiate the cells. Bacterial infections or immunological stimuli such as LPS induce macrophages to release a high concentration of NO, which is potentially cytotoxic and can destroy the surrounding cells and tissues. Before the NO inhibition test, the effects of PMLE, UA, and OA on cell viability was evaluated to determine the appropriate concentration for the NO inhibition tests (Fig. 4a). The viability of RAW264.7 cells did not significantly decrease after the addition of PMLE, UA, and OA at up to 500, 10, and 10 μg/ml, respectively. These results indicated that all samples at these concentrations were non-toxic to the cells and did not affect the regular cellular activity.

After treatment with PMLE, UA, and OA, the NO concentration in the cell culture media was measured using the Griess Reaction. All concentrations of PMLE and UA suppressed the NO production in RAW264.7 cells significantly after LPS induction (Fig. 4b), indicating that PMLE and UA have anti-inflammatory properties. OA (2.5–10 μg/ml) also inhibited the production of NO; however, the reduction was not significantly different from that of the control. On the other hand, previous research with normal media showed that OA inhibited some inflammatory mediators, such as COX-2, TNF-α, IL-1β, IL-6, and IFN-γ (Kartini et al., 2014, 2017; Ringbom et al., 1998; Stenholm et al., 2013). Therefore, further studies are needed to confirm whether or not OA is an active anti-inflammatory agent with hyperglycemic conditions using these other inflammatory mediators.

3.4. Effects of PMLE, UA, and OA on NIH/3T3 fibroblast proliferation

The wound healing cascade is a complex interaction of cellular and biochemical actions that differentiates into several phases, from healing to structural and functional integrity restoration to regain the strength of injured tissues. Fibroblast proliferation is one crucial step in tissue regeneration. A series of PMLE, UA, and OA concentrations were tested at the proliferation phase using NIH/3T3 fibroblast cells. Fig. 4c shows that PMLE at 31–500 μg/ml increased fibroblast proliferation, but the opposite was true for UA and OA administered at 7.81–125 μg/ml. These results were consistent with Kuonen et al. that the lipophilic extract of *Viscum album* L. and oleanolic acid did not produce any proliferation-stimulating effects in NIH/3T3 fibroblast cells at any concentration (0.01–100 and 0.001–10 μg/ml, respectively) (Kuonen et al., 2013). Therefore, UA and OA probably contributed to diabetic wound healing through other mechanisms.

3.5. Effects of PMLE, UA, and OA on fibroblast migration

Angiogenesis, another process contributing to wound healing, is important until the terminal stages of healing. Its mechanism involves proliferation, migration, and remodeling of endothelial cells, followed by subsequent tube formation. The effects of PMLE, UA, and OA on the migration activity were assessed using gingival fibroblast cells that had been isolated from the rats. Fig. 5 shows the photograph of the scratched cells before the addition of the samples. This figure also shows the open area of cells at the bottom of wells after incubation with serial concentrations of PMLE, UA, and OA. Fig. 6 shows that all concentrations of PMLE and UA did not increase cell migration; on the other hand, open areas were reduced as OA concentration increased. The percentage of closed area in treated cells to the control cells indicates the level of cell migration activity. Fig. 6 shows that OA (25–100 μg/ml) can increase the migration of rat gingival fibroblast cells.

3.6. Acute dermal irritation test

The results of the acute dermal irritation test of PMLE, UA, and OA gels are shown in Fig. 7. No dermal responses, including erythema or oedema, were found in animals treated with either UA or OA gels. In the group treated with PMLE gel, some rabbits showed erythema after 48 and 72 h. However, this is still included in the nonirritant category (Banerjee et al., 2013).

4. Conclusions

Plantago major leaf extracts and its phytochemicals (UA and OA) can increase the percentage of wound closure and accelerate wound healing time in hyperglycemic rats. The extent of these activities was determined using the inhibited NO production in the inflammation phase and enhanced fibroblast proliferation. While UA contributed to the wound healing process by inhibiting NO production, OA showed the same function by activating fibroblast cell migration. Topical applications of *P. major* leaf extracts, UA, and OA in gel dosage forms did not cause acute dermal irritation. This study showed that *P. major* leaf extracts, UA, and OA acid have the potential to improve wound healing with diabetes conditions.

Author statement

This study was designed, directed and coordinated by Kartini Kartini, Ridho Islamie and Sulistyo Emantoko Dwi Putra for all aspects of the project. Nina Wati, Rabbindra Gustav, and Risa Wahyuni partly contributed to the *in vivo* wound healing assay, whereas Yosua Fernaldi Anggada, Risna Hidayani, and Antoni Raharjo involved in the *in vitro* assay and safety evaluation. Ridho Islamie and Sulistyo Emantoko Dwi Putra suggested and commented on the design of the experiments. The manuscript was written by Kartini Kartini and commented by all authors. All authors have read and approved the final version of the manuscript.

Declaration of competing interest

The authors confirm that they have no conflicts of interest with respect to the work described in this manuscript.

Acknowledgements

This research was supported by the Indonesian Ministry of Research, Technology, and Higher Education (grant number 017/SP-Lit/LPPM-01/Ristekdikti/FF/III/2018).

References

Abdulghani, M. A., Hamid, I., Al-Naggar, R. A., & Osman, M. T. (2014). Potential antidiabetic activity of *Plantago major* leaves extract in streptozocin-induced diabetic rats. *Research Journal of Pharmaceutical, Biological and Chemical Sciences, 5*(2), 896–902.

Adom, M. B., Taher, M., Mutalabisin, M. F., Amri, M. S., Kudos, M. B. A., Sulaiman, M. W. A. W., Sengupta, P., & Susanti, D. (2017). Chemical constituents and medical benefits of *Plantago major*. *Biomedicine & Pharmacotherapy, 96*, 348–360.

Agra, L. C., Ferro, J. N., Barbosa, F. T., & Barreto, E. (2015). Triterpenes with healing activity: A systematic review. *Journal of Dermatological Treatment, 26*(5), 465–470.

Akbari, J., Saeedi, M., Morteza-Semnani, K., Zarrabi, B., Rostamkalaei, S. S., & Kelidari, H. R. (2016). The effect of *Plantago major* seed mucilage combined with carbopol on the release profile and bioadhesive properties of propranolol HCl buccoadhesive tablets. *Pharmaceutical and Biomedical Research, 2*(2), 84–100.

Amini, M., Kherad, M., Mehrabani, D., Azarpira, N., Panjehshahin, M., & Tanideh, N. (2010). Effect of *Plantago major* on burn wound healing in rat. *Journal of Applied Animal Research, 37*(1), 53–56.

Banerjee, S., Chattopadhyay, P., Ghosh, A., Pathak, M. P., Singh, S., & Veer, V. (2013). Acute dermal irritation, sensitization, and acute toxicity studies of a transdermal patch for prophylaxis against (±) anatoxin-A poisoning. *International Journal of Toxicology, 32*(4), 308–313.

Chanput, W., Mes, J. J., & Wichers, H. J. (2014). THP-1 cell line: An *in vitro* cell model for immune modulation approach. *International Immunopharmacology, 23*(1), 37–45.

Chiang, L. C., Chiang, W., Chang, M. Y., & Lin, C. C. (2003). *In vitro* cytotoxic, antiviral and immunomodulatory effects of *Plantago major* and *Plantago asiatica*. *American Journal of Chinese Medicine, 31*(2), 225–234.

Chiang, L. C., Chiang, W., Chang, M. Y., Ng, L. T., & Lin, C. C. (2002). Antiviral activity of *Plantago major* extracts and related compounds *in vitro*. *Antiviral Research, 55*(1), 53–62.

Gonçalves, S., & Romano, A. (2016). The medicinal potential of plants from the genus *Plantago* (Plantaginaceae). *Industrial Crops and Products, 83*, 213–226.

Hosseinkhani, A., Falahatzadeh, M., Raoofi, E., & Zarshenas, M. M. (2017). An evidence-based review on wound healing herbal remedies from reports of traditional Persian medicine. *Journal of Evidence-Based Complementary & Alternative Medicine, 22*(2), 334–343.

Houghton, P., Hylands, P., Mensah, A., Hensel, A., & Deters, A. (2005). *In vitro* tests and ethnopharmacological investigations: Wound healing as an example. *Journal of Ethnopharmacology, 100*(1–2), 100–107.

Hussan, F., Mansor, A. S., Hassan, S. N., Kamaruddin, T. N. E., Tasnim, T. N., Budin, S. B., & Othman, F. (2015). Anti-inflammatory property of *Plantago major* leaf extract reduces the inflammatory reaction in experimental acetaminophen-induced liver injury. *Evidence-based Complementary and Alternative Medicine, 2015*.

Ikeda, Y., Murakami, A., & Ohigashi, H. (2008). Ursolic acid: An anti- and pro-inflammatory triterpenoid. *Molecular Nutrition & Food Research, 52*(1), 26–42.

Jarić, S., Kostić, O., Mataruga, Z., Pavlović, D., Pavlović, M., Mitrović, M., & Pavlović, P. (2018). Traditional wound-healing plants used in the Balkan region (southeast Europe). *Journal of Ethnopharmacology, 211*, 311–328.

Jivad, N., Bahmani, M., & Asadi-Samani, M. (2016). A review of the most important medicinal plants effective on wound healing on ethnobotany evidence of Iran. *Der Pharmacia Lettre, 8*(2), 353–357.

Kartini, K., Fitriani, E. W., & Tansridjata, L. (2018). Formulation and physical stability test of oleanolic acid cream and gel. *Pharmaciana, 8*(1), 77–86.

Kartini, Islamie, R., & Handojo, C. S. (2018a). Wound healing activity of aucubin on hyperglycemic rat. *Journal of Young Pharmacists, 10*(2Suppl), s136–s139.

Kartini, Piyaviriyakul, S., Siripong, P., & Vallisuta, O. (2014). HPTLC simultaneous quantification of triterpene acids for quality control of *Plantago major* L. and evaluation of their cytotoxic and antioxidant activities. *Industrial Crops and Products, 60*, 239–246, 0.

Kartini, Piyaviriyakul, S., Thongpraditchote, S., Siripong, P., & Vallisuta, O. (2017). Effects of *Plantago major* extracts and its chemical compounds on proliferation of cancer cells and cytokines production of lipopolysaccharide-activated THP-1 macrophages. *Pharmacognosy Magazine, 13*(51), 393–399.

Kolak, U., Boğa, M., Uruşak, E. A., & Ulubelen, A. (2011). Constituents of *Plantago major* subsp. intermedia with antioxidant and anticholinesterase capacities. *Turkish Journal of Chemistry, 35*(4), 637–645.

