BJP

Bangladesh Journal of Pharmacology

Research Article

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A Journal of the Bangladesh Pharmacological Society (BDPS)
Journal homepage: www.banglajol.info
Abstracted/indexed in Academic Search Complete, Asia Journals Online, Bangladesh Journals Online, Biological Abstracts, BIOSIS Previews, CAB Abstracts, Current Abstracts, Directory of Open Access Journals, EMBASE/Excerpta Medica, Global Health, Google Scholar, HINARI (WHO), International Pharmaceutical Abstracts, Open J-gate, Science Citation Index Expanded, SCOPUS and Social Sciences Citation Index;
Bangladesh J Pharmacol 2018; 13: 106-113
ISSN: 1991-0088

Labisia pumila improves wound healing process in ovariectomized rat model

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Article Info

Received: 7 November 2017
Accepted: 26 March 2018
Available Online: 30 March 2018

DOI: 10.3329/bjp.v13i1.34473

Cite this article:
Ahmad SU, Shuid AN, Mohamed IN. *Labisia pumila* improves wound healing process in ovariectomized female rat model. Bangladesh J Pharmacol. 2018; 13: 106-13.

Abstract

The purpose of this study was to determine the wound healing effect of *Labisia pumila* in the ovariectomized rat model. Ninety-nine Sprague Dawley female rats equally divided into nine groups; where five were control groups, and four were treated groups. The dressing was changed daily, starting from the wound induction until complete healing. The percentage of wound contraction was measured on day 0, 2, 5, 8, 9, 10, 11, 12 and 13. Three rats were sacrificed from each group on day 2, 5 and 8 respectively for evaluating of histological assessments. Wounds dressed with extract showed considerable healing and significantly healed faster compared to all control groups ($p<0.05$). Moreover, histological analysis revealed remarkable reduction in the scar width correlated with the enhanced collagen content and fibroblast cells, accompanied by a reduction of inflammatory cells in the granulation tissues. In conclusion, *L. pumila* may promote wound healing in postmenopausal rat model.

Introduction

Labisia pumila (Blume) Fern.-Vill. synonym *Marantodes pumilum* (Blume) Kuntze is a flowering plant of the Primulaceae family. It is used traditionally by the Malay women to shrink the uterus, facilitate labor, improve menstrual irregularities and as post-partum medicine (Runi, 2000). The ethnopharmacological studies and clinical findings have illustrated that *L. pumila* possesses many biological functions, including phytoestrogenic, antimicrobial, anti-oxidative, anti-inflammatory properties and anti-aging (Choi et al., 2010; Nadia et al., 2012). Recent studies on *L. pumila* also demonstrated that it could also be used for osteoporosis, cardiovascular diseases, and metabolic disorders (Shuid et al., 2011). The ethnobotanical and therapeutic properties of *L. pumila* make it a potential source of novel medicine for the healing of wounds. Therefore, this study investigates the effectiveness of topical application of the aqueous extract of *L. pumila* on the rate of wound closure and the histology of the wound area.

Materials and Methods

Plant collection and extraction

The plant species were collected from their natural habitat in the rainforest of Taiping, Perak, Malaysia in October, 2014. Identification of the plant species was verified by a botanist from the Faculty of Science, Universiti Kebangsaan Malaysia (UKM). All voucher specimens were deposited in the Herbarium of UKM (voucher specimen number of *L. pumila* var. alata = UKMB 30006/SM 2622 and *var. pumila* = UKMB 30007/SM s.n.). The standardized aqueous extraction method was used according to Jamal et al. (2003) with minor modification to obtain extracts of leaf and roots of *L. pumila var. pumila* and *L. pumila var. alata*. This resulted in four standardized aqueous extracts, two for each species.

Ointment preparation

Plant extract materials were grinded by mortar and pestle to make it into a fine powder. Dried extract powder and cetomacrogol emulsifying ointment (Hovid Berhad, Malaysia) used as a vehicle were weighted for two different concentrations of 1.0% and 2.0%. 1.0% concentration was prepared for *L. pumila var. pumila* extracts and 2.0% concentration for *L. pumila var. alata* extracts. Concentrations of powder extracts were selected based on pilot study. The ointment was put on a cleaned glass plate followed with extract powder and mixed uniformly by spatula. Finally, the mixed extract ointment was put into a jar and covered properly.

