The Dose Dependence Analysis of the Water Fraction of *merremia mammosa (lour.)* Extract on Diabetic wound Healing Enhancement

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Table of Content

Hiroshima Journal of Medical Sciences Volume 67

sort: ID (asc)

Antioxidant Activity and Soluble Protein Content of Tempeh Gembus Hydrolysate

creator: Agustina, Rizki Kurnianti, Diany, Filah Fithria, Rustianti, Nindi, Anjani, Gamelia, Arifah, Diana Nur
journal title: Hiroshima Journal of Medical Sciences
volume: Volume 67
date of issued: 2015-03

Effect of Remote Ischemic Preconditioning on the Periprocedural Myocardial Injury Events during Elective Percutaneous Coronary Intervention

creator: Anggiyani, N. U. Samudin, Rifqi, S.
journal title: Hiroshima Journal of Medical Sciences
volume: Volume 67
date of issued: 2015-03

P-Selectin as Predictor Venous Thromboembolism in Cancer Patients Undergoing Chemotherapy

creator: Suhani, C., Santosa, Pangarso, Eko Adhi, Setiawan, Budi, Samekto, Gatot Hadi
journal title: Hiroshima Journal of Medical Sciences
volume: Volume 67
date of issued: 2015-03

The Impact of Antenatal Coping Skill Training (ACST) towards Cortisol and IgG Serum Level among Pregnant Women

creator: Runjati, Susanto, Hardhono, Savitri, Dian R., Thaufik, Syarif
journal title: Hiroshima Journal of Medical Sciences
volume: Volume 67
date of issued: 2015-03

The Dose Dependence Analysis of the Water Fraction of merremia mammosa (lour.) Extract on Diabetic wound Healing Enhancement

creator: Marchiani, Ancah Caesarina Novi, Ulfa, Evi Umayah, Sakinah, Elly Nurus
journal title: Hiroshima Journal of Medical Sciences
volume: Volume 67
date of issued: 2015-03

The Efficacy of Education with the WHO Dengue Algorithm on Correct Diagnosing and Triaging of Dengue-Suspected Patients: Study in Public Health Centre

creator: Pyl Pauwels, Patrick, Fm Meisenmakers, Job, Himawan, An Budi, Kristina, Tri Nur
journal title: Hiroshima Journal of Medical Sciences
volume: Volume 67
date of issued: 2015-03

The Effect of Green Tea Epigallocatechin-3-Gallate on Spatial Memory Function, Malondialdehyde and TNF-α Level in D-Galactose-Induced BALB/C Mice

creator: Gumay, Airun Rahmasari, Bikri, Syaikhul, Pudjonarko, Dwi, Supriharti
journal title: Hiroshima Journal of Medical Sciences
volume: Volume 67
date of issued: 2015-03

Muntingia calabura Leaves Extract, a Potential Gastroprotective Agent against Gastric Mucosal Damage Induced by Soft Drink and Alcoholic Beverages

creator: Nadirah, Ummi Chamidatun, Beturusman, Dimas, Ilalasi, Hermawan, Utomo, Astika Widy
journal title: Hiroshima Journal of Medical Sciences
volume: Volume 67
date of issued: 2018-03

Effect of Mangosteen (Garcinia mangostana) PEEL Extract towards CD4+, CD8+ T LYMPHOCYTES, CD38 Expression, NK Cells, IL-2 and IFNγ in HIV Patients with Antiretroviral Therapy

creator: Amanah, Amanah; Komala, Ika; Kumasari, Maria O; Charmana, Edi; Gasem, M. Hussein
journal title: Hiroshima Journal of Medical Sciences
volume: Volume 67
date of issued: 2018-03

Effects of Physical-Cognitive Therapy (PCT) on Critically Ill Patients in Intensive Care Unit

creator: Suwardianto, Heru; Prasetyo, Aviat; Utami, Rani Sulung
journal title: Hiroshima Journal of Medical Sciences
volume: Volume 67
date of issued: 2018-03

The Effectiveness of Merremia mammosa (Lour.) Extract Fractions as Diabetic Wound Healers on Diabetic Rat Model

creator: Sakinah, Elly Nurus; Ulfa, Evi Umayah; Marchianti, Ancah Caesarina Novi
journal title: Hiroshima Journal of Medical Sciences
volume: Volume 67
date of issued: 2018-03

Positive Correlation between Ferritin and Activated Monocyte in Iron Overloaded Major β-thalassemia Patients

creator: Ghozali, M.; Anggia, M. Fariz; Tjahjadi, Adi Imam; Rahayu, Latari; Ghrahani, Rini; Syamsunarno, MRAA.; Setiabudiawan, Budi; Pangaribuan, Ramdan
journal title: Hiroshima Journal of Medical Sciences
volume: Volume 67
date of issued: 2018-03