Kuonen, R., Weissenstein, U., Urech, K., Kunz, M., Hostanska, K., Estko, M., Heusser, P., & Baumgartner, S. (2013). Effects of lipophilic extract of *Viscum album* L. and oleanolic acid on migratory activity of NIH/3T3 fibroblasts and on HaCat keratinocytes. *Evidence-based Complementary and Alternative Medicine, 2013*.

Liu, J. (1995). Pharmacology of oleanolic acid and ursolic acid. *Journal of Ethnopharmacology, 49*(2), 57–68.

Lukova, P., Karcheva-Bahchevanska, D., Nikolova, M., Iliev, I., & Mladenov, R. (2017). Comparison of structure and antioxidant activity of polysaccharides extracted from the leaves of *Plantago major* L., P. media L. and P. lanceolata L. *Bulgarian Chemical Communications, 49*, 282–288.

Mansor, A. S., Budin, S. B., & Othman, F. (2014). Effect of *Plantago major* extract on plasma cytokine changes in paracetamol-induced liver injury. *Cytokine, 70*(1), 49.

Mazzutti, S., Riehl, C. A., Ibañez, E., & Ferreira, S. R. (2017). Green-based methods to obtain bioactive extracts from *Plantago major* and *Plantago lanceolata*. *The Journal of Supercritical Fluids, 119*, 211–220.

Moura-Letts, G., Villegas, L. F., Marçalo, A., Vaisberg, A. J., & Hammond, G. B. (2006). *In vivo* wound-healing activity of oleanolic acid derived from the acid hydrolysis of *Anredera diffusa*. *Journal of Natural Products, 69*(6), 978–979.

Najafian, Y., Hamedi, S. S., Farshchi, M. K., & Feyzabadi, Z. (2018). *Plantago major* in traditional Persian medicine and modern phytotherapy: A narrative review. *Electronic Physician, 10*(2), 6390.

Niknam, R., Ghanbarzadeh, B., Ayaseh, A., & Rezagholi, F. (2020). The hydrocolloid extracted from *Plantago major* seed: Effects on emulsifying and foaming properties. *Journal of Dispersion Science and Technology, 41*(5), 667–673.

OECD. (2015). *OECD Guideline for testing of chemicals. Acute dermal irritation/corrosion, series on testing and assessment (No. 404)*. Paris: Organisation for Economic Cooperation and Development.

Ozaslan, M., Didem Karagoz, I., Kalender, M. E., Kilic, I. H., Sari, I., & Karagoz, A. (2007). *In vivo* antitumoral effect of *Plantago major* L. extract on Balb/C mouse with Ehrlich ascites tumor. *American Journal of Chinese Medicine, 35*(5), 841–851.

Patel, S., Srivastava, S., Singh, M. R., & Singh, D. (2019). Mechanistic insight into diabetic wounds: Pathogenesis, molecular targets and treatment strategies to pace wound healing. *Biomedicine & Pharmacotherapy, 112*, Article 108615.

Phipps, M., & Mahmood, A. (2006). Gastroprotective activity of *P. major* in rats. *International Journal of Tropical Medicine, 1*(1), 36–39.

Poor, M. H. S., Khatami, M., Azizi, H., & Abazari, Y. (2017). Cytotoxic activity of biosynthesized Ag nanoparticles by *Plantago major* towards a human breast cancer cell line. *Rendiconti Lincei, 28*(4), 693–699.

Prakash, V., Bisht, H., & Prasad, P. (2011). Altitudinal variation in morpho-physiological attributes in *Plantago major*: Selection of suitable cultivation site. *Research Journal of Medicinal Plant, 5*(3), 302–311.

Ringbom, T., Segura, L., Noreen, Y., Perera, P., & Bohlin, L. (1998). Ursolic acid from *Plantago major*, a selective inhibitor of cyclooxygenase-2 catalyzed prostaglandin biosynthesis. *Journal of Natural Products, 61*(10), 1212–1215.

Samuelsen, A. B. (2000). The traditional uses, chemical constituents and biological activities of *Plantago major* L. A review. *Journal of Ethnopharmacology, 71*(1–2), 1–21.

Samuelsen, A. B., Paulsen, B. S., Wold, J. K., Otsuka, H., Yamada, H., & Espevik, T. (1995). Isolation and partial characterization of biologically active polysaccharides from *Plantago major* L. *Phytotherapy Research, 9*(3), 211–218.

Shirley, K. P., Windsor, L. J., Eckert, G. J., & Gregory, R. L. (2017). *In vitro* effects of *Plantago major* extract, aucubin, and baicalein on *Candida albicans* biofilm formation, metabolic activity, and cell surface hydrophobicity. *Journal of Prosthodontics, 26*(6), 508–515.

Stenholm, A., Goransson, U., & Bohlin, L. (2013). Bioassay-guided supercritical fluid extraction of cyclooxygenase-2 inhibiting substances in *Plantago major* L. *Phytochemical Analysis, 24*(2), 176–183.

Tam, J. C. W., Lau, K. M., Liu, C. L., To, M. H., Kwok, H. F., Lai, K. K., Lau, C. P., Ko, C. H., Leung, P. C., & Fung, K. P. (2011). The *in vivo* and *in vitro* diabetic wound healing effects of a 2-herb formula and its mechanisms of action. *Journal of Ethnopharmacology, 134*(3), 831–838.

Vasconcelos, M. A. L., Royo, V. A., Ferreira, D. S., Crotti, A. E. M., e Silva, M. L. A., Carvalho, J. C. T., Bastos, J. K., & Cunha, W. R. (2006). *In vivo* analgesic and anti-inflammatory activities of ursolic acid and oleanoic acid from *Miconia albicans* (Melastomataceae). *Zeitschrift für Naturforschung C, 61*(7–8), 477–482.

Velasco-Lezama, R., Tapia-Aguilar, R., Román-Ramos, R., Vega-Avila, E., & Pérez-Gutiérrez, M. S. (2006). Effect of *Plantago major* on cell proliferation *in vitro*. *Journal of Ethnopharmacology, 103*(1), 36–42.

Zubair, M., Ekholm, A., Nybom, H., Renvert, S., Widen, C., & Rumpunen, K. (2012). Effects of *Plantago major* L. leaf extracts on oral epithelial cells in a scratch assay. *Journal of Ethnopharmacology, 141*(3), 825–830.

Zubair, M., Nybom, H., Lindholm, C., Brandner, J. M., & Rumpunen, K. (2016). Promotion of wound healing by *Plantago major* L. leaf extracts – *Ex-vivo* experiments confirm experiences from traditional medicine. *Natural Product Research, 30*(5), 622–624.

Zubair, M., Nybom, H., Lindholm, C., & Rumpunen, K. (2011). Major polyphenols in aerial organs of greater plantain (*Plantago major* L.), and effects of drying temperature on polyphenol contents in the leaves. *Scientia Horticulturae, 128*(4), 523–529.

1-S20-_1.PDF

by Sulistyo Johan

Submission date: 08-May-2021 09:31AM (UTC+0700)
Submission ID: 1581060547
File name: 1-S20-_1.PDF (8.25M)
Word count: 6869
Character count: 36451

Food Bioscience 41 (2021) 100937

Contents lists available at ScienceDirect

Food Bioscience

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

Wound healing effects of *Plantago major* extract and its chemical compounds in hyperglycemic rats

Kartini Kartini [a,*], Nina Wati [a], Rabbindra Gustav [a], Risa Wahyuni [a], Yosua Fernaldi Anggada [a], Risna Hidayani [a], Antoni Raharjo [a], Ridho Islamie [b], Sulistyo Emantoko Dwi Putra [c]

[a] Department of Pharmaceutical Biology, Faculty of Pharmacy, University of Surabaya, Surabaya, 60293, Indonesia
[b] Department of Clinical and Community Pharmacy, Faculty of Pharmacy, University of Surabaya, Surabaya, 60293, Indonesia
[c] Department of Biology, Faculty of Biotechnology, University of Surabaya, Surabaya, 60293, Indonesia

ARTICLE INFO

Keywords:
Plantago major
Greater plantain
Ursolic acid
Oleanolic acid
Diabetic wound

ABSTRACT

Impaired wound healing is among the serious complications of diabetes that can lead to amputation and even death. *Plantago major* has been used empirically to improve wound healing. The main bioactive compounds of *P. major* extracts, ursolic acid (UA) and oleanolic acid (OA), have also been studied for their benefits with non-hyperglycemic wounds. This study was done to examine the *in vivo* wound healing effects of *P. major* leaf extracts (PMLE), UA, and OA in hyperglycemic rats, to evaluate their *in vitro* diabetic wound healing activity, and to observe possible dermal irritation after topical application. Wound closure, duration of epithelialization, and histopathological profiles of healed tissue were observed in the hyperglycemic rats with excision wounds for 21 days. An anti-inflammatory test using the NO inhibitory assay, a fibroblast proliferation assay, and a migration assay with high-glucose medium were done to investigate the mechanism of action of the tested samples in wound healing. The acute dermal irritation test followed the international guidelines. PMLE, UA, and OA increased the percentage of wound closure and accelerated wound healing time. PMLE activities were assessed for the inhibition of NO production in the inflammation phase and enhancement of fibroblast proliferation. UA may contribute to this wound healing process through inhibition of NO production, whereas OA through activation of migration of fibroblast cells. Topical applications of PMLE, UA, and OA did not cause acute dermal irritation. PMLE, UA, and OA have the potential to improve wound healing with diabetes conditions.

1. Introduction

Plantago major or greater plantain is the most widely used species of the genus Plantago and belongs to the Plantaginaceae family. In many parts of the world, *P. major* is ubiquitous and, therefore, has various local names, including *daun sendok* in Indonesia (Adom et al., 2017; Gonçalves & Romano, 2016; Kartini et al., 2017; Samuelsen, 2000). They are wild plants that have good adaptability to diverse conditions in the environment. Nevertheless, to minimize the exploitation of natural resources, prior studies have attempted to optimize its cultivation, harvest, and postharvest processing (Prakash et al., 2011; Zubair et al., 2011). These practices can promote sustainable and efficient production now that there is an increasingly high demand for *P. major*, both in the nutraceutical and pharmaceutical sectors.