Animals and treatment

Female Sprague-Dawley rats, aged 3–5 months and weighing between 200-250 g were obtained from the Universiti Kebangsaan Malaysia Laboratory Animal Research Unit. The rats were housed in plastic cages under standard environment at room temperature and natural day/night cycle. They were fed with commercial food pellets (Gold Coin, Port Klang, Malaysia) and deionized water *ad libitum*. They were allowed to acclimate to the laboratory conditions for a week before ovariectomized was done. After ovariectomized of rats, they were kept for a minimum of two weeks to observe for estrogen deficiency state (Kalu, 1991). Mixing solution of ketamine (100 mg/mL) and xylazil (20 mg/mL) as 1:1 ratio were injected intraperitoneally to anesthetize the rats before all surgical procedures (Ibrahim et al., 2014). After two weeks ovariectomy observation, wound treatment of rats was done by using excision wound model (Latif et al., 2015). Four full skin thickness wounds were made bilaterally using biopsy punch with 6 mm diameter on the dorsal surface of each rat. A total ninety-nine rats was equally divided into nine groups: Non-ovariectomized sham operated (SH), ovariectomized control (OC), ovariectomized and treated with vehicle dressing (OV), ovariectomized and treated with flavine dressing (OF), ovariectomized and treated with estrogen (OE), ovariectomized and treated with *L. pumila var. pumila*, leaf extract (PL), ovariectomized and treated with LPvp, root extract (PR), ovariectomized and treated with LPva, leaf extract (AL) and ovariectomized and treated with LPva, root extract (AR). The rats of SH and OC groups did not receive any treatment and OC group acts as a negative control. Three positive control groups including OV, OF and OE groups were treated with vehicle only (cetamacrogol emulsifying ointment), flavin (acriflavine) and estrogen (17β estradiol) respectively. Of the remaining four treatment groups; PL, PR, AL and AR groups were treated with 1.0% of aqueous extract of LPvp leaf and root and 2.0% of aqueous extract of LPva leaf and root respectively. The dressing was changed daily, starting from the wound induction until complete healing. All treatments were administered topically once a day with 0.1 gm on every wound. Three rats were sacrificed from each group on day 2, 5 and 8 respectively. After sacrificed, wounded skin tissue was taken for the histological analysis.

Microscopic observation and wound contraction measurement

The wound size was measured on 0, 2, 5, 8, 9, 10, 11, 12 and 13 post-wounding day and at the same time, the photograph of wounded skin was taken of each rat for macroscopic observation. Wounds were measured using digital caliper (General, China) in mm according to clock method (Fernandes et al., 2015).

Histological analysis

The skin biopsy 1.0 x 1.0 cm was excised from the center of the wound area and fixed in 10% neutral buffer formalin for histological analysis. After fixation, skin tissues were dehydrated through graded alcohol series, cleared in xylene, tissue infiltration and embedded in paraffin wax. The tissues were sectioned 5 µm thick perpendicular to the wound. These were dewaxed in xylene and followed by staining with standard hematoxylin and eosin (H & E) and Masson-Goldner trichrome (MGT). The section of the skin was stained with Hematoxylin and Eosin for general morphological observations and Masson's Trichrome for detection of collagen fibers. Slides observed under light microscope (Olympus CX31, Philippines) and photomicrographs were taken for examinations. Stained sections were scored by histological expert, Dr. Elvy Suhana Mohd Ramli (Anatomy, PPUKM) in a blind fashion using the modified 0 to 3 numerical scale as described by Abramov et al. (2007) (Table I).

Statistical analysis

Experimental results are presented as means ± SD of eight animals in each group. Statistical analysis was performed by using SPSS (23 version) statistical software. The effect of extracts on the wound was analyzed by one-way ANOVA test. P values ≤0.05 were considered significant.