The Sensitivity of Human Breast Cancer Stem Cells (ALDH+) Against Doxorubicin Treatment is Associated with PCNA and BIRC5 Gene Expressions

creator: Dewi, Syarifah; Syamsiah, Reeza Akhria; Sadikin, Mohammad; Wanandi, Septelia Inawati
journal title: Hiroshima Journal of Medical Sciences
volume: Volume 67
date of issued: 2018-03

Effects of Engineered Stimulation of Oxytocin on Hormonal Status of Postpartum Women

creator: Anggorowati, Anggorowati; Susilowati, Dwi; Zubaidah
journal title: Hiroshima Journal of Medical Sciences
volume: Volume 67
date of issued: 2018-03

The Correlation between Serum Vascular Endothelial Growth Factor (VEGF) Levels and Size of Colorectal Cancer Tumors

creator: Pangaribuan, Elko A.; Suharti, C. Santoso; D. Setiawan, Budi; Utthofah, Anq
journal title: Hiroshima Journal of Medical Sciences
volume: Volume 67
date of issued: 2018-03

TNF - α Gene Polymorphism is Likely to be a Risk Factor for NASH in Indonesia

creator: Purnomo, Hary Dagat; Sp Mundhofir, Fahmadiya; Kasno, Sudjarno; Edi, Darmono; Dadiyono, Mh Farazz; Suhana
journal title: Hiroshima Journal of Medical Sciences
volume: Volume 67
date of issued: 2018-03

The Effect of Stress on IL-17 Levels in an OVA-Immunized Mice Allergic Model

creator: Santosa, Yanuar Iman; Supriadi; Charmana, Edi
journal title: Hiroshima Journal of Medical Sciences
volume: Volume 67
date of issued: 2018-03

Correlation between HPV Vaccination and Cervical Cancer Incidence in Southeast Asian Population

creator: Lestari, Sheila; Kuno, Mika; Fadlolia, Gaofanny; Aisyah, Siti; Kemal, Rahmat Azhari; Agusta, Isnjoman; Sumargo, Anton
journal title: Hiroshima Journal of Medical Sciences
volume: Volume 67
date of issued: 2018-03

Polymorphism in 4'-UTR Region of PITX2 in Vertical Mandibular Symmetry

creator: Sofyanti, Ervina; Boel, Thella; Soegiharto, Benny; Nasruddin, Ilyas; Syafruddin, Bachtiar; Hanna; Bachtiar, Adang; Auerkari, Eiza Ibrahim
journal title: Hiroshima Journal of Medical Sciences
volume: Volume 67
date of issued: 2018-03

Effect of Chronic Organophosphate Poisoning on Attention Deficit and Memory Impairment

creator: Helmi, Irsana Andriani; Emi; Gumay, Airun Rahmasari; Bakri, Saekhol; Maharani, Nani; Munirah, Multhatul; Bakhtiar, Yunir; Herdian
journal title: Hiroshima Journal of Medical Sciences
volume: Volume 67
date of issued: 2018-03

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The Dose Dependence Analysis of the Water Fraction of *merremia mammosa (lour.)* Extract on Diabetic wound Healing Enhancement

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ABSTRACT

Introduction: Diabetic wounds or ulcers happened in Indonesia's hospitalized diabetes patients range from 17.3 to 32.9%. The high cost of treatment, the high risk of amputation and the difficulty of handling diabetic wounds, make it necessary to look for alternative medicine derived from plants e.g. *Merremia mammosa (Mm)*. This study aimed to analyze the potential dose of the water fraction of *Mm (Lour.)* extract on diabetic wound healing enhancement. **Method:** This study used fifty -seven male wistar rats that were made diabetic by intraperitoneal injection of 40 mg/kg body weight streptozotocin. Rats divided into six groups equally, which consist of positive control (gentamicin 0.1%), negative control (aquadest) and water fraction of *Mm (Lour.)* extract dose 12.5 mg, 25 mg, 50 mg and 100 mg. Wound was made by Morton method and treatment applied on the wound every other day for 21 days. Wound healing process were observed by percent wound healing and histopathological changings on day 0, 3, 10 and 25, representing each healing phase. **Results:** The percentage of reduction in wound size comparison at day 10 showed no significant different when compared with positive control started from dose 50 mg. This result is consistent with the histopathological changings parameter (angiogenesis, macrophage, fibroblast and collagen density). **Conclusion:** Water fraction of *Mm (Lour.)* extract was dose-dependently enhanced the process of wound healing in diabetic rat model and the most effective dose was 100 mg, which looks similar with positive control. Therefore, it is potential to be developed further as a topical drug.