Plantago major contains high concentrations of mucilaginous carbohydrates that are active as immunostimulants and antioxidants and are important as excipients in food processing, tablets and emulsions formulations (Akbari et al., 2016; Lukova et al., 2017; Niknam et al., 2020). Its mucilaginous property underlies the use of *P. major* as a nutraceutical, especially to improve intestinal health (Gonçalves & Romano, 2016; Samuelsen, 2000; Samuelsen et al., 1995). In some regions, fresh leaves of *P. major* are also used as ingredients in salads and soups, and the seeds can also be processed into snacks, cakes, and breads (Gonçalves & Romano, 2016). The medicinal properties of *P. major* are attributable to its various biologically active compounds such as terpenoids, phenolic acids, flavonoids, alkaloids, and iridoids. It is traditionally used in the treatment of a number of diseases, such as wounds and other skin diseases, infectious diseases, problems concerning the digestive and respiratory organs, reproduction and circulation issues, and tumors, and for pain relief and fever reduction (Adom et al., 2017; Gonçalves & Romano, 2016; Najafian et al., 2018; Samuelsen, 2000). Many studies have been carried out to confirm these practical applications (Chiang et al., 2002, 2003; Kartini et al., 2014; Kolak et al., 2011; Mansor et al., 2014;

* Corresponding author.
E-mail address: kartini@staff.ubaya.ac.id (K. Kartini).

https://doi.org/10.1016/j.fbio.2021.100937
Received 24 June 2020; Received in revised form 21 February 2021; Accepted 22 February 2021
Available online 27 February 2021

Abbreviations

DFU	diabetic foot ulcer
HPTLC	high performance thin layer chromatography
NO	nitric oxide
OA	oleanolic acid
OECD	Organisation for Economic Cooperation and Development
PMLE	*Plantago major* leaves extract
UA	ursolic acid

Ozaslan et al., 2007; Poor et al., 2017).

These lead to the conclusion that *P. major* may have promise for the management of various chronic clinical disorders, including diabetes mellitus and its complications, particularly diabetic foot ulcers (DFU). Abdulghani et al. (2014) found that *P. major* methanol extracts at 500 and 1000 mg/kg bw lowered blood glucose levels in streptozotocin-induced diabetic rats. The mechanism underlying this activity was studied by increasing the control of the glycemic mechanism of the remaining pancreatic cells in diabetic rats (Abdulghani et al., 2014; Adom et al., 2017). *P. major* also has a long history of wound healing. Many countries in Europe, Asia, and the Middle East (e.g., Iran) have long used this herb either for acute or chronic wounds (Adom et al., 2017; Gonçalves & Romano, 2016; Hosseinkhani et al., 2017; Jarić et al., 2018; Jivad et al., 2016). Moreover, both *in vitro* and *in vivo* tests have been done to show the wound healing effect of *P. major* and its constituents, such as UA, OA, and aucubin (Agra et al., 2015; Amini et al., 2010; Kartini et al., 2018a; Moura-Letts et al., 2006; Phipps & Mahmood, 2006; Velasco-Lezama et al., 2006; Zubair et al., 2012, 2016).

However, there has been no report on *P. major* activity for diabetic wound healing. The wound healing process involves a series of overlapping phases, namely, hemostasis, inflammation, proliferation, and remodeling, that may be inhibited by the presence of oxygen free radicals, microbial infection, and high blood glucose (Houghton et al., 2005; Patel et al., 2019). Chemical or herbal medicines that can modulate one or more of those phases can be examined as candidates for wound healing agents with normal or hyperglycemic conditions. Previous studies showed that *P. major* and its phytochemicals are active anti-inflammatory, antioxidant, and antimicrobial agents (Hussan et al., 2015; Ikeda et al., 2008; Kartini et al., 2014, 2017; Liu, 1995; Mazzutti et al., 2017; Shirley et al., 2017; Stenholm et al., 2013; Vasconcelos et al., 2006). Therefore, this study was intended to determine the *in vivo* wound healing effects of *P. major* extracts and chemical compounds, namely UA and OA, in hyperglycemic rats (i), to evaluate their *in vitro* diabetic wound healing activity through the anti-inflammatory process, fibroblast proliferation, and fibroblast migration (ii), and to determine possible dermal irritation after topical application (iii).

2. Materials and methods

2.1. Plant materials and chemicals

Wholes plants of *P. major* was collected from the cultivation area of the Balai Materia Medica, Batu City, Jawa Timur Province, Indonesia in September 2016 (Global Positioning System (GPS) coordinates 7°52′2.262″ S 112°31′11.719″ E; 875 m above sea level) and promptly transported within 1 h to the laboratory. Authentication of the plant was done by the Center for Traditional Medicine Information and Development, University of Surabaya, Surabaya, Indonesia, with accession certificate number: 1212/UN.IX/2016. UA, OA, alloxan monohydrate, methylthiazolyldiphenyl-tetrazolium bromide (MTT), and hematoxylin-eosin were obtained from Sigma Aldrich Co. (St. Louis, MO, USA), whereas absolute ethanol, toluene, acetone, formic acid,

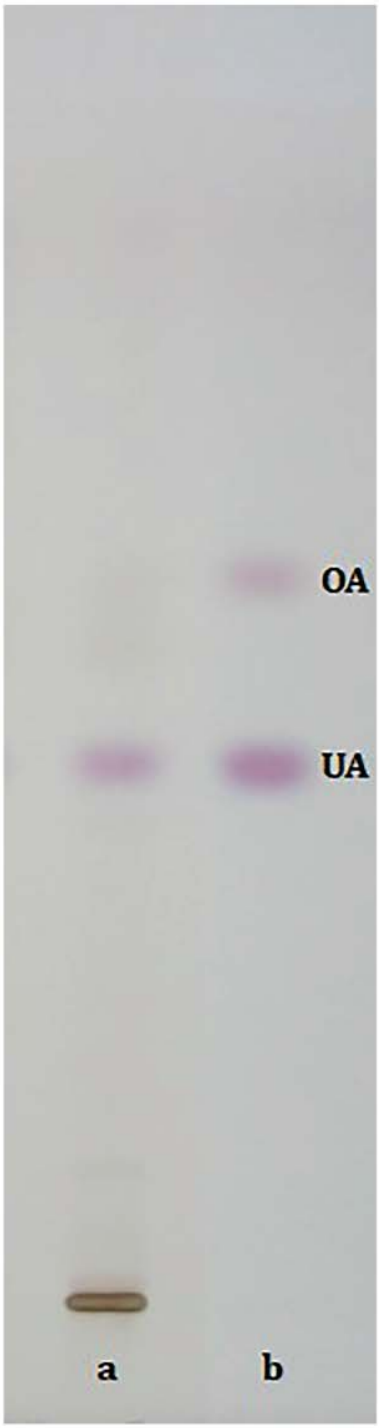


Fig. 1. High performance thin layer chromatography profile of PMLE. Stationary phase: silica gel 60 F_{254}, mobile phase: toluene:acetone:formic acid (78:22:0.15). a: PMLE, b: ursolic acid (UA) - oleanolic acid (OA) standard.

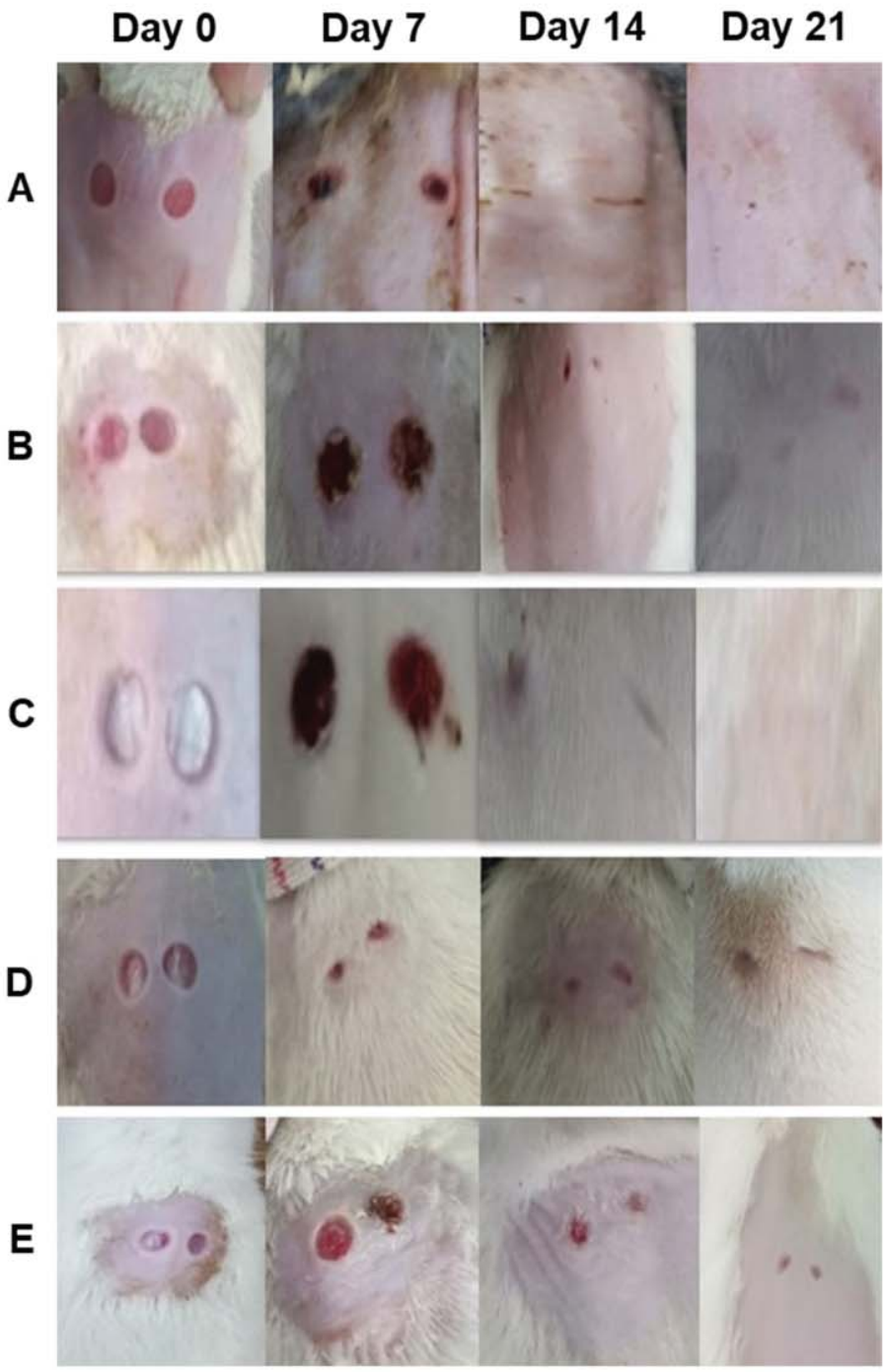


Fig. 2. Wound morphology of hyperglycemic rats from Day 0–21. A: PMLE, B: UA, C: OA, D: Positive control, E: Negative control.

sulfuric acid, methanol, chloroform, iodine, and DMSO were from Merck KGaA (Darmstadt, Germany). Carbomer, sodium hydroxide, methylparaben, propylparaben, and propylene glycol were pharmaceutical grade and obtained from Tristar Chemical Co. (Surabaya, Jawa Timur, Indonesia).