Results

Macroscopic observation of wound

Figure 1 shows the macroscopic view of wounds of nine ovariectomized female rat experimental groups: SH, OC, OV, OF, OE, PL, PR, AL and AR on day 0, 2, 5, 8, 9, 10, 11, 12 and 13. In the macroscopic view, wounds treated with *L. pumila* extracts (PL, PR, AL and AR) healed on day 9, whereas, three control groups such as SH, OC and OV healed on the day 11. Wounds treated

Table I

Scoring for histomorphological study of skin tissue

Variable	Scoring			
	0	1	2	3
Re-epithelialization	None	Partial	Complete but immature or thin	Complete and mature
Inflammation cell infiltration	None	Scant	Moderate	Abundant
Fibroblast proliferation	None	Scant	Moderate	Abundant
Neo-vascularization	None	Up to five vessels per HPF (High-Power Field)	6–10 vessels per HPF	More than 10 vessels per HPF
Granulation tissue formation	Immature	Mild maturation	Moderate maturation	Fully matured
Collagen deposition	None	Scant	Moderate	Abundant

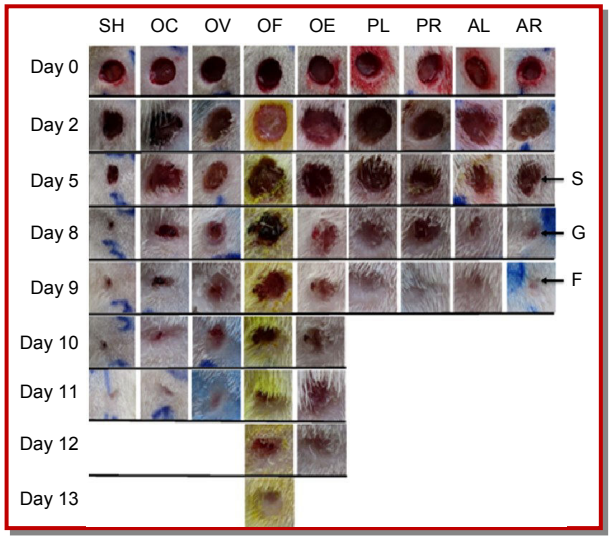

Figure 1: Macroscopic view of wounds of nine ovariectomized female rat experimental groups: SH, OC, OV, OF, OE, PL, PR, AL and AR against treatment day 0, 2, 5, 8, 9, 10, 11, 12 and 13. S = Scab, G = Granulation tissue and F = Fibrous tissue

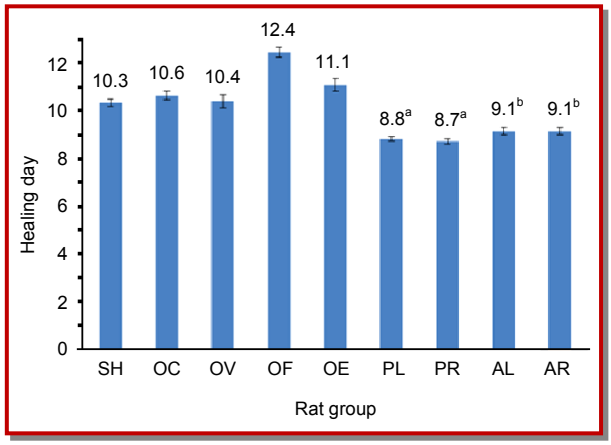

Figure 2: Bar chart represents the mean value of wounds healed day of nine ovariectomized female experimental groups: SH, OC, OV, OF, OE, PL, PR, AL and AR. Statistically significant results indicated as [a]p<0.001 versus control groups (SH, OC, OV, OF and OE) and [b]p<0.05 versus control groups (SH, OC, OV, OF and OE)

with estrogen (OE) and flavine (OF) healed on day 12 and 13 respectively. On day 2, wounds treated with LP extracts showed dried wound and on day 5, the formation of a scab. The scab starts to drop off on day 8 for wounds treated with all *L. pumila* extracts. After the falling off of scab, granulation tissue characterized by pinkish color (day 8) could be seen and slowly replaced by fibrous tissue that was whitish (day 9). In contrast, granulation tissue in control groups is seen later at day 9-10 and fibrous tissue seen on day 11-13.