Keywords: *Merremia mammosa (Lour), wound healing, diabetic ulcers*

The process of wound healing in Diabetes Mellitus (DM) patients are longer than normal injuries due to disruption of all processes of wound healing (1). Continuous hyperglycaemic conditions, pro-inflammatory environments, peripheral arterial disease, and peripheral neuropathy simultaneously cause impaired immune function, ineffective inflammatory responses, endothelial cell dysfunction, and neovascularization disorders (2). Increased sugar levels in collagen synthesis, worsening epithelization, decreased angiogenesis in the proliferation phase and fibroblasts caused extracellular matrix was not formed maximally because the circulatory and oxygen distribution to the region were disrupted (3). Diabetic injuries that do not treat well will rapidly expand to bacterial infections and in further circumstances will cause diabetic gangrene. Diabetic gangrene is a form of tissue in patients with DM due to reduced or cessation of blood flow to the tissue.

The management of diabetic injuries is complex because it requires comprehensive and multidisciplinary handling. Wound care in patients with DM is done with the aim to prevent the occurrence of infection, accelerate wound healing and reduce the risk of amputation. The effectiveness of wound care can be seen through changes in wound area, repair of wound severity, wound healing time and complete wound healing (4). Management of diabetic injuries include overcoming comorbid diseases (hypertension and atherosclerosis), reducing the burden (offloading), keeping the wound always moist (moist), handling infections and debridement. Skin moisture is needed to accelerate the process of tissue re-epithelization through stimulation of

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proliferation and epithelial cell migration and growth factor growth (5).

Mm (Lour.) is one of medicinal plants from Indonesia that can be found in Meru Betiri National Park. Derived from Convolvuraceae family, it can be used as an anti-inflammatory, analgesic, wound healer, treating snake bites, cancer, leprosy, syphilis, typhoid, diphtheria, inflammation and diabetes (6, 7). Upper luminous tubs contain polysaccharides, flavonoids and glycoside resin compounds such as meremocides A, E, J, mammosa that have activity as antibacterial by hollowing the cell membrane (8). Agil et al, 2011 and Sugijanto et al, 2012 reported research on extract of the *Mm* (Lour.) showed an ability to inhibit the growth of pathogenic bacteria of Microbacterium tuberculosis, Salmonella typhi, Staphylococcus aureus (research report). It is also capable on lowering blood sugar levels in diabetic rats with impaired glucose tolerance (Tilaqza, 2009, unpublished script).

The same genus with *Mm* namely *Merremia tridenta* has been shown to have activity as wound healer, (9) anti-diabetic, anti-hyperlipidemic, antioxidant and anti-inflammatory (10, 11). Our preliminary study with the *Mm* (Lour.) extract has shown that healing process significantly enhance at day 7 of the treatment and water fraction was the most effective fraction of *Mm* (Lour.) extract among the ethyl acetat, n hexane and water fraction on diabetic wound healing enhancement (unpublished data). Using chemotaxonomic approach with the plant in the same family or genus of similar compounds and the possibility of having almost the same efficacy and empirical usage of *Mm* (Lour.), this research aims to analyze the potential dose of the water fraction of *Mm* (Lour.) extract on diabetic wound healing enhancement.

MATERIALS AND METHODS

Experimental animals

Albino Wistar rats of male sex in early adulthood weighing between 150-200 g were used in the present study. Rats kept in an individual cage with a standard feed of ad libitum food and water. Mice adapted to the condition for 1 week. The mice were induced using Streptozotocin (STZ) dissolved in 0.05 mol / L buffer citrate (pH 4.5) with a single dose of 40 mg / kg body weight. The mice had diabetes when blood glucose levels exceeded 300mg/dL on the fifth day after STZ injection (12). Blood glucose levels were measured using Glucose meter (Easytouch, Taiwan) once a week. The experimental protocol was approved by Institutional Animal Ethics Committee and animals were maintained under standard conditions in an animal house approved by Ethic Committee.

Formulation of water fraction of Mm (Lour.) extract

The viscous ethanol extract of *Mm (Lour.)* was fractionated by partition using 3 different solvents of polarity i.e. n hexane, ethyl acetate and water. 50 g of condensed extract added with 100 mL of water and stirred until homogeneous. This water fraction is subsequently in a successive partition using n hexane and ethyl acetate with a ratio of 2: 3. The fractionation is performed by 3 repetitions. The water fraction is then concentrated by a freeze dryer.