Table 1
Effect of gels on wound closure and time of epithelialization.

Group	Percentage of wound closure			Time of epithelialization (day)
	Day 7	Day 14	Day 21	
A	69 ± 3	98 ± 1	100 ± 0	16 ± 1*
B	70 ± 3	99 ± 1*	100 ± 0	15 ± 1*
C	84 ± 8*	100 ± 0*	100 ± 0	12 ± 1*
D	81 ± 6*	99 ± 2	100 ± 0	15 ± 2*
E	67 ± 2	95 ± 4	100 ± 0	19 ± 1

Group A: PMLE, B: UA, C: OA, D: Positive control (Mebo®), E: Negative control. Data represent means ± SD (n = 5), *p < 0.05 against negative control (Kruskal-Wallis, followed by Mann-Whitney test).

2.2. Preparation of *P. major* extract and determination of UA and OA

The leaves of *P. major* were separated from the other parts of the plant, then air-dried for 120 h, powdered into 2 mm particles using a household Panasonic MX-J1G blender (Panasonic Corp., Kadoma, Osaka, Japan), and stored at room temperature (31 ± 2 °C) for a maximum of 12 wk until used. Three hundred g of these leaves were macerated with ethanol (3 × 800 ml, 24 h) at room temperature. The extracts were filtered using Whatman® qualitative filter paper grade 1 (Merck) and evaporated under vacuum using a rotavapor R-200 (BÜCHI Labortechnik AG, Flawil, Switzerland) to yield viscous extracts. High performance thin layer chromatography (HPTLC) was done to determine OA and UA as described previously (Kartini et al., 2014). Chromatography was done on a pre-coated HPTLC plate with silica gel 60 F_{254}, 0.20 mm layer thickness (Merck), using toluene:acetone:formic acid (78:22:0.15) as the mobile phase. PMLE and a mixture of OA and UA (1:1 w/w) were spotted as 5 mm bands using a Linomat 5 sample applicator (Camag, Muttenz, Switzerland) and then prederivatized using iodine vapor (1% iodine in chloroform). Development was then carried out using 10 ml of the mobile phase in a twin trough chamber (Camag) previously equilibrated with the mobile phase for 20 min at room temperature. Following development, the plate was dried in a fume hood and subjected to derivatization by spraying the plate with 5% sulfuric acid in methanol and then heating at 120 °C for 3 min. The chromatogram was obtained using Reprostar 3 documentation densitometry (Camag) with a VH-C20 3CCD color video camera (Hitachi Denshi Ltd., Shinjuku City, Tokyo, Japan).

2.3. Preparation of the gel dosage form

P. major leaf extract (PMLE), UA, and OA were formulated into gel dosage forms as described previously using carbomer, sodium hydroxide, methylparaben, propylparaben, and propylene glycol (Kartini, et al., 2018). Carbomer (2 g) was dispersed over 40 ml water, stirred (1000 rpm) using a high shear mixer-HSM 2003 SV/DV (CKL Multimix (M) Sdn Bhd, Puchong, Selangor Darul Ehsan, Malaysia) while adding 1% sodium hydroxide gradually until it formed a gel. PMLE (5 g), UA (15 mg), or OA (15 mg) were dissolved in 2 ml ethanol. Methyl paraben (180 mg) and propyl paraben (20 mg) were mixed and dissolved in propylene glycol (16 g). These two solutions were then mixed and poured into the gel with 100 g water and stirred until homogeneous. The gels were administered up to 0.5 g/test animals. Every 0.5 g PMLE gel contained 25 mg extract, whereas 0.5 g UA or OA gels contained 75 μg of the respective active compounds.

2.4. *In vivo* wound healing assay

Male Wistar rats (180–200 g) were from the Integrated Research and Testing Laboratory, Gadjah Mada University, Yogyakarta, Indonesia and housed at room temperature, 70% RH, and a 12 h light-dark cycle. These test animals were put in plastic cages (1 rat/cage) and fed with a standard diet (water max. 13%, crude protein 21–23%, fat min. 5%, fiber max. 5%, ash max. 7%, calcium min. 0.9%, and phosphorus min 0.6%), given water *ad libitum*, and acclimatized for 2 wk before the experiment. All handling procedures were according to the institutional rules on animal experiments (approval No: 721-KE; dated June 15, 2017). Before the induction of hyperglycemia, test animals were weighed, their blood was collected from the tail vein, and the fasting blood glucose levels were measured. Then, they were intraperitoneally injected with a single dose of alloxan monohydrate (160 mg/kg bw) in normal saline. Two days later, the fasting blood glucose levels were measured to confirm their hyperglycemic status. Hyperglycemia was indicated if the random blood glucose levels were ≥200 mg/dl.

Hyperglycemic rats were intraperitoneally anesthetized with ketamine HCl (Ketalar®, Pfizer Indonesia, Jakarta, Indonesia) at 100 mg/kg bw. An excision wound was induced on the dorsal area of the animals using a biopsy punch from Medax Srl Unipersonale (San Possidonio, MO, Italy) with a diameter of 5 mm. Test animals were then divided into 5 groups, each consisting of 5 rats. Three groups were treated with PMLE, UA, and OA gels, respectively; whereas two other groups were treated with positive and negative control, respectively. A commercially available gel product containing Copitidis Rhizoma, *Phellodendri chinensis* Cortex, and Scutellariae Radix was used as a positive control (Mebo®, from Shantou MEBO Pharmaceutical Co., Ltd. (Shantou, Guangdong, China)), while the gel without active ingredient was applied as a negative control. All treatments were carried out topically once daily. Parameters of the *in vivo* wound healing were the percentage and time of wound closure. Wound diameter was measured using a digital caliper (resolution 0.01 mm) on Day 0, 7, 14, and 21. The percentage of wound closure was determined using the following equation:

$$((D_1 - D_2) / D_1) \times 100 \quad (1)$$

where D_1 = the largest wound diameter (on Day 0), and D_2 = wound diameter on the day of observation. Healing time was measured as the

Table 2
Effect of gels on wound healing phase on Day 21.

Group	Healing score
A	2.2 ± 0.3
B	2.7 ± 0.0*
C	3.0 ± 0.7*
D	3.3 ± 0.1*
E	2.0 ± 0.0

Group A: PMLE, B: UA, C: OA, D: Positive control (Mebo®), E: Negative control. Data represent the means ± SD (n = 3), *p < 0.05 against negative control (Kruskal-Wallis, followed by Mann-Whitney test).

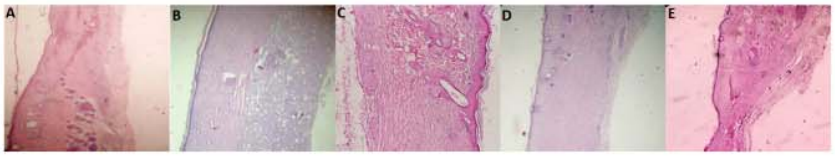


Fig. 3. Histological profiles of healing tissue on Day 21. A: PMLE, B: UA, C: OA, D: Positive control, E: Negative control.

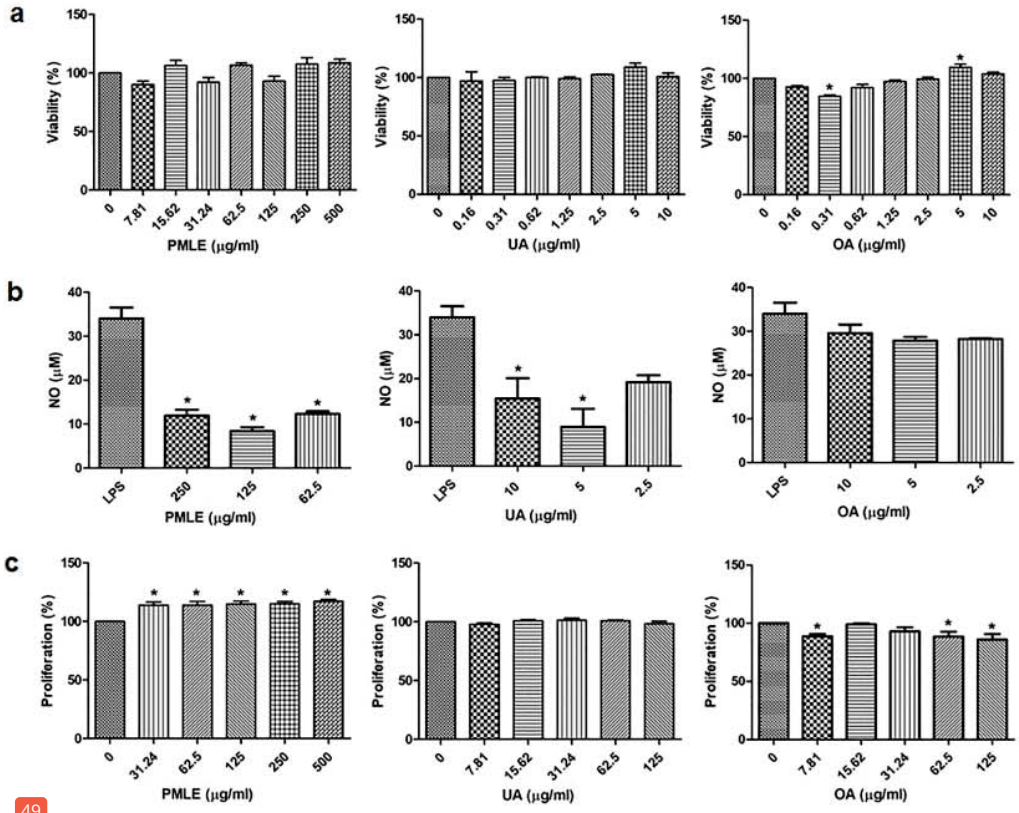


Fig. 4. The effects of PMLE, UA, and OA on the viability of the RAW 264.7 cell line (a), NO production of RAW 264.7 cell line (b), and proliferation of NIH/3T3 fibroblast cells. Data are expressed as mean ± SD (n = 3), *p < 0.05 against control (one-way ANOVA, followed by Tukey's multiple comparison test).

day when the wound closed fully. Also, healing tissues were collected for a histopathological study on Day 21, in which skin tissues were fixed in 10% formalin, sliced using an automatic rotary microtome (Thermo Fisher Scientific, Waltham, MA, USA) and prepared on a glass slide, stained with hematoxylin-eosin, and then observed under a light microscope (Olympus Corp., Shinjuku City, Tokyo, Japan). Histopathological study was done to evaluate the condition of healed tissue by observing the presence of inflammatory cells, angiogenesis, fibrosis, fibroblasts, and collagen, on which a score (0–4) of each sample was based. Score 0 means the wound was still open. Score 1 indicated the presence of bleeding (++), inflammation (++), angiogenesis (++), and epithelialization (-); while at score 2, there were signs of inflammation (+), angiogenesis (+), epithelialization (+), and fibrosis (+). Score 3 or early remodeling was represented by the presence of fibroblasts and collagen (+), as well as epithelialization (+); and score 4 or the complete remodeling phase was the formation of collagen (++) and epithelialization (++).