Determination of wound contraction

Figure 2 shows the mean value of wounds healed day for nine ovariectomized female experimental groups: SH, OC, OV, OF, OE, PL, PR, AL and AR. In the figure, wounds dressed with extracts healed significantly faster compared to all control groups. On the complete healed day, the length to complete healed wound in the treated groups with *L. pumila var. pumila* ($p<0.001$) and LPva ($p<0.05$) were significantly faster than control groups (SH, OC, OV, OF and OE). There were no significant differences among three control groups (SH, OC and OV) and among the four treated groups (PL, PR, AL and AR).

Histopathological examinations

The scores obtained from the histological evaluation of nine experimental groups by H & E and MGT staining (Table II). The histopathological view of skin wound of H & E and MGT stain is illustrated in Figure 3 and Figure 4 respectively. Histological findings in re-epithelialization, the proliferation of fibroblast cells, neovascularization, granulation tissue formation and collagen deposition in each group were showed similar results, whereas, inflammatory cell infiltration was antipodal. At the early stage, all parameters of skin histology obtained by H & E stain except inflammatory

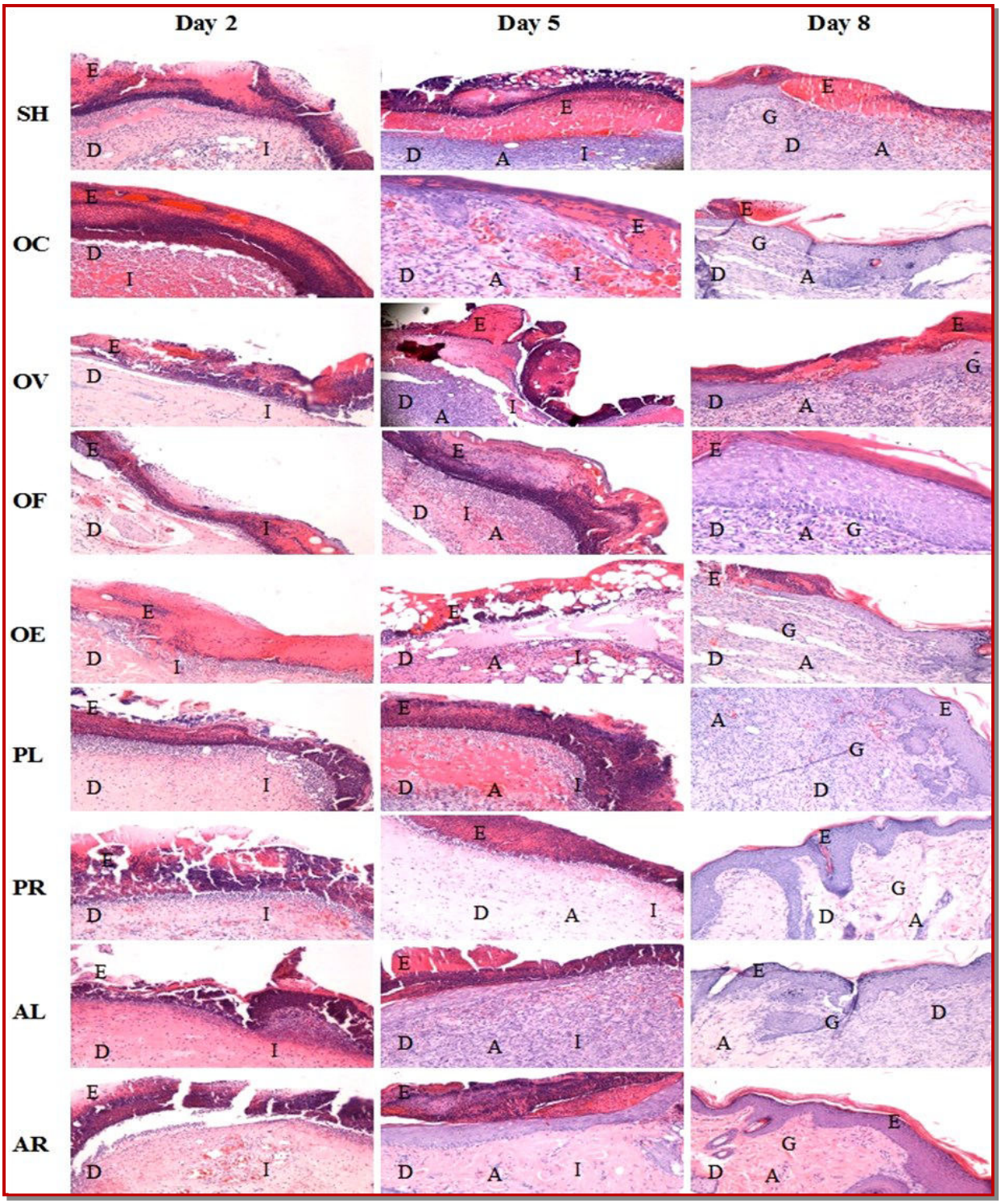

Figure 3: Histopathological view of skin wound by H & E staining of nine ovariectomized female experimental groups: SH, OC, OV, OF, OE, PL, PR, AL and AR at day 2, day 5 and day 8 after the formation of the wound. Pictures of stain are at x10 magnification. Alphabets indicate the events during wound healing; E = Epidermis, D = Dermis, I = Inflammatory cells, A = Angiogenesis, G = Granulation tissue