Effect on excision wound

Animals were anesthetized using ketamin (50 mg/kg) and xylazine (10 mg/kg) injected intramuscular. An impression was made on the dorsal thoracic region 1 cm away from vertebral column on the anaesthetized rat. Particular skin area was shaved and the skin of impressed area was excised to the full thickness to obtain a rectangle wound area of about 25x25 mm.13 Animals were then grouped (n = 9 per group) and treated topically as follows: Group C(+): Control positive with gentamycin 0.1%, Group C(-): Control negative with aquadest, and the rest are with water fraction of *Mm (Lour)* extract dose 12.5 mg (T1), 25 mg (T2), 50 mg (T3) and 100 mg (T4) every other day for 21 days. The wound was left undressed to the open environment. Wound area was measured by tracing the wound on a millimeter scale graph paper. Percentage of wound healing was calculated as original wound size as for each animal of the group on predetermined days i.e, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25 days post-wounding.

Histopathology

Histopathological parameter observed on days 0, 3, 10 and 25 (n=3 per group per day) representing the wound healing phase (inflammation, proliferation and remodeling). The mice were sacrificed on those days with cervical dislocation and excision was performed to obtain tissue for histopathologic examination with hematoxylin-eosin stain. The tissue was

observed under a light microscope (Olympus trinocular type 31X) at 5 large fields (400X) and photographed to assess angiogenesis, macrophage, fibroblast density and collagen density degree and expressed in the scoring system. The scoring of the parameters mentioned above was determined as absent 1. when it was between 0-10%; mild (1) when it was between 10-40 %; moderate (2) when it was between 40-70% and severe (3) when it was between 70-100%.[5] In addition, extracellular matrix (ECM) represent collagen fiber density was also being assessed using ImageJ software, described as percentage [13].

Statistical analysis

The data obtained were analyzed using unpaired Student's t-test to identify significant differences between group using Excel software from Microsoft Office Profesional Plus 2016. Values are shown as the mean ± standard error (SE).

RESULTS

Body weight and non-fasting blood glucose

The data obtained showed that each group has similar body weight changing pattern. As shown in Figure 1A, after induction the rat body weight increase for a week, slightly decrease on day 3 of the excision and return to gain weight until the day of sacrificed. As shown in Figure 1B, the non -fasting blood glucose increased to more than 300 mg/dl five days after injection of STZ and maintain above 300 mg/dl until the day of sacrificed.

Wound healing

The average measurement of percent wound healing from day to day on a macroscopic basis among the wound given gentamycin 0.1%, aquadest and the water fraction of *Mm (Lour)* extract several doses can be seen in the table 1 where the percent wound healing on day 10 showed no significant different with positive control starting from dose 50 mg. For a clearer result, Figure 1C showed the percent wound healing measured every two days of the rats kept until day 25 (n=3).

Histopathology

Histopathological parameter in all experimental group during the experimental period summarizes in table 2. It showed that on day 10 and 25 the collagen fiber have significant different in all group except T1 on day 10 when comparing with C(-) and no significant different when comparing with C(+). Description of histopathological parameter by photomicrograph in each healing phase can be seen in figure 2 which there were no significant different yet on day 0 and 3 of wound excision.

DISCUSSIONS

Wound healing is a complex process that involves interactions between cells, ECM, growth factor, and cytokines. Wound healing process consists of three stages i.e. inflammation, angiogenesis (proliferation) and remodeling [14]. Diabetic wound possibly developing in difficult-to-heal wounds (wounds still proceeding through the wound healing stages but at an exceedingly slow rate), although such wounds have not been well studied [11, 15]. This study demonstrated that water fraction of *Mm (Lour)* extract enhances excisional diabetic wound healing, as defined by a more rapid of healing phase showed by faster percent wound healing and faster formation of ECM that mostly consist of collagen. In particular, the assessment of histopathological parameter revealed an advantage for the groups primed with water fraction of *Mm (Lour)* extract in comparison to the control animals. The time needed to achieve 50% closure of the wound decreased from 7.7 ± 1.0 days in control negative to 5.6 2.0.6 in water fraction of *Mm (Lour)* extract dose 100 mg (data not shown). This is consistent with our previous study on *Mm (Lour)* extract.[8] Therefore, the findings in this study allow the presumption that local *Mm (Lour)* treatment may be a novel and clinically applicable treatment for enhancing wound healing in diabetic wounds.

A number of studies have reviewed the use of natural plants as local treatment for wound healing but to our knowledge this is the first to be done with water fraction of *Mm (Lour)* extract [8, 16, 17]. The ethanol extract used in the previous study was still contained all the components of *Mm (Lour)* which gave effect as well as those not on wound healing. The amount of extract required is still large (200 mg) because the active components are still mixed with other components that are not active. Fractionation or stratified purification of uptake extract performed to increase the yield of the active component and decrease the amount of non-active material contaminant in the extract. Non-active material removal is also expected to facilitate in the formulation phase to improve

the acceptability of the preparation. It can also be used to minimize unwanted effects from impurities. In an immunomodulatory preparation contained active *meniran* extract, fractionation is proven to be able to eliminate the unwanted effects of diuretics.