2.5. In vitro wound healing assay

2.5.1. Nitric oxide (NO) inhibitory assay

The *in vitro* anti-inflammatory test was done following the method used in a previous study (Tam et al., 2011). RAW264.7 cells (mouse macrophage, ATCC® TIB-71™, 4 × 10^5/well, counted using a counting chamber with v-slash (Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany)) were seeded in a 24-well plate overnight. The cells were provided by the Parasitology Laboratory, Medical Faculty, Gadjah Mada University. PMLE, UA, or OA were added at various concentrations together with 0.1 μg LPS/ml of the medium and incubated for 24 h. The cell culture supernatant was taken out and added into Griess Reagent (Promega Corp., Madison, WI, USA) at a 1:1 ratio in 96-well plate, followed by incubation in the dark for 10 min. The absorbance was measured at 540 nm using a Bio-Rad Benchmark microplate reader (Bio-Rad Laboratories Inc., Hercules, CA, USA). The $NaNO_2$ was used to prepare a standard curve and linear regression equation.

2.5.2. Fibroblast proliferation assay

The procedures used for this assay were according to the method described previously, with a slight adjustment (Tam et al., 2011). NIH/3T3 cells (mouse fibroblasts, ATCC® CRL-1658™; provided by the same parasitology laboratory) were seeded at 10^3 cells/well in a 96-well plate in high-glucose DMEM (4500 mg/l; Fisher Scientific International Inc., Waltham, MA, USA). Then, the cells were exposed to different concentrations of PMLE, UA, and OA for 48 h at 37 °C. MTT solution (5 mg/ml) in PBS (Fisher Scientific) was added to the medium in each well, and the plate was then incubated at 37 °C for 4 h. All medium was then removed and replaced with DMSO. The optical density was measured at 540 nm to determine the relative amounts of viable cells and expressed as the percentage of control samples without treatment.

2.5.3. Migration assay

This test, which followed Tam et al. (2011) with adjustment for the type of cell (Tam et al., 2011), was started by seeding rat gingival

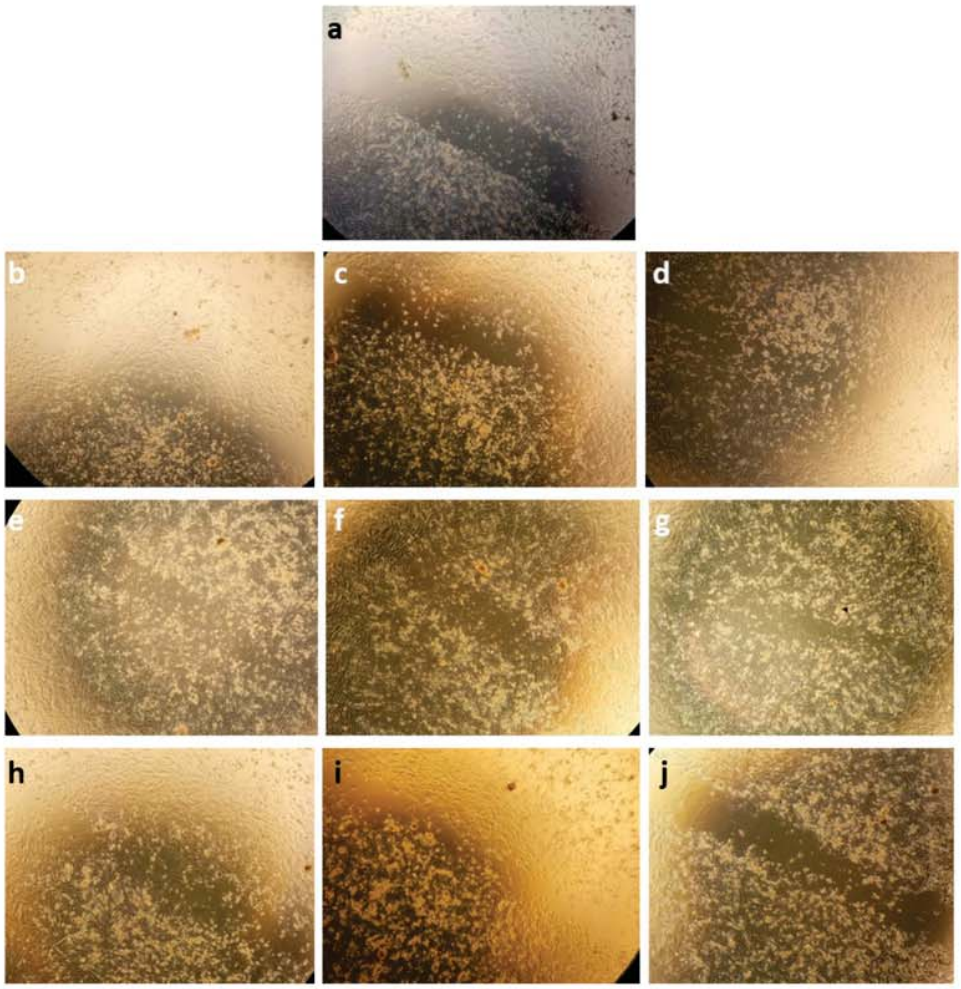


Fig. 5. Representative microphotograph of gingival fibroblast cells before the addition of the test samples (a) and after 24 h of incubation. b, c, d were induced with PMLE 250, 500, 1000 μg/ml, respectively; e, f, g with UA 25, 50, 100 μg/ml, respectively; h, i, j with OA 25, 50, 100 μg/ml, respectively.

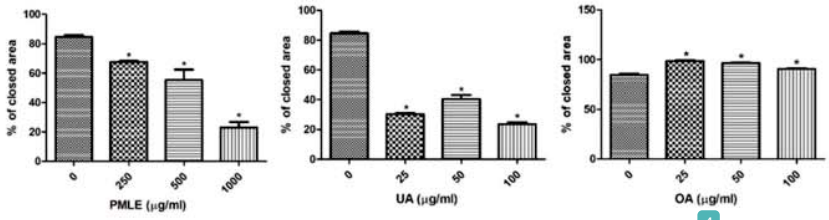


Fig. 6. The effects of PMLE, UA, and OA on closed areas observed in the gingival fibroblast migration test on rats. Data are expressed as mean ± SD (n = 3), *p < 0.05 against control (one-way ANOVA, followed by Tukey's multiple comparison test).

fibroblast cells at 10^5 cells/well in a 24-well plate in high-glucose DMEM. These cells were obtained from the Institute of Tropical Disease, Airlangga University (Surabaya, Indonesia). Cells were incubated for 24 h at 37 °C in a 5% CO_2 humidified incubator. Cells were then observed under a microscope to ensure that they had adhered to the well and achieved a confluent state. Fibroblast cells in the well were then scratched in one direction using a 1000 μl pipette tip, then observed under a light microscope, and photographs were taken. A series of concentrations of PMLE, UA, or OA solutions were added into each well, then incubated overnight. The results were observed under a light microscope and photographed. The photographs were compared and analyzed using the TScratch program (CSElab, Zurich, Switzerland) to

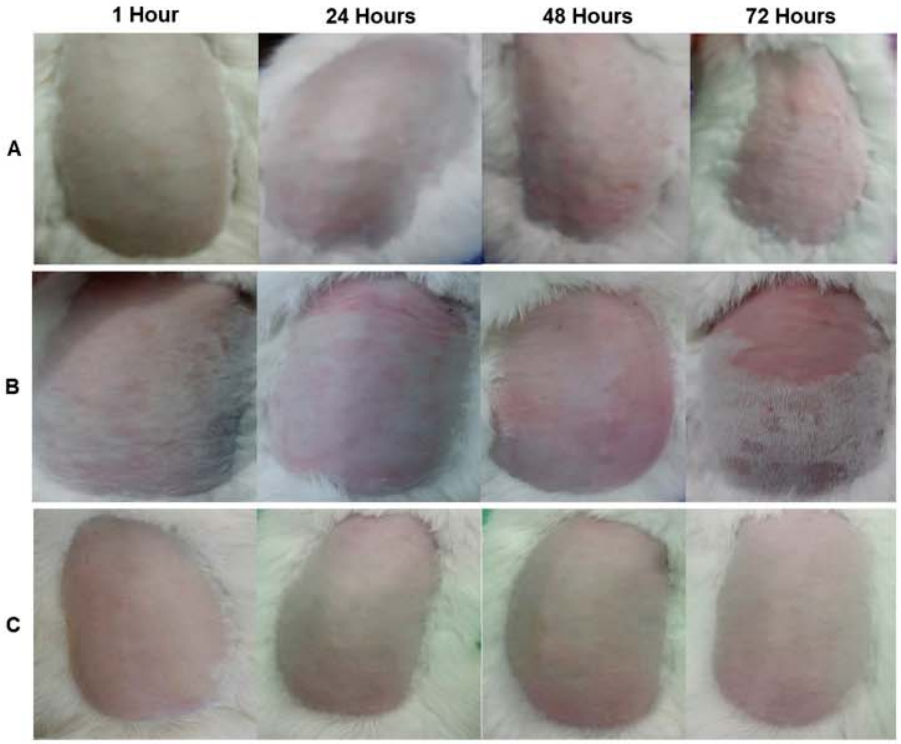


Fig. 7. The results of the 72-h acute irritation dermal test. A: PMLE, B: UA, C: OA.

determine the migration. This program measures open areas in the images. Percentage of the closed area before and after treatments were then calculated. An increase of the percentage of closed area indicated the migration.