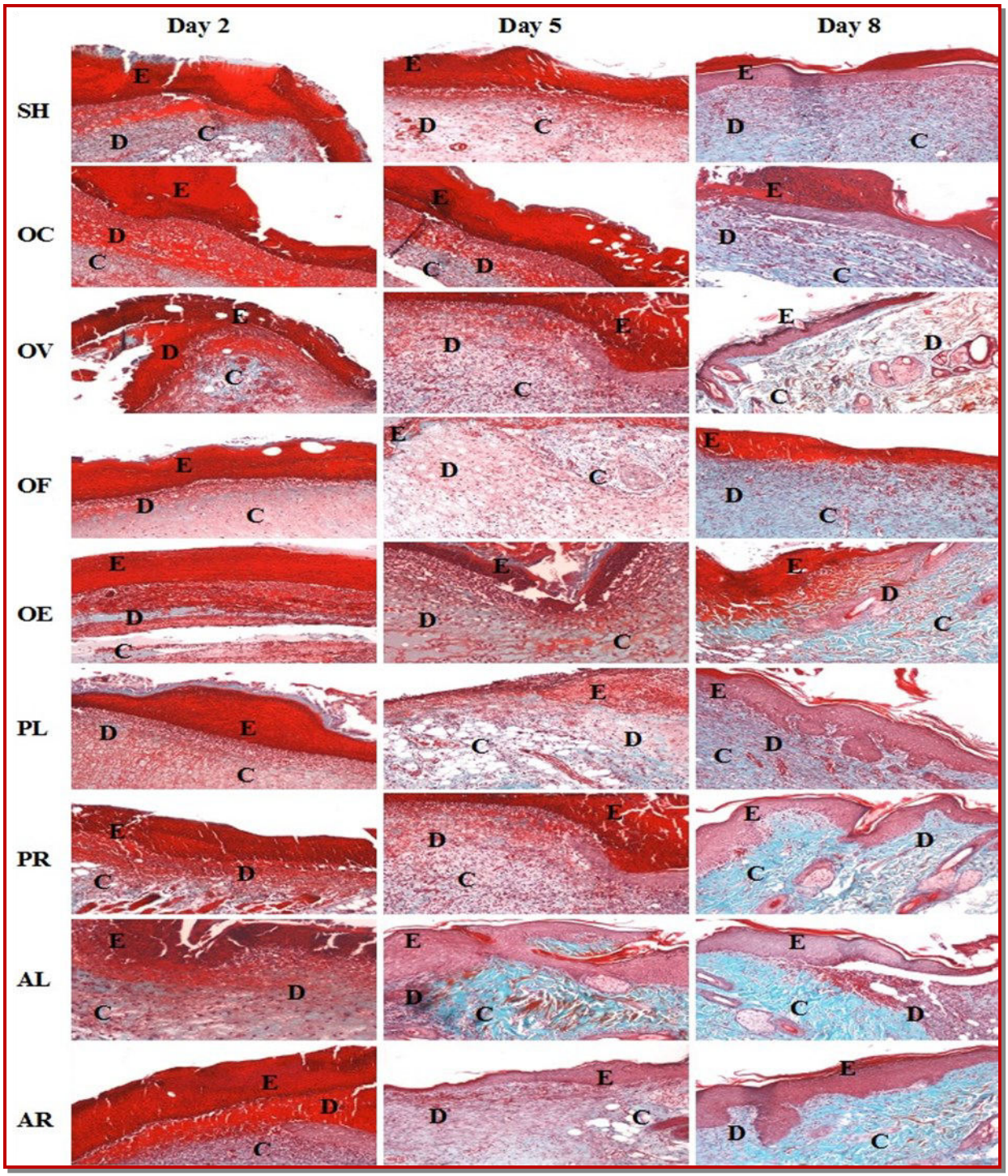

Figure 4: Histopathological view of collagen deposition in wounded skin tissue evaluated by MGT staining of nine ovariectomized female experimental groups: SH, OC, OV, OF, OE, PL, PR, AL and AR at day 2, day 5 and day 8 after the formation of the wound. Pictures of stain are at x10 magnification. Arrows pointing indicate the events during wound healing; E = Epidermis, D = Dermis and C = Collagen

Table II

Score from histological observations of nine experimental groups: SH, OC, OV, OF, OE, PL, PR, AL and AR at day 2, day 5 and day 8 after the formation of the wound

Groups	Re-epithelialization			Inflammatory cell infiltration			Fibroblast cell proliferation			Neo-vascularization			Granulation tissue formation			Collagen deposition		
Day	2	5	8	2	5	8	2	5	8	2	5	8	2	5	8	2	5	8
SH	0	1	2	2	2	1	1	2	2	1	2	3	1	2	2	1	2	2.5
OC	0	1	3	2	2	1	1	2	2	0	2	2	1	2	2	0	2	2
OV	1	1	3	2	1	1	1	2	3	1	1	2	1	2	3	1	2	2
OF	0	1	2	2	2	1	1	2	2	0	1	2	0	1	2	0	1	2
OE	0	1	3	2	1	0	1	1	2	1	1	2	1	1	2	1	2	2.5
PL	1	1	3	2	1	1	1	2	2	1	2	3	1	1	3	1	2	3
PR	0	1	3	2	1	1	1	2	3	1	2	3	1	1	3	1	2	3
AL	1	2	3	2	1	0	1	2	2	1	2	3	1	2	3	1	2	3
AR	1	3	3	2	1	1	1	2	2	1	1	2	1	2	3	1	2	3

cell infiltration were immature, and the score was around 0-1. On day 5, the cells were abundant, but not mature. At a later stage of the healing process, the cells were abundant and mature as well. The score was approximately 2-3. In contrast, the score of inflammatory cell infiltration followed the opposite trend. On day 2, the score of inflammatory cells was 2 and the score was 1 at a later stage. However, from the histopathological view of skin wound stained with MGT, collagen deposition was weak, and scores were 1 in all groups. Collagen