Wound healing percentage showed that the effective dose started from 50 mg (no significant different with the positive control at day 10, n=3) and the effect increased by increasing the dose into 100 mg. But when we examine histopathology parameter, it is described that dose 25 mg already showed wound healing enhancement by evaluating the ECM enhancement on day 10. There is no significant different can be seen in other histopathology parameters probably due to the limited number of samples and the data were not fully quantitative. While the other groups showed low density of all examined histopathology parameters, negative control group showed a still high density of ECM, at day 25, that significantly different with all other group. This is because the negative control has a prolonged phase of healing, relevant with the result of Ackermann et al study on diabetic wound.1 However, although showed trend of delayed healing process at day 10, negative control wound healing percentage was not reached any significant different (n=3) when comparing with all other group, including the positive control (p=0.08), probably due to high variation in control negative data that shown by higher standard error. This variation may be the result of individual non-treatment inflammatory response differences that related to various factors, i.e. epidermal barrier function, growth factor production, etc (1).

Some of the content of *Mm (Lour)* are polyphenols such as flavonoids. Flavonoids have antioxidant effects that accelerate the inflammatory phase by capturing free radicals and prevent oxidation reactions by increasing the activity of the enzyme Superoxide dismutase (SOD) and glutathione transferase (Subandi, et al., research report). In addition, flavonoids have anti-inflammatory activity that inhibits the important phase in biosynthesis that is on the path of cyclooxygenase and an antibacterial activity by inhibited bacterial DNA gyrase function so that the replication and translation of bacteria is inhibited (Gunawan, 2009, unpublished script). Flavonoids with their anti-inflammatory activity can stimulate cells such as macrophages to produce growth factors and cytokines such as EGF, TGF-ß, IL-1, IL-4, IL-8 to accelerate the proliferation and other wound healing phase. The content of flavonoids in *Mm (Lour)* can also stimulate cellular immunity by proliferating lymphocytes and production of reactive oxygen intermediate macrophages (Farizal, 2012, unpublished script).

Diabetic rats model in this study were maintained to have severe diabetes because the STZ dose administered at 40 mg/kg body weight and the non-fasting blood glucose higher than 300 mg/dL through all the experiment period (Figure 1) (12). Body weight in all group showed a slightly decreased right after STZ injection and then increased gradually, this is relevant with DM pathogenesis (18, 19).

Although the researcher has tried maximally to control every step in conducting this research, there were still few limitations such as the method in measuring wound size and grading the density of angiogenesis, macrophage, fibroblast and collagen that was done by manual measurement. The usage of ImageJ software also has a limit to only differentiate the ECM and cannot be specific in collagen structure.

CONCLUSIONS

Diabetic wound possibly developing in difficult-to-heal wounds, therefore, it need a special treatment. Based on the result of data analysis, water fraction of *Mm* (Lour.) extract was dose-dependently enhancing the process of wound healing in diabetic rat model and the most effective dose was 100 mg, which looks similar with positive control. When comparing with negative control there is no group significantly different including the positive control, probably due to high variation in control group related to severity variation of DM. According to the purpose of this research, it is suggested to develop the effective dose of *Mm* (Lour) extract water fraction as a topical drug in diabetic wound or other prolonged wound healing conditions. However, to overcome the limitation of this study, it is suggested to conduct future research with larger number of samples and a more specific parameter on wound healing phase.

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We declare that we have no conflict of interest.

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Appendix

*Table 1 Effect of different dose of water fraction of Mm (Lour) extract on percent wound healing.**