2.6. Acute dermal irritation assay

The test followed the method recommended in the Organisation for Economic Cooperation and Development Guideline No. 404 (2015) (OECD, 2015). Healthy male albino rabbits aged 8–9 months (adult) and weighing around 1.8–2.3 kg were used as test animals. They were individually housed and acclimatized to the experimental conditions for 1 wk, fed with conventional laboratory diets (crude protein 18–19%, crude fiber 13%, fat 5–6%, calcium 0.9%, phosphorous 0.7%, water 11%, flumequin antibiotic and vitamins), and given *ad libitum* access to drinking water. Approximately 24 h before the test, fur was removed by carefully clipping the dorsal area of the trunk of the animals using an electrical hair clipper. The test samples were applied to a small area (±6 cm^2) of skin and covered with a gauze pad, which was held in place with non-irritating tape (Beiersdorf Indonesia, Malang, Jawa Timur, Indonesia). For the initial test, one animal was used. Three test patches were applied sequentially to the animal. The first patch was removed after 3 min. If there was no apparent serious skin reaction, a second patch was applied at a different site and removed after 1 h. If the observations at this stage indicated that exposure could humanely be allowed to extend to 4 h, a third patch was applied and removed after 4 h, and the response was graded. If a corrosive effect appeared after any of the three sequential exposures, the test was immediately terminated. However, if no corrosive effect (irritation) was formed after the last patch was removed, the animal was observed for 14 days, unless corrosion developed at an earlier point of time. If there was no serious skin reaction in the initial test, the irritant or negative response was confirmed using up to two additional animals, each with one patch, for an exposure period of 4 h. The animals were observed for 14 days after the removal of patches. All animals were examined for erythema and oedema, and the responses were scored at h 1, 24, 48, and 72 after patch removal. Score 0 was indicated by no erythema and no oedema; score 1 was very slight erythema and oedema; score 2 was well defined erythema and slight oedema; score 3 was moderate to severe erythema and moderate oedema; and score 4 was severe erythema and oedema.

2.7. Statistical analysis

All data were reported as mean ± SD, then analyzed by descriptive statistics, one way ANOVA and Tukey's multiple comparison test in the statistical program, GraphPad Prism (GraphPad Software Inc., San Diego, CA, USA, Windows Version 5.01). If the data did not meet the requirements of a parametric statistical test, then they were processed with the Kruskal-Wallis test and the Mann-Whitney post hoc test. $p < 0.05$ distinguished a statistically significant difference.

3. Results and discussion

3.1. High performance thin layer chromatography of PMLE

The presence of UA and OA in the PMLE was identified using HPTLC (Fig. 1). The chromatogram showed that PMLE contains UA, but the presence of OA cannot be detected. This is consistent with the previous studies which showed that OA was present in the petiole, seed, and aerial parts, but not in the leaves and roots (Kartini et al., 2014, 2017).

3.2. In vivo wound healing

The wound healing efficacy of PMLE, UA, and OA was investigated from the wound closure, time of epithelialization, and gross examination for 21 days. Also, at the end of the test, the histopathological profile of healed tissue was documented. Wound closure and time of epithelialization were examined to determine the rate of reduction of the unhealed area during the healing process. A faster rate of wound closure indicates a better efficacy. The shrinking of the wound area in different groups of treated animals over 21 days due to PMLE, UA, and OA is shown in Fig. 2 and Table 1.

Overall, the topical application of PMLE and its chemical constituents (UA and OA) increased wound closure significantly ($p < 0.05$), particularly on Day 7 and 14. On Day 21, although complete wound closure was observed in all groups, no significant differences were detected. Table 1 also shows that time of epithelialization of the groups treated with PMLE and UA was not significantly different from that of the positive control (Mebo®). It suggested that PMLE and UA are as active as the positive control. Animals treated with OA gel showed the fastest epithelialization, indicating that OA had higher efficacy for wound healing with hyperglycemic conditions. A previous study on wound healing in mice with normal conditions confirmed that OA is 42.9% faster and more effective than the placebo, and at the dose of 40 μg/g bw, it produces the highest wound healing effect (Moura-Letts et al., 2006). Histological profiles of healing tissue (Fig. 3 and Table 2) show that on Day 21, animals treated with OA and positive control gels had started the remodeling phase, approaching complete remodeling. However, groups treated with PMLE, UA, and negative control were still in phase 2 and progressing to early remodeling, as characterized by ongoing angiogenesis and inflammation, and early development of fibrosis and epithelialization. These results were consistent with OA producing the best outcome in wound closure and the fastest epithelialization.

3.3. Effect of PMLE, UA, OA on NO production by RAW264.7 cells

Repair needs the coordination of various cells, growth factors, and cytokines. In diabetic patients, the normal wound healing process is interrupted by high blood glucose. Diabetic wounds show a persistent inflammatory phase resulting in the inhibited formation of mature granulation tissue and reduction in wound tensile strength, which may be caused by ischemic damage to blood vessels (Patel et al., 2019).

The anti-inflammatory activity of PMLE, UA, and OA was investigated using the NO inhibition test on RAW264.7 macrophage cells. The cells were cultured in high-glucose medium to mimic a diabetic condition. Apart from RAW264.7, macrophage-like cells such as THP-1 can also be used to evaluate *in vitro* anti-inflammatory activity. These monocytes will differentiate into macrophage after induction with phorbol 12-myristate 13-acetate (PMA) (Chanput et al., 2014). The use of RAW264.7 cells was preferred since this research did not need to differentiate the cells. Bacterial infections or immunological stimuli such as LPS induce macrophages to release a high concentration of NO, which is potentially cytotoxic and can destroy the surrounding cells and tissues. Before the NO inhibition test, the effects of PMLE, UA, and OA on cell viability was evaluated to determine the appropriate concentration for the NO inhibition tests (Fig. 4a). The viability of RAW264.7 cells did not significantly decrease after the addition of PMLE, UA, and OA at up to 500, 10, and 10 μg/ml, respectively. These results indicated that all samples at these concentrations were non-toxic to the cells and did not affect the regular cellular activity.

After treatment with PMLE, UA, and OA, the NO concentration in the cell culture media was measured using the Griess Reaction. All concentrations of PMLE and UA suppressed the NO production in RAW264.7 cells significantly after LPS induction (Fig. 4b), indicating that PMLE and UA have anti-inflammatory properties. OA (2.5–10 μg/ml) also inhibited the production of NO; however, the reduction was not significantly different from that of the control. On the other hand, previous research with normal media showed that OA inhibited some inflammatory mediators, such as COX-2, TNF-α, IL-1β, IL-6, and IFN-γ (Kartini et al., 2014, 2017; Ringbom et al., 1998; Stenholm et al., 2013). Therefore, further studies are needed to confirm whether or not OA is an active anti-inflammatory agent with hyperglycemic conditions using these other inflammatory mediators.

3.4. Effects of PMLE, UA, and OA on NIH/3T3 fibroblast proliferation

The wound healing cascade is a complex interaction of cellular and biochemical actions that differentiates into several phases, from healing to structural and functional integrity restoration to regain the strength of injured tissues. Fibroblast proliferation is one crucial step in tissue regeneration. A series of PMLE, UA, and OA concentrations were tested at the proliferation phase using NIH/3T3 fibroblast cells. Fig. 4c shows that PMLE at 31–500 μg/ml increased fibroblast proliferation, but the opposite was true for UA and OA administered at 7.8–125 μg/ml. These results were consistent with Kuonen et al. that the lipophilic extract of *Viscum album* L. and oleanolic acid did not produce any proliferation-stimulating effects in NIH/3T3 fibroblast cells at any concentration (0.01–100 and 0.001–10 μg/ml, respectively) (Kuonen et al., 2013). Therefore, UA and OA probably contributed to diabetic wound healing through other mechanisms.

3.5. Effects of PMLE, UA, and OA on fibroblast migration

Angiogenesis, another process contributing to wound healing, is important until the terminal stages of healing. Its mechanism involves proliferation, migration, and remodeling of endothelial cells, followed by subsequent tube formation. The effects of PMLE, UA, and OA on the migration activity were assessed using gingival fibroblast cells that had been isolated from the rats. Fig. 5 shows the photograph of the scratched cells before the addition of the samples. This figure also shows the open area of cells at the bottom of wells after incubation with serial concentrations of PMLE, UA, and OA. Fig. 6 shows that all concentrations of PMLE and UA did not increase cell migration; on the other hand, open areas were reduced as OA concentration increased. The percentage of closed area in treated cells to the control cells indicates the level of cell migration activity. Fig. 6 shows that OA (25–100 μg/ml) can increase the migration of rat gingival fibroblast cells.

3.6. Acute dermal irritation test

The results of the acute dermal irritation test of PMLE, UA, and OA gels are shown in Fig. 7. No dermal responses, including erythema or oedema, were found in animals treated with either UA or OA gels. In the group treated with PMLE gel, some rabbits showed erythema after 48 and 72 h. However, this is still included in the nonirritant category (Banerjee et al., 2013).

4. Conclusions

Plantago major leaf extracts and its phytochemicals (UA and OA) can increase the percentage of wound closure and accelerate wound healing time in hyperglycemic rats. The extent of these activities was determined using the inhibited NO production in the inflammation phase and enhanced fibroblast proliferation. While UA contributed to the wound healing process by inhibiting NO production, OA showed the same function by activating fibroblast cell migration. Topical applications of *P. major* leaf extracts, UA, and OA in gel dosage forms did not cause acute dermal irritation. This study showed that *P. major* leaf extracts, UA, and OA acid have the potential to improve wound healing with diabetes conditions.

Author statement

This study was designed, directed and coordinated by Kartini Kartini, Ridho Islamie and Sulistyo Emantoko Dwi Putra for all aspects of the project. Nina Wati, Rabbindra Gustav, and Risa Wahyuni partly contributed to the *in vivo* wound healing assay, whereas Yosua Fernaldi Anggada, Risna Hidayani, and Antoni Raharjo involved in the *in vitro* assay and safety evaluation. Ridho Islamie and Sulistyo Emantoko Dwi Putra suggested and commented on the design of the experiments. The manuscript was written by Kartini Kartini and commented by all authors. All authors have read and approved the final version of the manuscript.

Declaration of competing interest

The authors confirm that they have no conflicts of interest with respect to the work described in this manuscript.

Acknowledgements

This research was supported by the Indonesian Ministry of Research, Technology, and Higher Education (grant number 017/SP-Lit/LPPM-01/Ristekdikti/FF/III/2018).