deposition increased with time. Though collagen was slightly increased on day 5; it was still premature. Collagen was deposited rapidly after day 5. Collagen was abundant and prominent in all treated groups on day 8, however, there were more mature collagen in the treated groups.

Discussion

Wound contraction is an essential process in healing which leads to wound closure. It depends on the ability to repair damaged tissue and the angiogenesis process, the type and extent of tissue damage and the general condition of the tissue itself (Priya et al., 2004). Thus, visible appearances and measurements of wound contraction become reliable parameters in macroscopic evaluation for wound healing (Gal et al., 2008). Results obtained from our macroscopic study emphatically agree with our initial assessment. *L. pumila* stimulate the surrounding cells of the wound to enhance the proliferation during wound healing process (Usui et al., 2013). Though wounds treated with *L. pumila* on day 2 and day 5 showed less wound healing process compared to the normal control group, the healing process was more rapid after day 5. During the proliferation phase, wounds treated with *L. pumila* contract more rapidly and possessed a better wound healing effect. The results of our observation correlates very well with the anti-inflammatory properties of *L. pumila*. During the second phase of wound healing which is the inflammatory phase (day 2 to 5), wound healing may appear to have slowed due to the inflammation (Korkina et al., 2006). The reduction of inflammation is not up to marked as to reduce the healing time of wounds and have beneficial effects of reducing redness, swelling, and pain during the early stage of wound healing.