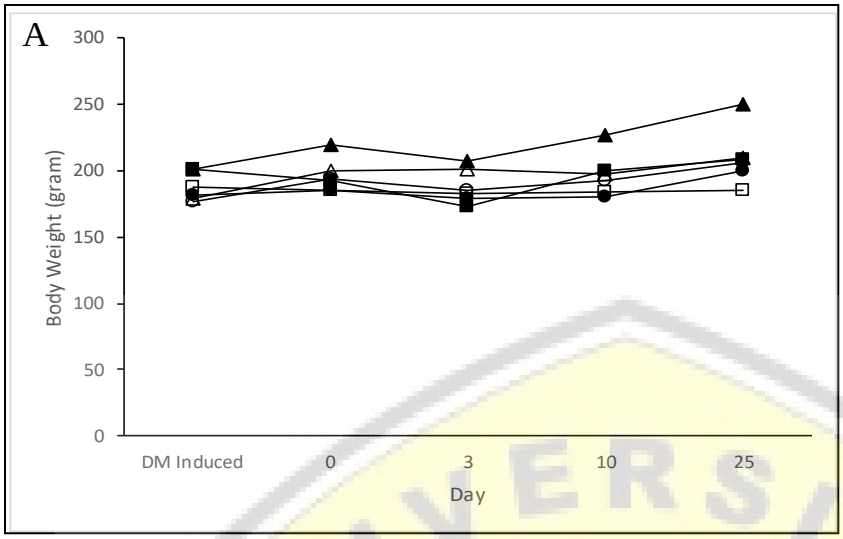
Percent wound healing	n	Groups					
		C(-)	C(+)	T1	T2	T3	T4
Day 3	9	16.3 ± 3.3	10.3 ± 3.1	15.9 ± 1.1	19.4 ± 4.0	22.8 ± 3.3	20.5 ± 3.8
Day 5	6	29.5 ± 6.2	26.2 ± 3.3	37.4 ± 3.2	35.5 ± 4.9	41.1 ± 6.0	38.3 ± 7.7
Day 7	6	46.7 ± 10.1	45.3 ± 5.8	60.1 ± 1.8	60.4 ± 6.9	64.1 ± 6.2	68.1 ± 6.7
Day 9	6	56.5 ± 10.8	74.9 ± 8.0	76.7 ± 4.0	73.7 ± 5.8	76.2 ± 4.1	83.1 ± 3.7
Day 11	3	64.9 ± 7.1	81.4 ± 3.5	77.4 ± 7.9	87.3 ± 5.9	80.1 ± 8.4	89.2 ± 3.9
Day 13	3	76.9 ± 6.2	87.8 ± 2.9	86.9 ± 3.7	93.7 ± 4.8	84.0 ± 7.1	93.2 ± 3.1
Day 15	3	88.3 ± 1.9	92.6 ± 2.1	89.0 ± 3.3	94.6 ± 4.4	88.6 ± 6.2	95.1 ± 2.2
Day 17	3	92.2 ± 3.1	93.5 ± 2.2	92.6 ± 3.0	95.6 ± 3.4	90.0 ± 5.3	98.3 ± 0.7
Day 19	3	96.1 ± 1.6	96.9 ± 1.1	97.5 ± 1.4	96.1 ± 3.4	92.9 ± 3.5	99.5 ± 0.2
Day 21	3	98.5 ± 0.8	97.9 ± 1.2	98.8 ± 0.6	96.6 ± 3.1	93.9 ± 3.1	99.7 ± 0.2
Day 23	3	99.3 ± 0.2	99.1 ± 0.3	99.5 ± 0.4	97.7 ± 2.0	96.5 ± 1.8	99.8 ± 0.1
Day 25	3	99.6 ± 0.1	99.6 ± 0.3	99.6 ± 0.3	99.7 ± 0.0	98.9 ± 0.9	99.8 ± 0.1

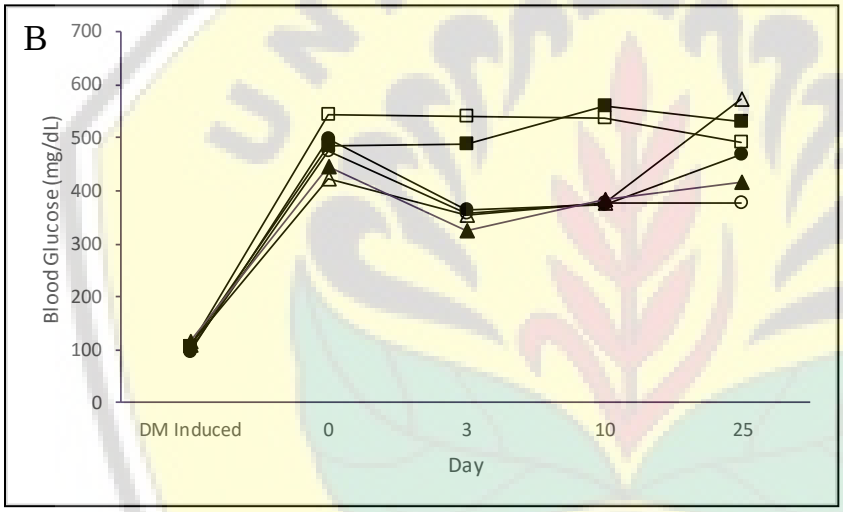
*All measurements are reported in % mean ± Standard Error (SE). C(-): Aquadest, C(+): Gentamycin 0.1%, T1,T2,T3,T4 were water fraction of *Mm (Lour)* extract dose 12.5, 25, 50 and 100 mg.