References

Abdulghani, M. A., Hamid, I., Al-Naggar, R. A., & Osman, M. T. (2014). Potential antidiabetic activity of *Plantago major* leaves extract in streptozocin-induced diabetic rats. *Research Journal of Pharmaceutical, Biological and Chemical Sciences, 5*(2), 896–902.

Adom, M. B., Taher, M., Mutalabisin, M. F., Amri, M. S., Kudos, M. B. A., Sulaiman, M. W. A. W., Sengupta, P., & Susanti, D. (2017). Chemical constituents and medical benefits of *Plantago major*. *Biomedicine & Pharmacotherapy, 96*, 348–360.

Agra, L. C., Ferro, J. N., Barbosa, F. T., & Barreto, E. (2015). Triterpenes with healing activity: A systematic review. *Journal of Dermatological Treatment, 26*(5), 465–470.

Akbari, J., Saeedi, M., Morteza-Semnani, K., Zarrabi, B., Rostamkalaei, S. S., & Kelidari, H. R. (2016). The effect of *Plantago major* seed mucilage combined with carbopol on the release profile and bioadhesive properties of propranolol HCl buccoadhesive tablets. *Pharmaceutical and Biomedical Research, 2*(2), 84–100.

Amini, M., Kherad, M., Mehrabani, D., Azarpira, N., Panjehshahin, M., & Tanideh, N. (2010). Effect of *Plantago major* on burn wound healing in rat. *Journal of Applied Animal Research, 37*(1), 53–56.

Banerjee, S., Chattopadhyay, P., Ghosh, A., Pathak, M. P., Singh, S., & Veer, V. (2013). Acute dermal irritation, sensitization, and acute toxicity studies of a transdermal patch for prophylaxis against (±) anatoxin-A poisoning. *International Journal of Toxicology, 32*(4), 308–313.

Chanput, W., Mes, J. J., & Wichers, H. J. (2014). THP-1 cell line: An *in vitro* cell model for immune modulation approach. *International Immunopharmacology, 23*(1), 37–45.

Chiang, L. C., Chiang, W., Chang, M. Y., & Lin, C. C. (2003). *In vitro* cytotoxic, antiviral and immunomodulatory effects of *Plantago major* and *Plantago asiatica*. *American Journal of Chinese Medicine, 31*(2), 225–234.

Chiang, L. C., Chiang, W., Chang, M. Y., Ng, L. T., & Lin, C. C. (2002). Antiviral activity of *Plantago major* extracts and related compounds *in vitro*. *Antiviral Research, 55*(1), 53–62.

Gonçalves, S., & Romano, A. (2016). The medicinal potential of plants from the genus *Plantago* (Plantaginaceae). *Industrial Crops and Products, 83*, 213–226.

Hosseinkhani, A., Falahatzadeh, M., Raoofi, E., & Zarshenas, M. M. (2017). An evidence-based review on wound healing herbal remedies from reports of traditional Persian medicine. *Journal of Evidence-Based Complementary & Alternative Medicine, 22*(2), 334–343.

Houghton, P., Hylands, P., Mensah, A., Hensel, A., & Deters, A. (2005). *In vitro* tests and ethnopharmacological investigations: Wound healing as an example. *Journal of Ethnopharmacology, 100*(1–2), 100–107.

Hussan, F., Mansor, A. S., Hassan, S. N., Kamaruddin, T. N. E., Tasnim, T. N., Budin, S. B., & Othman, F. (2015). Anti-inflammatory property of *Plantago major* leaf extract reduces the inflammatory reaction in experimental acetaminophen-induced liver injury. *Evidence-based Complementary and Alternative Medicine, 2015*.

Ikeda, Y., Murakami, A., & Ohigashi, H. (2008). Ursolic acid: An anti- and pro-inflammatory triterpenoid. *Molecular Nutrition & Food Research, 52*(1), 26–42.

Jarić, S., Kostić, O., Mataruga, Z., Pavlović, D., Pavlović, M., Mitrović, M., & Pavlović, P. (2018). Traditional wound-healing plants used in the Balkan region (southeast Europe). *Journal of Ethnopharmacology, 211*, 311–328.

Jivad, N., Bahmani, M., & Asadi-Samani, M. (2016). A review of the most important medicinal plants effective on wound healing on ethnobotany evidence of Iran. *Der Pharmacia Lettre, 8*(2), 353–357.

Kartini, K., Fitriani, E. W., & Tansridjata, L. (2018). Formulation and physical stability test of oleanolic acid cream and gel. *Pharmaciana, 8*(1), 77–86.

Kartini, Islamie, R., & Handojo, C. S. (2018a). Wound healing activity of aucubin on hyperglycemic rat. *Journal of Young Pharmacists, 10*(2Suppl), s136–s139.

Kartini, Piyaviriyakul, S., Siripong, P., & Vallisuta, O. (2014). HPTLC simultaneous quantification of triterpene acids for quality control of *Plantago major* L. and evaluation of their cytotoxic and antioxidant activities. *Industrial Crops and Products, 60*, 239–246, 0.

Kartini, Piyaviriyakul, S., Thongpraditchote, S., Siripong, P., & Vallisuta, O. (2017). Effects of *Plantago major* extracts and its chemical compounds on proliferation of cancer cells and cytokines production of lipopolysaccharide-activated THP-1 macrophages. *Pharmacognosy Magazine, 13*(51), 393–399.

Kolak, U., Boğa, M., Urusak, E. A., & Ulubelen, A. (2011). Constituents of *Plantago major* subsp. intermedia with antioxidant and anticholinesterase capacities. *Turkish Journal of Chemistry, 35*(4), 637–645.

Kuonen, R., Weissenstein, U., Urech, K., Kunz, M., Hostanska, K., Estko, M., Heusser, P., & Baumgartner, S. (2013). Effects of lipophilic extract of *Viscum album* L. and oleanolic acid on migratory activity of NIH/3T3 fibroblasts and on HaCat keratinocytes. *Evidence-based Complementary and Alternative Medicine, 2013*.

Liu, J. (1995). Pharmacology of oleanolic acid and ursolic acid. *Journal of Ethnopharmacology, 49*(2), 57–68.

Lukova, P., Karcheva-Bahchevanska, D., Nikolova, M., Iliev, I., & Mladenov, R. (2017). Comparison of structure and antioxidant activity of polysaccharides extracted from the leaves of *Plantago major* L., P. media L. and P. lanceolata L. *Bulgarian Chemical Communications, 49*, 282–288.

Mansor, A. S., Budin, S. B., & Othman, F. (2014). Effect of *Plantago major* extract on plasma cytokine changes in paracetamol-induced liver injury. *Cytokine, 70*(1), 49.

Mazzutti, S., Riehl, C. A., Ibañez, E., & Ferreira, S. R. (2017). Green-based methods to obtain bioactive extracts from *Plantago major* and *Plantago lanceolata*. *The Journal of Supercritical Fluids, 119*, 211–220.

Moura-Letts, G., Villegas, L. F., Marçalo, A., Vaisberg, A. J., & Hammond, G. B. (2006). *In vivo* wound-healing activity of oleanolic acid derived from the acid hydrolysis of *Anredera diffusa*. *Journal of Natural Products, 69*(6), 978–979.

Najafian, Y., Hamedi, S. S., Farshchi, M. K., & Feyzabadi, Z. (2018). *Plantago major* in traditional Persian medicine and modern phytotherapy: A narrative review. *Electronic Physician, 10*(2), 6390.

Niknam, R., Ghanbarzadeh, B., Ayaseh, A., & Rezagholi, F. (2020). The hydrocolloid extracted from *Plantago major* seed: Effects on emulsifying and foaming properties. *Journal of Dispersion Science and Technology, 41*(5), 667–673.

OECD. (2015). *OECD Guideline for testing of chemicals. Acute dermal irritation/corrosion, series on testing and assessment (No. 404)*. Paris: Organisation for Economic Cooperation and Development.

Ozaslan, M., Didem Karagoz, I., Kalender, M. E., Kilic, I. H., Sari, I., & Karagoz, A. (2007). *In vivo* antitumoral effect of *Plantago major* L. extract on Balb/C mouse with Ehrlich ascites tumor. *American Journal of Chinese Medicine, 35*(5), 841–851.

Patel, S., Srivastava, S., Singh, M. R., & Singh, D. (2019). Mechanistic insight into diabetic wounds: Pathogenesis, molecular targets and treatment strategies to pace wound healing. *Biomedicine & Pharmacotherapy, 112*, Article 108615.

Phipps, M., & Mahmood, A. (2006). Gastroprotective activity of *P. major* in rats. *International Journal of Tropical Medicine, 1*(1), 36–39.

Poor, M. H. S., Khatami, M., Azizi, H., & Abazari, Y. (2017). Cytotoxic activity of biosynthesized Ag nanoparticles by *Plantago major* towards a human breast cancer cell line. *Rendiconti Lincei, 28*(4), 693–699.

Prakash, V., Bisht, H., & Prasad, P. (2011). Altitudinal variation in morpho-physiological attributes in *Plantago major*: Selection of suitable cultivation site. *Research Journal of Medicinal Plant, 5*(3), 302–311.

Ringbom, T., Segura, L., Noreen, Y., Perera, P., & Bohlin, L. (1998). Ursolic acid from *Plantago major*, a selective inhibitor of cyclooxygenase-2 catalyzed prostaglandin biosynthesis. *Journal of Natural Products, 61*(10), 1212–1215.

Samuelsen, A. B. (2000). The traditional uses, chemical constituents and biological activities of *Plantago major* L. A review. *Journal of Ethnopharmacology, 71*(1–2), 1–21.

Samuelsen, A. B., Paulsen, B. S., Wold, J. K., Otsuka, H., Yamada, H., & Espevik, T. (1995). Isolation and partial characterization of biologically active polysaccharides from *Plantago major* L. *Phytotherapy Research, 9*(3), 211–218.

Shirley, K. P., Windsor, L. J., Eckert, G. J., & Gregory, R. L. (2017). *In vitro* effects of *Plantago major* extract, aucubin, and baicalein on *Candida albicans* biofilm formation, metabolic activity, and cell surface hydrophobicity. *Journal of Prosthodontics, 26*(6), 508–515.

Stenholm, A., Goransson, U., & Bohlin, L. (2013). Bioassay-guided supercritical fluid extraction of cyclooxygenase-2 inhibiting substances in *Plantago major* L. *Phytochemical Analysis, 24*(2), 176–183.

Tam, J. C. W., Lau, K. M., Liu, C. L., To, M. H., Kwok, H. F., Lai, K. K., Lau, C. P., Ko, C. H., Leung, P. C., & Fung, K. P. (2011). The *in vivo* and *in vitro* diabetic wound healing effects of a 2-herb formula and its mechanisms of action. *Journal of Ethnopharmacology, 134*(3), 831–838.