In dermal tissues, shortly after the formation of a stable clot, inflammatory cells infiltrate at the injured site (Henry and Garner, 2003). The inflammatory cells would create an environment in which macrophage and neutrophils infiltrates remove necrotic tissue, debris, and bacterial contaminants as well as secreting inflammatory mediators and growth factors that will activate fibroblasts. The positive proliferative properties of *L. pumila* start to work on day 5 and the closure of wound accelerates from here on. The net effect of *L. pumila* is reduction in swelling, redness, and pain due to wounds while expedites the time for wound closure.

A study by Jamal et al. (2003) identified the phytoestrogens constituents in this plant that is chemically similar to estrogen. This study use the ovariectomized female rat model because our initial hypothesis was the phytoestrogens would be the main component stimulating wound healing through increase proliferation. We postulated the positive control estrogen dressing group will demonstrate similar results to the treated groups. However, this was not the case. The estrogen treated control group was not significantly different compared to the other positive or negative control groups. Therefore, we postulate that estrogen and phytoestrogens may not play a vital role in wound healing process as initially postulated. This reinforce that there may be inherent properties or other active compounds in *L. pumila* that is beneficial for the wound healing process. The result of flavine dressing

was worse than the normal healing process. Flavine usually is used for the treatment of minor wounds, infected wounds or minor burns. However, prolonged treatment of flavine may delay the healing of wounds. This delay in wound healing may be due to flavine being a disinfectant and therefore kill new immature cells while having no inherent proliferative properties. As a comparison, *L. pumila* is an excellent wound healing agent with proliferative properties and has inherent antimicrobial properties to prevent wound infection (Agra et al., 2013).

The histological analysis further revealed that wounds treated with *L. pumila* extracts significantly increased in re-epithelialization, fibroblast cells growth, angiogenesis, and granulation tissue formation at the later stage of wound healing process (day 8), whereas, the inflammatory cells infiltration decreased at the same time. During the proliferative phase of wound healing, wound contraction enhances closure of the defect by pulling the edges of the wound towards the center (Tang et al., 2007). Contraction facilitates re-epithelization by migrating keratinocytes in the epidermis layers (Strodtbeck, 2001). Collagen is a major component of connective tissue. Fibroblast cells produce the collagen. Collagen deposition is important because it increases the strength of the wound. It acts as a structural scaffold in skin connective tissues. It controls many cellular functions, including cell shape and differentiation, migration, and synthesis of some proteins (Hynes, 1992). The findings in the study demonstrated that collagen had more density at the late stage in the wound healing process. The MGT analysis showed the collagen deposition was higher in the *L. pumila*-treated groups compared to the control groups. Infection can seriously delay healing process by causing impaired epithelization, poor quality granulation tissue formation, lower vascularization and reduced tensile strength of connective tissue (Annan and Houghton, 2008). Therefore, biological properties of *L. pumila* has a very important role in facilitating wound healing (Nagori and Solanki, 2011). Higher scoring of all parameters in *L. pumila* extracts treated groups compared to the control groups indicated *L. pumila* has a very good potential application as a wound healing agent.

Conclusion

In the ovariectomized female rat model, *L. pumila*-treated groups healed faster by up to 30% compared to the standard treatment (flavine). There was no positive effect with estrogen dressing compared to the normal healing (SH) and negative control (OC) groups. The histological analysis of all *L. pumila*-treated groups showed better re-epithelialization, angiogenesis, accompanied with an abatement of inflammatory cells in the granulation tissues. *L. pumila*-treated wounds significantly healed faster compared with all other control groups and therefore demonstrate huge potential as an effective wound healing agent.

Ethical Issue

The study was approved by Universiti Kebangsaan Malaysia Animal Ethics Committee (ethical approval number: FP/FAR/2014/ISA/26-NOV./637-JAN.-2015-DEC.-2016).

Conflict of Interest

The authors declare to have no conflict of interests whatsoever.

Acknowledgement

Authors would like to thank the Faculty of Medicine, Universiti Kebangsaan Malaysia for providing the resources and funding, the NRGS grant (NH1014D034) by the Ministry of Agriculture and Agro-based Industry, Malaysia to conduct the research.

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