*Table 2 Comparison of histopathological parameters on day 0, 3, 10 and 25 of wound excision, representing each of healing phase.**

Time (day)	Variable	Groups					
		C(-)	C(+)	T1	T2	T3	T4
0	Angiogenesis	0 (0-0)					
	Fibroblast	1 (0-1)					
	Macrophage	0 (0-0)					
	Collagen	1 (1-1)					
	ECM	27.2 ± 2.9					
3	Angiogenesis	0 (0-0)	0 (0-1)	1 (0-1)	1 (0-1)	0 (0-1)	0 (0-0)
	Fibroblast	1 (0-1)	0 (0-1)	1 (1-1)	1 (0-2)	1 (0-1)	0 (0-0)
	Macrophage	2 (1-3)	1 (0-1)	1 (1-2)	1 (1-1)	1 (1-1)	1 (1-2)
	Collagen	1 (1-1)	2 (1-2)	1 (1-2)	1 (1-2)	2 (1-2)	1 (1-2)
	ECM	31.4 ± 2.8	37.7 ± 2.9	40.6 ± 2.5	40.3 ± 2.4	41.7 ± 0.5	37.7 ± 4.7
10	Angiogenesis	0 (0-1)	0 (0-0)	0 (0-1)	0 (0-1)	0 (0-0)	0 (0-1)
	Fibroblast	0 (0-1)	1 (1-2)	2 (1-2)	1 (1-2)	1 (1-2)	2 (1-2)
	Macrophage	0 (0-1)	0 (0-0)	0 (0-1)	0 (0-0)	0 (0-0)	0 (0-1)
	Collagen	1 (1-1)	2 (1-2)	1 (1-2)	1 (1-2)	2 (1-2)	2 (2-2)
	ECM	35.9 ± 2.3	50.9 ± 3.8[a]	32.1 ± 5.7	45.9 ± 2.0[a]	47.8 ± 0.6[a]	48.3 ± 2.2[a]
25	Angiogenesis	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)
	Fibroblast	1 (0-1)	0 (0-0)	1 (1-1)	1 (0-1)	1 (1-1)	1 (1-1)
	Macrophage	0 (0-0)	0 (0-0)	0 (0-1)	0 (0-1)	0 (0-0)	0 (0-0)
	Collagen	2 (2-2)	1 (1-1)	1 (1-1)	1 (1-2)	1 (1-2)	1 (1-2)
	ECM	48.9 ± 1.2	26.5 ± 4.0[a]	34.1 ± 2.8[a]	32.8 ± 4.9[a]	31.2 ± 6.2[a]	31.3 ± 5.1[a]

Data are expressed as Median (Min-Max) except for ECM (Extra Cellular Matrix) is in mean percentage ± SE. C(+): Gentamycin 0.1%, C(-): Aquadest, T1,T2,T3,T4 were water fraction of *Mm (Lour)* extract dose 12.5, 25, 50 and 100 mg. Angiogenesis, fibroblast, macrophage and collagen density were evaluated in accordance with the percentage of the densities in a 400X magnified dissected area of the lession region. The scoring of the parameters mentioned above was determined as absent (0) when it was between 0-10%; mild (1) when it was between 10-40 %; moderate (2) when it was between 40-70% and severe (3) when it was between 70-100%. ECM density was determined as % mean ± Standard Error (SE) using ImageJ software. [a]$p<0.05$ v.s. C(-) group using unpaired Student t-test analysis.