Vasconcelos, M. A. L., Royo, V. A., Ferreira, D. S., Crotti, A. E. M., e Silva, M. L. A., Carvalho, J. C. T., Bastos, J. K., & Cunha, W. R. (2006). *In vivo* analgesic and anti-inflammatory activities of ursolic acid and oleanoic acid from *Miconia albicans* (Melastomataceae). *Zeitschrift für Naturforschung C, 61*(7–8), 477–482.

Velasco-Lezama, R., Tapia-Aguilar, R., Román-Ramos, R., Vega-Avila, E., & Pérez-Gutiérrez, M. S. (2006). Effect of *Plantago major* on cell proliferation *in vitro*. *Journal of Ethnopharmacology, 103*(1), 36–42.

Zubair, M., Ekholm, A., Nybom, H., Renvert, S., Widen, C., & Rumpunen, K. (2012). Effects of *Plantago major* L. leaf extracts on oral epithelial cells in a scratch assay. *Journal of Ethnopharmacology, 141*(3), 825–830.

Zubair, M., Nybom, H., Lindholm, C., Brandner, J. M., & Rumpunen, K. (2016). Promotion of wound healing by *Plantago major* L. leaf extracts – *Ex-vivo* experiments confirm experiences from traditional medicine. *Natural Product Research, 30*(5), 622–624.

Zubair, M., Nybom, H., Lindholm, C., & Rumpunen, K. (2011). Major polyphenols in aerial organs of greater plantain (*Plantago major* L.), and effects of drying temperature on polyphenol contents in the leaves. *Scientia Horticulturae, 128*(4), 523–529.

1-S20-_1.PDF

ORIGINALITY REPORT

12%	8%	9%	2%
SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS

PRIMARY SOURCES

1	atvb.ahajournals.org Internet Source	<1%
2	Nathalie Alépée, Marie-Hélène Grandidier, José Cotovio. "Usefulness of the EpiSkin™ reconstructed human epidermis model within Integrated Approaches on Testing and Assessment (IATA) for skin corrosion and irritation", Toxicology in Vitro, 2019 Publication	<1%
3	Zeineb Mzoughi, Hatem Majdoub. "Pectic polysaccharides from edible halophytes: Insight on extraction processes, structural characterizations and immunomodulatory potentials", International Journal of Biological Macromolecules, 2021 Publication	<1%
4	Zhang, Qi, Chi Chun Fong, Wai Kin Yu, Yao Chen, Fan Wei, Chi Man Koon, Kit Man Lau, Ping Chung Leung, Clara Bik San Lau, Kwok Pui Fung, and Mengsu Yang. "Herbal formula Astragali Radix and Rehmanniae Radix	<1%

exerted wound healing effect on human skin fibroblast cell line Hs27 via the activation of transformation growth factor (TGF-β) pathway and promoting extracellular matrix (ECM) deposition", Phytomedicine, 2012.
Publication

---

5 G.P. Dillon, A. Yiannikouris, C.A. Moran. "Toxicological evaluation of a glycan preparation from an enzymatic hydrolysis of Saccharomyces cerevisiae", Regulatory Toxicology and Pharmacology, 2021
Publication

<1 %

---

6 Riazul Haque Tuhin, Mst. Marium Begum, Md. Sohanur Rahman, Rubaba Karim et al. "Wound healing effect of Euphorbia hirta linn. (Euphorbiaceae) in alloxan induced diabetic rats", BMC Complementary and Alternative Medicine, 2017
Publication

<1 %

---

7 mdpi.com
Internet Source

<1 %

---

8 Submitted to Bronx High School of Science
Student Paper

<1 %

---

9 Rupesh Thakur, Nitika Jain, Raghvendra Pathak, Sardul Singh Sandhu. "Practices in Wound Healing Studies of Plants", Evidence-Based Complementary and Alternative Medicine, 2011

<1 %

	Publication	
10	Www.oecd.org Internet Source	<1 %
11	livrepository.liverpool.ac.uk Internet Source	<1 %
12	www.pvj.com.pk Internet Source	<1 %
13	www.wjgnet.com Internet Source	<1 %
14	Ah, Y.C.. "A novel transdermal patch incorporating meloxicam: In vitro and in vivo characterization", International Journal of Pharmaceutics, 20100129 Publication	<1 %
15	Satish Patel, Shikha Srivastava, Manju Rawat Singh, Deependra Singh. "Mechanistic insight into diabetic wounds: Pathogenesis, molecular targets and treatment strategies to pace wound healing", Biomedicine & Pharmacotherapy, 2019 Publication	<1 %
16	Submitted to Universitas Sebelas Maret Student Paper	<1 %
17	www.innspub.net Internet Source	<1 %
	Submitted to Birla Institute of Technology	

18	Student Paper	<1 %
19	joe.bioscientifica.com Internet Source	<1 %
20	www.fs.fed.us Internet Source	<1 %
21	Kotíková, Zora, Miloslav Šulc, Jaromír Lachman, Vladimír Pivec, Matyáš Orsák, and Karel Hamouz. "Carotenoid profile and retention in yellow-, purple- and red-fleshed potatoes after thermal processing", Food Chemistry, 2016. Publication	<1 %
22	Monika Lazarová, Juraj Lábaj, Peter Eckl, Grigorij Kogan, Darina Slameňová. "Effects of Dietary Intake of a Fungal β-D-Glucan Derivative on the Level of DNA Damage Induced in Primary Rat Hepatocytes by Various Carcinogens", Nutrition and Cancer, 2006 Publication	<1 %
23	Submitted to Queen Mary and Westfield College Student Paper	<1 %
24	Ziwen Liu, Yumei Fan, Yu Wang, Cui Han, Yu Pan, Huang Huang, Ying Ye, Lan Luo, Zhimin Yin. "Dipyrithione inhibits lipopolysaccharide-	<1 %

induced iNOS and COX-2 up-regulation in macrophages and protects against endotoxic shock in mice", FEBS Letters, 2008
Publication

---

25 Dong-Kyoo Kim, Jin Hyen Baek, Chang-Mo Kang, Mi-Ae Yoo et al. "Apoptotic activity of ursolic acid may correlate with the inhibition of initiation of DNA replication", International Journal of Cancer, 2000
Publication

<1 %

---

26 Elaine Reina, Nouf Al-Shibani, Eman Allam, Karen S. Gregson, Michael Kowolik, L. Jack Windsor. "The Effects of Plantago major on the Activation of the Neutrophil Respiratory Burst", Journal of Traditional and Complementary Medicine, 2013
Publication

<1 %

---

27 Min Jung Kwon, He Min Shin, Haribalan Perumalsamy, Xue Wang, Young-Joon Ahn. "Antiviral effects and possible mechanisms of action of constituents from Brazilian propolis and related compounds", Journal of Apicultural Research, 2019
Publication

<1 %

---

28 oalib.com
Internet Source

<1 %

---

29 Fatima Benaoun, Cédric Delattre, Zakaria Boual, Alina V. Ursu et al. "Structural

<1 %

characterization and rheological behavior of a heteroxylan extracted from Plantago notata Lagasca (Plantaginaceae) seeds", Carbohydrate Polymers, 2017
Publication

30	Kumar, B.. "Ethnopharmacological approaches to wound healing-Exploring medicinal plants of India", Journal of Ethnopharmacology, 20071101 Publication	<1 %
31	Nina Dewi Oktaviyanti, Kartini, Abdul Mun'im. "Application and optimization of ultrasound-assisted deep eutectic solvent for the extraction of new skin-lightening cosmetic materials from Ixora javanica flower", Heliyon, 2019 Publication	<1 %
32	Thongchai Taechowisan, Chunhua Lu, Yuemao Shen, Saisamorn Lumyong. "Anti-inflammatory Effects of 4-Arylcoumarins in LPS-induced Murine Macrophage RAW 264.7 Cells", Pharmaceutical Biology, 2008 Publication	<1 %
33	app.trdizin.gov.tr Internet Source	<1 %
34	tel.archives-ouvertes.fr Internet Source	<1 %

35	www.botanicalauthentication.org Internet Source	<1 %
36	"Poster Presentation - Others : Abstract", Respirology, 2014. Publication	<1 %
37	Adetutu, A.. "Ethnopharmacological survey and in vitro evaluation of wound-healing plants used in South-western Nigeria", Journal of Ethnopharmacology, 20110901 Publication	<1 %
38	Luming Wan, Huan Yang, Huilong Li, Jing Gong et al. "GP73 is a glucogenic hormone regulating SARS-CoV-2-induced hyperglycemia", Cold Spring Harbor Laboratory, 2021 Publication	<1 %
39	Samuelsen, A.B.. "The traditional uses, chemical constituents and biological activities of Plantago major L. A review", Journal of Ethnopharmacology, 200007 Publication	<1 %
40	brage.bibsys.no Internet Source	<1 %
41	insight.jci.org Internet Source	<1 %
42	rep.bioscientifica.com Internet Source	<1 %

43	scitepress.org Internet Source	<1 %
44	theses.gla.ac.uk Internet Source	<1 %
45	www.carahealth.com Internet Source	<1 %
46	www.journal.uad.ac.id Internet Source	<1 %
47	www.repository.cam.ac.uk Internet Source	<1 %
48	www.semanticscholar.org Internet Source	<1 %
49	Furtado, R. A., E. P. Rodrigues, F. R. R. Araujo, W. L. Oliveira, M. A. Furtado, M. B. Castro, W. R. Cunha, and D. C. Tavares. "Ursolic Acid and Oleanolic Acid Suppress Preneoplastic Lesions Induced by 1,2-Dimethylhydrazine in Rat Colon", Toxicologic Pathology, 2008. Publication	<1 %
50	Zahra Zamani, Seyed M.A. Razavi. "Physicochemical, rheological and functional properties of Nettle seed (Urtica pilulifera) gum", Food Hydrocolloids, 2020 Publication	<1 %
51	ejournal.uin-malang.ac.id Internet Source	<1 %

52	eprints.skums.ac.ir Internet Source	<1 %
53	ijarnp.org Internet Source	<1 %
54	revistas.ufcg.edu.br Internet Source	<1 %
55	Ahmed F. Al-obaidi. "Chapter 7 Phytotoxicity of Extracts on Germination and Seedling Growth of Purslane ( ) ", IntechOpen, 2020 Publication	<1 %
56	journals.iau.ir Internet Source	<1 %

Exclude quotes	Off	Exclude matches	< 4 words
Exclude bibliography	On		