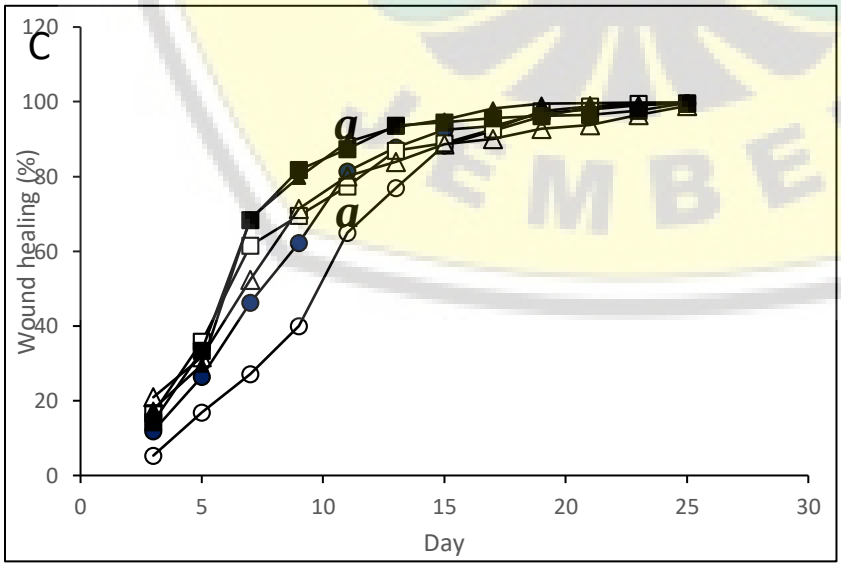


Figure 1

Body weight, non-fasting blood glucose and percent wound healing during the experimental period. Amount of body weight (A), non-fasting blood glucose (B) and percent wound healing (C) are shown. Opened and closed circles represent negative and positive control. Opened and closed squares and triangle represent water fraction of *Merremia mamossa (Lour)* extract dose 12.5, 25, 50 and 100 mg in a sequent. Percent wound healing significances (n=3) at 3, 10 and 25 days of wound excision showed by Greek letter a, $p<0.01$ compare with positive control.

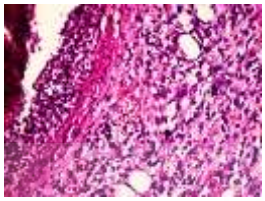
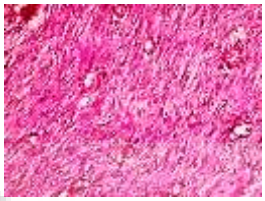
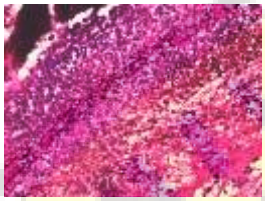
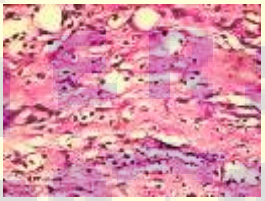
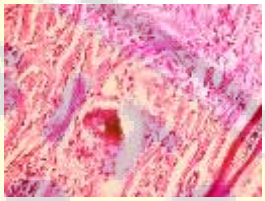
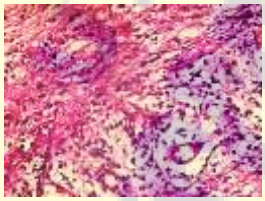
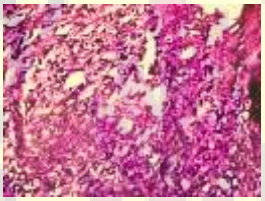
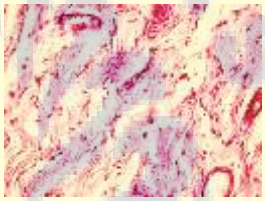
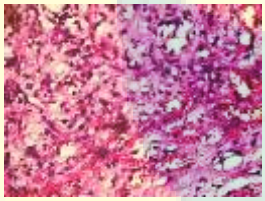
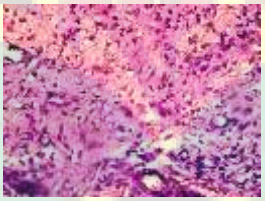
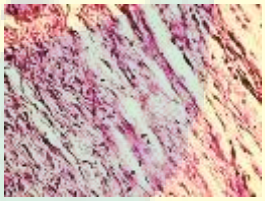
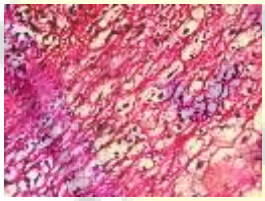
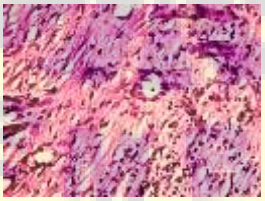
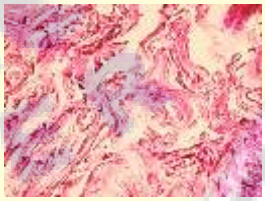
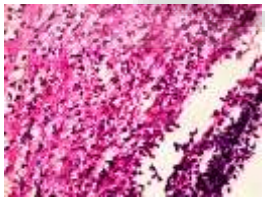
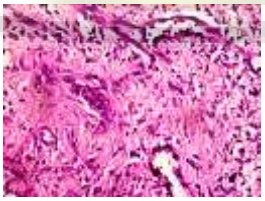
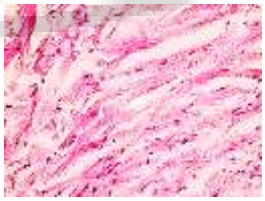
Groups (n=3)	3rd Day	10th Day	25th Day
Aquadest			
‘ Gentamycin 0.1%			
12.5 mg			
25 mg			
50 mg			
100 mg			

Figure 2

Photomicrographs of rat skin sample on hematoxylin-eosin stain: (A) At 3rd day post excision showing similar density of angiogenesis, fibroblast, macrophage and collagen in each group at high power view (magnification 400x); (B) 10th day post excision group; and (C) 25th day post excision group.